FIELD AND LABORATORY STUDIES OF *Culex erraticus* (Diptera: Culicidae) ABILITY TO DETECT HOSTS, HABITAT IDENTIFICATION AND ATTEMPTS AT COLONIZATION

By

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To my wife, Vicki

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FIELD AND LABORATORY STUDIES OF *Culex erraticus* (Diptera: Culicidae) ABILITY TO DETECT HOSTS, HABITAT IDENTIFICATION AND ATTEMPTS AT COLONIZATION

By

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Chair: Jerry F. Butler
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Four artificial diets were used in attempt to rear a colony of *Culex erraticus* mosquitoes for transmission of *Plasmodium floridense* to *Anolis carolinensis*. There was a 90% egg hatch rate with a 51% larval mortality and 31% pupal survival rate. Fifty-eight percent of surviving pupae were adult males and 42% were adult females. The artificial diets that contained powdered yeast performed the best. However, all four diets failed to produce progeny after the F_3 generation, therefore wild *Culex erraticus* mosquitoes were trapped using modified CDC light traps with CO_2 and lizard extract.

The lizard extract was prepared by washing deceased colony *A. carolinensis* lizards in filtered hexane for 24 hours to remove skin compounds that may be used by *Cx. erraticus* as attractants during host location. Four dosage amounts 5, 10, 15, 20 and 25 micro-liters of lizard extract were evaluated using 160 *Cx. erraticus* mosquitoes in an open chamber 10 port olfactometer. The 10 microliters was found to be the most attractive. The 10 microliters was then evaluated in 15 paired-*t* replications with hexane

as a control and the lizard extract performed better than the hexane in the laboratory and in the CDC light traps with CO_2 and hexane in the field trapping study.

Anolis carolinensis lizards were caught in the wild and brought into the laboratory where small blood samples drawn by toe clippings were performed to determine if the lizards were infected with *P. floridense*. One in four wild lizards proved to be infected with *P. floridense*, and these lizards were used in seven transmission studies to successfully transmit the reptilian malaria parasite in two non-infected *A. carolinensis* lizards by bite from *Cx. erraticus* with evidence of positive infections with *P. floridense* in red blood cells and phanerozoites in the tissues of the kidneys, liver and pancreas.

Anolis carolinensis lizards caught in the wild and purchased from Texas were used in the olfactometer, and it was shown that *Cx. erraticus* could detect the lizard's presence. However no significant difference was found in *Cx. erraticus* having a preference for infected lizards over apparently non-infected lizards.

CHAPTER 1
INTRODUCTION

There are 39 genera and 135 subgenera containing approximately 3,000 mosquito species in the world (Clements 1992, Reinert 2000c, 2001). *Culex (Melanoconion) erraticus* Dyar and Knab is one of approximately 250 species of mosquitoes in the subgenus *Melanoconion* (Pecor et al. 1992, Peyton and Harbach 1991). *Culex erraticus* is found in many parts of the world including the southeastern United States especially in all areas of Florida (Darsie and Ward 2005, Reinert 2001).

Florida has 13 genera, 22 subgenera and 77 species of mosquitoes (Darsie and Ward 2005, Reinert 2001). Of the 77 species found in Florida, there are 13 United States species that only occur in Florida (Darsie and Ward 2005). The semi-tropical environment of Florida and its location near other tropical communities may possibly be a contributing factor to the large diversity of mosquitoes found in Florida.

The genus *Culex* (*Cx*.) found in Florida has 15 different species of mosquitoes that are divided into four subgenera. Species of the Subgenus *Culex* are *Culex bahamensis* Dyar and Knab, *Cx. nigripalpus* Theobald, *Cx. quinquefasciatus* Say, *Cx. restuans* Theobald, *Cx. salinarius* Coquillett *and Cx. tarsalis* Coquillett. Subgenera Neoculex and Micraedes contain only one member each, *Cx. territans* Walker and Cx. *biscaynensis*, respectively. *Culex atratus* Theobald, *Cx. cedecei* Stone and Hair, *Cx. iolambdis* Dyar, *Cx. mulrennani* Basham, *Cx. peccator* Dyar and Knab, *Cx. pilosus* Dyar and Knab *and Cx. erraticus* Dyar and Knab are members of the subgenus *Melanoconion* (Darsie and Ward 2005).

Culex iolambdis and *Culex cedecei* are usually found in central Florida throughout South Florida. *Culex atratus* is found in the Western side of the peninsula of South Florida joining *Culex mulrennani* in the Florida Keys. *Culex peccator* is found primarily from central Florida throughout the northern part of Florida. *Culex pilosus* and *Cx. erraticus* are indigenous throughout Florida (Darsie and Ward 2005). However, with the recent hurricanes in Florida redistribution of these melanoconion mosquitoes may bring about some confusion with identification because their color and size is very similar (Pratt 1959).

The origin of *Cx. erraticus* is still in question but it is believed to have come from South America making its way through Panama into and along the Gulf States. *Culex erraticus* was first trapped in California in a habitat less favorable for this mosquito leading to speculation that entrance into that part of the United States was more likely from Ixtapa in the state of Guerrero located on the pacific coast of Mexico (Aldrete and Pletsch 1976, Lothrop et al. 1995). *Culex erraticus* is one of the smallest mosquitoes found in the family Culicidae and this species has an extensive and somewhat confusing synonymy (King and Bradley 1937).

Early identification of *Cx. erraticus* was vexatious because of the constant change being made in the subgenera *Mochlostyrax* or *Melanoconion* (King and Bradley 1937). Most of the changes were due to the coloration, markings and lack of specific characters in the female mosquitoes (King and Bradley 1937). Because of the color and size of the female Melanoconion mosquitoes, there has been some confusion with identification between species (Pratt 1989). Various mutations especially body color pigments, melanotic or dark color mutants are common in many mosquito species especially those

in the genus *Culex* (Dennhofer 1973, Iltes et al. 1965, Seawright et al. 1985, Shetty et al. 1976, 1985, Suguna 1986, Suguna and Vaidyanathen 1983, Tadano and Barrl 1975).

King and Bradley in 1937, addressed the terminology published by Dyar in 1928, that suggested *Cx. inhibitator* Dyar and Knab from Santo Domingan was identical to *Cx. erraticus.* The original specimen materials along with the published descriptions were closely investigated, and both *inhibitator* and *erraticus* were in fact two separate species. During the study changes were made to the male terminalia of *Cx. inhibitator* and the characteristics of the larval stages of *Cx. peccator* were changed to correctly reflect the differences from *Cx. erraticus*. Dyar and Knab reported that materials found in the National Museum for *Cx. egberti* Dyar and Knab, *Cx. peribleptus* Dyar and Knab, *Cx. pose* Dyar and Knab, *Cx. degustator* Dyar and *Cx. homoeopas* Dyar and Ludlow all found in the United States were the same as *Cx. erraticus*. Dyar finally concluded that the tropical Americas species *Cx. leprincei* Dyar and Knab, *Cx. invocator* Pazos, *Cx. trachycampa* Dyar and Knab, *Cx. borinqueni* Root, *Cx. moorei* Dyar and *Cx. tovari* Evans are the same as *Cx. erraticus* thus creating a wide geographical distribution for *Cx. erraticus* in the United States.

The wide geographical distribution of *Cx. erraticus* makes it one of the most common mosquitoes found throughout the southeastern United States and in many parts of the world (Cupp et al. 2003, Lothrop et al.1995, Pecor et al. 2002). *Culex erraticus* is found in low to moderately high numbers in various habitats and has adapted to moderate as well as extreme conditions (Afolabi et al. 1989, Barber et al. 1924, Bradley and Fritz 1945, Carter 1915, Crans 2004, Cupp et al. 2003, Furlow and Hays 1972, Gage 1925,

Gartrell et al. 1981, Hess and Crowell 1949, Lothrop et al. 1995, Lounibos and Escher 1985, McNelly and Crans 1989, Pecor et al. 2002, Robertson et al. 1993, TVA 1947).

Bates (1949) and Pratt (1959) proposed two similar classification methods that put mosquitoes in shared life cycle strategies in the different ecological environments found throughout the United Kingdom and the United States. However, both models did not take into account that the same species of mosquito can undergo various life cycle types depending on what part of the geographical United States it lives in. Merging of Pratt's and Bates classification systems brought about our modern day system of taking into account the geographical locations of *Cx. erraticus,* where adult female oviposits her eggs, the number of generations per year and the stage in which *Cx. erraticus* lives during diapause (over-wintering). Therefore, utilization of different trapping methods validated the presence of *Cx. erraticus* in many areas of the United States and foreign countries (Ali et al. 1989, Carestia and Savage 1967, Carestia and Horner 1968, Cupp et al. 2003, Gladney and Turner 1970, Kline 2002, Kline and Mann 1998, Kline et al. 1990, Love and Goodwinn 1963).

Culex erraticus Trapping Methods

Trapping methods used to collect adult *Cx. erraticus* include Centers for Disease Control (CDC) light traps baited with CO_2, CDC light traps baited with dry-ice (CO_2), Animal-baited traps, New Jersey light traps with six incandescent light sources of different colors and wattages (intensities), Gravid traps, Mosquito Magnet™ Pro Model and the American Biophysics Corp Counterflow Geometry trap generating CO_2 by combustion of propane (Ali et al. 1989, Bates 1944, Bellamy and Reeves 1952, 1963; Carestia and Savage 1967, Carestia and Horner 1968, Cupp et al. 2003, Evans and Willis

2002, Jones and Meisch 1993, Kent et al. 2001, Kline 2002, Kline and Mann 1998, Kline et al. 1990, Love et al. 1963, Pinger 1985, Strickman et al. 2000).

A field study conducted by Ali et al. (1989) in Florida tested New Jersey light traps in combination with six different 100 watt color light bulbs (red, orange, yellow, green, blue and white) to see which species of mosquitoes are attracted to what color. In all, *Psorophora* and *Culex* were the two most dominant number of mosquitoes trapped. Interestingly, the white and blue bulbs were more instrumental as an attractant than the other four colors. Other field and laboratory studies involving the use of artificial lights and mosquito attractancy are numerous and the results from each of these are similar in that they increased the number of mosquitoes and species of mosquitoes (Barr et al. 1963, Breyev 1963, Brown 1956, Burkett et al. 1998, Dennett et al. 2004, Gillett 1972, Herbert et al. 1972, Kusakabe and Ikeshoji 1990, Lehane 1991, Muir et al. 1992, Sippel and Brown 1953, Thurman and Thurman 1955, Wood and Wright 1968).

It was not until the introduction of carbon dioxide as an attractant in light traps that mosquito indices rose significantly for both species and population numbers (Baily et al. 1965, Brockway et al. 1962, Carestia and Horner 1968, Carestia and Savage 1967, Headlee 1933, 1934, Newhouse et al.1966, Reeves 1951, 1953, Reeves and Hammon 1942, Rudolphs 1922, Roberts 1972). To help validate mosquito olfaction, Van Thiel (1974) tested carbon dioxide in combination with air in an air current olfactometer and showed a 76% increase in mosquito attraction. Van Thiel's research in the olfactometer help to support earlier findings that carbon dioxide was found to serve two purposes when mosquitoes are actively seeking a host. First mosquitoes use CO_2 as an activator that raises the awareness that a host is close, and second, the CO_2 combined with host

odors induces the mosquito to land and probe the host for feeding purposes (Kahn and Maibach 1966).

Different environmental concentrations of CO_2 can also be a factor for attracting different species of mosquitoes (Reeves 1951). Carbon dioxide is a natural component present in the atmosphere at 0.03% to 0.05% and naturally found in the breath of humans, animals and emanating from the skin of vertebrate host (Black 1968, Gillies 1980, Lehane 1991, Wessenling 1962). An infrared analyzer revealed that carbon dioxide continuously emanated from the human hand at 1.0 to 1.8 ml/h (Carlson et al. 1992). Whitsel and Schoeppner (1965) reported that approximately 12 humans exhale the equivalent of CO_2 released from a pound of dry ice. Kline and Mann (1998) showed that five species of *Culex* mosquitoes' *salinarius, nigripalpus, pilosus, restuans* and *erraticus*, responded to CO_2 and light. Of the five *Culex* species, *pilosus* and *erraticus* did not respond to light-only traps. In addition to CO_2, most synthropic flies and mosquitoes use visual stimuli and body odors to locate their host (Davis and Sokolove 1976). Because of minimal information published on the host feeding patterns of the 250 species found in the subgenus *Melanoconion,* it is presumed that *Cx. erraticus* is an opportunistic feeder favoring avian hosts (Edman 1979)

Mosquito Activation and Host Location

Most adult female mosquitoes, including *Cx. erraticus*, use various methods and signals when locating a host to sequester a blood meal. There are few mosquitoes that produce one generation per year, but many species of mosquitoes can produce multiple generations per year. A huge increase in these species of mosquitoes creates more opportunities for vectoring of disease organisms. Knowing what populations of wildlife

reside in certain ecological habitats and what species of mosquitoes are present in that habitat helps researchers to better understand vector-host relationships.

Many mosquito larval species can grow rapidly in the hotter months of the year and emerge as flying adults in as little as a week. This has the potential to increase the number of vectors in a habitat and to increase the number of vector-host contacts. Adult mosquito activation and orientation behaviors utilize signals generated by physiological conditions, environmental conditions and signals from a host that provoke and inhibit host-seeking behaviors (Adams 1999, Takken 1996).

During the first two days after most adult mosquitoes emerge, they are concerned with mating and acclimating to their environment and generally do not demonstrate host seeking behavior (Klowden 1994). Finding a host is a critical element in the life cycle of most adult female mosquitoes; therefore, after the adult female mosquito finishes her acclimation period, her juvenile hormones initiate the host seeking behavior for acquisition of a blood meal to procure the necessary proteins and cholesterols to develop her eggs (Adams 1999, Lehane 1991).

The process of ovarian development is part of the physiological equation that initiates an adult female in locating a host for a blood meal. Sutcliffe (1987) addressed three areas of concern for host location, appetitive searching, activation of orientation and attraction towards a selected host.

Appetitive searching by female mosquitoes is nonorientated behavior driven by hunger. If the female mosquito is in the right place at the right time and if the mosquito is an opportunistic feeder, such as *Cx. erraticus*, then she has many different hosts available from which to select. Once the female mosquito perceives a host in the

appetitive-searching mode, she uses visual and/or chemical cues to change her search behavior to activation of orientation.

Activation of orientation is stimulated by the presence of carbon dioxide, host odors and other chemical compounds existing in the environment (Gillies 1980). Carbon dioxide has been shown to have synergistic properties when combined with host odors (Gillies 1980, Costantini et al. 1996) leading to a stronger attraction to isolating and locating the host (Khan 1966).

Bidlingmayer (1994) found in his study that artificial host shape and size along with motion, trap color, color contrast, patterning, light color and intensity can serve as stimulation factors for mosquitoes in locating a host. Fairly large unpainted plywood-covered suction traps produced large silhouettes that were highly attractive to various species of nocturnal feeding mosquitoes (Bidlingmayer and Hem 1980). Allan et al. (1987) reported that mosquitoes with diurnal feeding times might locate their host most of the time with visual stimuli as opposed to nocturnally feeding mosquitoes. Allan also reported that visual perceptions of the approaching female mosquito could include color and movement of the host. Certain *Culex* mosquitoes in Africa were found to be more visually attracted to large ramp traps in comparison to small suction traps (Gillies and Wilkes 1974). Olfaction (sense of smell) cues utilized by female mosquitoes include detection of small amounts of carbon dioxide, pheromones, methane, octenol, urine components, lactic acid, acetone, butane and phenolic compounds.

When female mosquitoes are detecting odors in an atmospheric plume, their level of stimulation is increased as the odor concentrations increase (Sutton 1953). Farkas and Shorey (1972) reported that host seeking insects could detect a host when they are outside

the plume or host recognition zone. When the female mosquito perceives the right conditions, such as moist warm air along with or in combination with chemical signals from the host, the mosquito becomes more deliberate in its approach and possibly committed to feed on the host (Davis and Sokolove 1976, Lehane 1991). Once the blood meal is successfully sequestered from the host(s), stretch receptors in the mosquito gut turn off this search mode until the blood meal is digested and the eggs are deposited. After the female mosquito deposits her eggs a small period of rest takes place before she searches for another blood meal (Adams 1999).

The host stimulates the mosquitoes by emitting carbon dioxide through respiration, body heat in combination with sweat (moisture) and various chemical cues that are yet to be understood completely (Bar-Zeev et al. 1977, Knols 1996, McKenzie 2003). After mating some species of female mosquitoes such as *Aedes taeniorhynchus* Wiedemann, *Aedes atropalpus* Coquillett and *Culex pipens molestus* Forskal are autogenous, not requiring a blood meal to develop their first clutch of eggs, but they will require blood meals for subsequent egg development (Klowden 1997).

Autogenous mosquitoes decrease their vector capacity and chances of vectoring diseases because they do not acquire a blood meal by biting an infected host prior to depositing their first clutch of eggs. Anautogenous mosquitoes, such as *Cx. erraticus*, create an increased chance of vectoring a disease because of taking blood meals before oviposition of their first eggs, thus increasing their vector host contact capacity. Published research on the feeding habit of most *Melanoconion* mosquitoes, specifically *Cx. erraticus*, are minimal and need further investigation.

Culex erraticus Host Preference

Host feeding studies of mosquitoes has been conducted for many years and the data on the feeding habits of mosquitoes is vast (Bertsch and Norment 1983, Crans 1965, Edman 1971, 1974, 1979, Edman and Haeger 1977, Edman and Taylor 1968, Edman et al. 1972, Joseph and Bickley 1969, LeDuc et al. 1972, Magnarelli 1977, Nasci 1982, 1984, Nasci and Edman 1981, Suyemoto et al. 1973, Taylor et al. 1971, Tempelis 1975).

The different ecological conditions and control measures used against other mosquitoes have not been a deterrent against the ability of *Cx. erraticus* to survive (Levy et al. 1982, Nayar and Ali 2003). The epidemiology of mosquito-borne diseases starts with the understanding of the host-feeding patterns of mosquitoes. What little information is available on *Cx. erraticus* points to it being an opportunistic feeder sequestering a blood meal from the nearest suitable host.

Identifying the period of day or night when mosquitoes are the most active is important for understanding population studies and disease transmission by individual species (Blakeslee et al. 1959, Harcourt and Cass 1958, Horsfall 1962, Hutchins 1940, Love et al.1963, MacCreary 1941, Meyers 1959, Platt 1955, Platt and Witherspoon 1957, Snow 1955, Standfast 1965, Williams 1935). *Culex erraticus* is implicated as primarily an avian feeder, but many published papers concerning *Melanonocion* mosquito host preferences show blood meal sources to be varied (Edman 1979, Edman and Taylor 1968, Edman et al. 1972, Gorgas Memorial Laboratory 1972, Robertson et al. 1993).

Gladney and Turner (1970) were first to use malaise traps to determine diel flight rhythms of mosquitoes versus conventional light traps. Gladney's trapping data resulted in the light traps capturing 16 different species of mosquitoes and the malaise traps captured 12 species of mosquitoes. The malaise trap did not yield significant numbers of

Cx. erraticus throughout the trapping regime, but the light traps captured higher numbers of *Cx. erraticus* from dusk to 11:00 p.m. with a steady decline thereafter to a low level.

Love et al. (1963) researched in two habitats in southern Georgia, open field and woodland. In these two habitats the same elevations were sampled during the same periods of time. Elevations in both habitats were six, twenty-five and forty feet. The time periods were in three-hour blocks starting at 6:00 p.m. until 6:00 a.m. for a total of 12 hours. The largest activity was reported from 6:00 p.m. until 9:00 p.m. at the six-foot elevation with reported numbers of *Cx. erraticus* declining steadily thereafter. The 25-foot elevation reported a low activity from 6:00 p.m. till 9:00 p.m., then increased significantly from 9:00 p.m. until 12:00 a.m., then drastically declined thereafter. The 40-foot elevation reported similar activity as the 6-foot elevation, starting at 6:00 p.m. and rose steadily until 12:00 a.m., then declined thereafter. Also, *Cx. erraticus* was trapped in higher populations in the wooded area than in the open fields, and the number of *Cx. erraticus* trapped at the 25-foot level was significantly more than at the six- and 40-foot elevated traps. Love et al. (1963) stated in their paper, "the *C. (Melanoconion)* group was most abundant before midnight. The *C. (Melanoconion)* group was predominantly *Cx. erraticus* but contained some *Cx. peccator* and some *Cx. pilosus.*"

Bidlingmayer et al. (1985) performed a field test comparing two traps situated on rafts in an open water-filled borrow pit and two traps situated along the shore line in dense vegetation and two traps placed under a canopy of mixed trees and shrubbery in the same location. They found that females that are primarily avian feeders, such as *Cx. erraticus*, fly high in the canopy of trees when searching for hosts, which supports the finding of the previously mentioned research of Love et al. (1963).

Robertson et al. (1993) performed precipitin test on previous blood meals taken in by *Cx. erraticus* and found that *Cx. erratiucus* fed on 111 different mammals, including deer, fox or dog, six human, and 31 unidentified mammals. Robertson et al. (1993) also reported that *Cx. erraticus* fed on 45 reptiles or amphibians, with 23 on turtle, 11 on snakes, three on frogs and seven unidentified reptiles. The number of avian blood meals totaled 69, with 10 Passeriformes, 39 Columbiformes, one Strigiformes and 18 unidentified hosts. Edman (1979) reported that out of 757 blood meal analyses, 511 were Ciconiiform birds, 142 were mammals ranging from rabbits to rodents, and 18 were amphibians and reptiles with none feeding on lizards. These results are important because the different habitats trapped show that *Cx. erraticus* may prefer avian host, but it will feed on other host in order to sequester its blood meal to complete the requirements for egg development. Having the option of taking different types of blood indicates that this species of mosquito should do well on laboratory bovine blood (Garrett 2005, Klein 1985)

Conflicting opinions about the feeding habits of *Cx. erraticus* is still being debated because of its ability to feed on equine, canine, bovine, feline, human, mammal and various kinds of reptiles. *Culex erraticus* is known to feed primarily on avians in most aquatic areas but has been found to be an opportunistic feeder in many outlying areas that have a number of different types of wildlife to feed upon. Few previous blood meal studies have been repeatedly performed on this mosquito; therefore, little is known about *Cx. erraticus* and its preference for selecting specific host(s) and what stimulants it uses, including visual and host odors. How *Cx. erraticus* deliberately locates an *Anolis*

carolinensis lizard respiring carbon dioxide at 1.5 ml/hour (W.A. Hopkins, personal communication) is unclear.

The previous feeding styles of *Cx. erraticus* suggest that many of the blood meals were taken opportunistically from encountered hosts rather than from highly attractive host being actively searched on a consistent basis (Washino and Tempelis 1983). Because *Cx. erraticus* is an opportunistic feeder, it has a greater chance of testing positive for various disease organisms and/or being a capable vector of disease organisms and parasites.

Most mosquitoes are specific as competent vectors of viruses, bacteria, nematodes and protozoan organisms (Afolabi et al. 1989, Cupp et al. 2003, Jones and Meisch 1993, Klein et al. 1987). However, published research on the bionomics including the biology, ecology and vectorship of *Cx. erraticus* is minimal and needs more exploration because of its potential to vector the previously mentioned pathogenic organisms to different hosts including avian, equine, canine, fowl and reptiles (Bigler et al. 1976, Chamberlain et al. 1954, Cupp et al 2004, Day et al.1996, Edman 1979, King et al.1960, Klein et al. 1987, Lothrop et al. 1995, Mitchell et al. 1996, Morris 1988, Robertson et al. 1993, Ross 1947, Sudia et al. 1968, Wozniak et al. 2001). Although *Cx. erraticus* has been reported as more of a nuisance to man than as a vector of diseases, investigation into the life cycles of this mosquito is warranted.

Culex erraticus Bionomics

Culex erraticus Egg Raft

The shape and size of mosquito eggs are species specific. Most *Culex* mosquitoes lay their eggs on the water surface and do not require specific amounts of rainfall for the eggs to hatch, as do mosquitoes in certain other genera.

Réaumur (1738) reported the first description of the adult female *Culex* mosquito's behavior during oviposition. During the first 20 eggs being oviposited by the female, Réaumur (1738) observed that during this time the female mosquito was the most sensitive to all disturbances. This sensitivity behavior during oviposition by *Culex* mosquitoes has been well documented by other researchers (Beaument and Corbet 1981, Suleman and Shirin 1981, Pappas et al. 1982, Pile 1987, 1989). Pile (1989) also observed during egg deposition that an existing *Culex* egg raft was utilized by a second adult *Culex* female (same species) to attach 132 of her own eggs. There is no recorded evidence of *Cx. erraticus* utilizing previously laid egg rafts to attach newly deposited eggs, however, Pile found that there was a low frequency of this behavior, at approximately 1%, and this behavior could have important implications, especially in the area of aggregation pheromones produced by egg rafts already laid (Bruno and Laurence 1979, Laurence and Pickett 1985).

There are many well documented physical factors such as temperature, light and humidity, and chemical cues including attractants, arrestants, and oviposition stimulants, that are used by many species of gravid female mosquitoes to locate a suitable site for ovipositing their eggs (Benzon and Apperson 1988). Most *Culex* mosquitoes leave an apical droplet on the pointed apices of the eggs which function as an oviposition aggregation pheromone that encourages other *Culex* mosquitoes to lay their eggs in the same area (Aharoni and Zweig 1973, Bruno and Laurence 1979, Christophers 1945, Dadd and Kleinjan 1974, Goeldi 1905, Iltis and Zweig 1962, McLintock 1951, Osgood 1970, Starratt and Osgood 1972,). No previous information about apical droplets on *Cx.*

erraticus eggs has been published; however, this does not mean that the oviposition pheromone does not exist on the target mosquito's eggs.

Adult female *Cx. erraticus* deposit egg rafts on the water surface near, under or attached to aquatic vegetation (Carpenter and LaCasse 1955, Dyar 1921, Gorgas Memorial Laboratory 1978) that serve several important functions. First, aquatic plants aid in protecting and concealing the egg and larval stages of the life cycles against predation (Bradley 1932). Second, it anchors the egg rafts against the ecological and environmental conditions that would be unfavorable to the survivability of the mosquito eggs (Furlow and Hays 1972, Rueger et al. 1964, Zetek 1920). Third, aquatic vegetation helps control the water temperature, affecting evaporation, oxygen supply and chemical content (Horsfall 1967). *Culex erraticus* favors aquatic habitats with a diverse fauna of submersed and floating plants, perhaps utilizing the above important functions of aquatic vegetation.

Culex erraticus eggs are deposited in rafts with double or triple zigzagged rows and a curved base for flotation. There is a very small inconspicuous corolla on the anterior end that has an elevated floor to the egg. The anterior end of each egg also has an area that is sclerotized and translucent. Each egg is cylindrical with the ventral surface flattened and the dorsal surface tapering, creating a sharply pointed apice turned outwards from the major axis at the posterior end (Mattingly 1970).

***Culex erraticus* Larva**

The initial morphological characteristics used in the identification of *Cx. erraticus* were based on larval specimens from Baton Rouge, Louisiana (King and Bradley 1937). The main morphological characteristics used to identify the fourth instar of *Cx. erraticus* are single c-5 and c-6 setae on the head capsule and the siphon tube has 5 or more pairs

of branched setae extending beyond the pectin spines in a uniform manner. The fourth abdominal segment is clear and the antennae are white with both apical and basal ends being dark. The seta 2-S at the end of the siphon is strongly curved. The larva takes on a green color, possibly contributed by feeding on algae, bacteria and other plant matter in the water and even possibly using the green color as a camouflage to blend in with the environment for predatory reasons. Laboratory research has shown that some *Anopheline* mosquitoes have the ability to adapt their color to match the surrounding backgrounds; however, this color change has not been shown to actually take place in the field with *Anopheline* mosquito larva or other genera of mosquitoes (Benedict and Seawright 1987, Fuzeau-Breasch 1972, Seawright et al. 1979).

Culex erraticus is often found cohabiting with *Anopheles quadrimaculatus* Say and other larval mosquito species, including *Uranotaenia sapphirina* Osten and Sacken, An. *punctipennis* Say, *Cx. nigripalpus* Theobald and *Cx. territans* Walker (Horsfall 1972).

Klein et al. (1987) reported that *Cx. erraticus* was found in shallow water levels with grassy margins and other aquatic plants such as *Spirodela polyrhiza* (L.) Schleiden (Arales: Lemnaceae), *Lemna valdiviana* Philippi (Alismatales: Araceae) and *Pistia stratiotes* L. (Arales: Araceae) also known as giant duckweed, small duckweed and water lettuce, respectively. Although *Cx. erraticus* favors aquatic habitats with various vegetations, it has been shown that if the water surface is densely matted with aquatic plants, the increase in some mosquito species, specifically *Cx. erraticus,* can be inhibited (Irby and Apperson 1988, Robertson et al. 1993, Thurman et al. 1945).

***Culex erraticus* Pupa**

The *Cx. erraticus* pupa is one of the smallest in the Culicidae family and has short to moderately long thick trumpets, usually with an index of 5-8, sometimes longer. Seta

9-VII has at least 4 branches. The meatus of the trumpet is thick with much uniformity in width and generally not swollen in apical lower half. The apical margin of the trumpet is truncated. Both seta 8-C and 5-8 is branched; 6-III-VI generally has 3 or 4 branches (King and Bradley 1937). Trumpets are used by mosquitoes to access oxygen at the water surface.

Culex erraticus Adults

Initially the larval stage of *Cx. erraticus* was described from material collected in Baton Rouge, Louisiana, in 1906 (King and Bradley 1937). The adult female was described in 1915 and the male was described in 1917 (King and Bradley 1937).

The adult male *Cx. erraticus* has broad flat scales on the occiput (margin of the eye) and has a patch of 6 or more scales on the mesoepimeron. The female has the same morphological characteristics without the plumose antennae. Both male and female are dark brown in color and prefer to breed in marshlands, lakes, permanent pools, shallow grassy ponds or pond edge including areas that have grassy margins (Robertson et al. 1993). *Culex erraticus* is one of the most common Florida mosquitoes, and it is found in various habitats with aquatic vegetation starting in early summer with highest populations in the summer declining in the early fall (Barber et al. 1924, Carter 1915, Bradley and Fritz 1945, Cupp et al. 2003, Gage 1925, Gartrell et al. 1981, Hess and Crowell 1949, Robertson et al. 1993, TVA 1947).

Veterinary and Medical Importance of *Culex erraticus*

Culex erraticus has been identified in many earlier studies as being mostly an avian feeder, and it is not considered a nuisance to man. Therefore, past research has not considered this mosquito as a dangerous vector. However, this small Culicidae mosquito

may have the potential to be the next multiparasite vector, which should be taken very seriously.

Dirofilaria immitis Leidy, the dog heartworm is estimated to be vectored by as many as 30 different species of mosquitoes including *Cx. erraticus* (Afolabi et al. 1989, Bemrick and Sandholm 1966, Ludlam et al. 1970; Villavaso and Steelman 1970). Human infections are not likely, but there are reported cases (Beaver 1965, Dashiell 1961, Gershwin et al. 1974, Hock et al. 1974, Jones and Meisch 1993, Orihel and Schlotthauer et al. 1969, Navarrette 1972). Humans are considered a dead end host because the larvae do not normally develop into adults, and those that have been found are during autopsies and did not contribute to cause of death (Abadie et al. 1965, Faust 1961, Schlotthauer et al. 1969).

Culex erraticus has been implicated as a possible vector of West Nile Virus because *Cx. erraticus* larvae have been found in high numbers in many of the pools that have tested positive for the disease (Center for Disease Control 2003).

Culex erraticus was incriminated as the vector of the saurian malaria *Plasmodium floridense* Thompson and Huff when it successfully transmitted the malaria organism to an *Anolis carolinensis* lizard by bite under laboratory conditions (Klein et al.1987).

Anolis carolinensis

Anolis carolinensis (Squamata: Iquania: Polychrotidae) Voight, is a small beautifully radiant-green lizard weighing an average of three grams. The length of an *A. carolinensis* averages about 48 millimeters measuring from the snout to the tip of the tail. The average body temperature of an *A. carolinensis* lizard caught in the wild ranges between 27.7 and 32.4 degrees Celsius (ºC) temperature (Greenberg 2002). This temperature range takes into account the time of day when the lizards were caught and

tested, if the lizard was metabolizing its meal, and handling causes stress, which increases the body temperature of the lizards (Greenberg 2002).

Anolis carolinensis is a poikilotherm and uses the green color while basking in the sun to absorb heat to raise its metabolism, aiding in the digestion process of its newly acquired meal. It also uses the green color to signify dominance over competitors (Jenssen, T. A.1977). *Anolis carolinensis* changes to a brown and/or gray color for camouflage purposes while hiding from predators and also while waiting for prey to enter its territory. Males have a brightly colored dewlap that is pink in color, which is extended during aggression or courtship. The brown and/or gray coloration is usually associated with lower body temperatures and the submissive behavior of a dominated lizard (Jenssen et al. 2000).

The Cuban brown anole, *Anolis sagrei* Dumeril and Bibron, is presently out-competing the green anole throughout southern Florida, causing the green anole to spend more time in trees, shrubs, fences and other structures with only a short time to lay their eggs in the soil before returning to the higher elevations (Lovern and Jenssen 2001). *Anolis carolinensis* feed mainly on insects, including adult flies and crickets as well as the larval stages of both (Stamps and Krishnan 1994). Because of heavy predation most members of this species live one or two years in the wild. In domesticated environments, they can often live an average of 4 years with several being reported to have survived for up to 8 years.

Behler and King (1979) studied both species and determined that the competition of the brown anole throughout southern Florida is making the green anole less common in many places and is causing it to be mostly arboreal. Both sexes of the green anole are

very territorial although this quality is reduced in females because the reproductive benefit increases (Jenssen et al. 2000). Orell and Jenssen (2002) reported that green anoles recognize new lizards to their area, which results in aggressive behavior toward new males or courtship displays toward females. Stamps et al. (1997) also found that aggression between strangers helps to maintain the territorial rights of those lizards that have previously established boundaries.

The green anole usually deposits one egg every two weeks starting in the early spring until early fall. The green anole eggs hatch after five to seven weeks, which is considerably slower than the reproductive rate of the Cuban brown anole (Booden 1970). Many species of *Anolis* lizards from around the globe have tested positive for various malarial parasites but the identification of the natural host has eluded researchers for years.

Malaria

Background Information

Malaria is one of the most prevalent diseases that has plagued mankind since the dawn of civilization. Malaria is thought to have originated in Africa some 30 million years ago. Fossils of the *Anopheles* mosquito, the main vector of human malaria, show that the mosquito was present even before the existence of mankind. Early depictions of malaria's effects were recorded on various scrolls of papyrus as tremors caused by chills with accompanying fevers and splenomegaly (swollen spleen). History books are filled with accounts of this mysterious disease that was known as "bad air" (mala aria = Italian) because it was thought that the stagnant waters of marshlands, ponds and swamps produced poisonous gases that brought about the cyclic chills, fever and death. Malaria is caused by a protozoa belonging to the phylum Apicomplexa (Levine 1988). There are

four species of *Plasmodium* that cause malaria in humans, over 450 species of avian malaria and approximately 90 species of saurian malaria (S. R. Telford, personal communication). The development of the *Plasmodium* parasite in the mosquito and in the host is basically the same whether in humans, birds or reptiles.

There are four human malaria species, *Plasmodium vivax, Plasmodium malariae, Plasmodium ovale,* and *Plasmodium falciparum*, which are chiefly vectored by *Anopheles* mosquitoes (Kreier and Baker 1987). The malaria organism requires two hosts to complete its life cycle. The invertebrate (mosquito) is where the *Plasmodium* organism reaches sexual maturity, and the vertebrate host provides the environment where asexual reproduction and multiplication takes place, and male and female gametocytes are produced.

Garnham (1966) refers to the mosquito as the definitive host, and the vertebrate is the intermediate host. Garnham's application of the word "definitive" basically relates directly to the mosquito's ability to serve and provide a final solution, making sure the parasite has the correct environment to fully differentiate and/or develop. The intermediate host is one that is used by the parasitic organism in the course of its life cycle where it can multiply asexually rather than sexually.

When a malaria-infected mosquito with sporozoites takes a blood meal from a host to satisfy the requirements for egg development, an exchange takes place. First, the mosquito inoculates the host with the infective stage known as the sporozoites. Second, if the host has microgametocytes (male) and macrogametocytes (female), they are taken up by the mosquito during the blood meal.

The sporozoites immediately infect the hepatocytes in the liver and mature into schizonts. In *P. falciprum* and *P. malariae*, when the schizonts are mature they will be released from the liver as merozoites. In *P. vivax* and *P. ovale*, the merozoites may remain in the liver in a dormant stage known as hypnozoites and may cause relapse when released into the bloodstream, often time's years later. When the merozoites initially replicate in the liver is termed exoerythrocytic schizogony. The schizont ruptures and the merozoites enter the red blood cells (RBC), which is the erythrocytic cycle. The RBC is where the *Plasmodium* organism undergoes asexual multiplication in the erythrocytes, which is referred to as erythrocytic schizogony. It is here that the merozoites infect RBC and the ring stage, known as trophozoites, matures into schizonts, which rupture, releasing merozoites. At this stage of the parasite life cycle, some parasites differentiate into sexual erythrocytic stages known as gametocytes. It is the erythrocytic stage of the parasite that is responsible for the clinical manifestation of the malaria symptoms.

Once the mosquito has successfully taken its blood meal and sequestered the micro- and macrogametes, the parasite goes through a multiplication cycle referred to as the sporogonic cycle. This cycle is where the microgametes (male) unflagellate and penetrate the macrogametes (female), causing zygotes to form. The zygotes penetrate the midgut wall of the mosquito as ookinetes on the surface of the gut and eventually change into oocysts, which is where the sporozoites mature. When the oocysts rupture, the sporozoites (infective stage) travel to the salivary glands of the mosquito and wait for the next blood meal so the exchange of the sporozoites and gametocytes may take place. The life cycle of the *Plasmodium* organism is basically the same whether in avian or reptilian malaria.

Reptilian Malaria

Wenyon (1909) found the first saurian malarias, *P. mabuiae* Wenyon and *P. agamae* Wenyon, in the common skink, *Mabuya quinquetaneiata* Lichenstein, and the rainbow lizard, *Agama agama* L., from Africa. In Brazil during this same time, Aragao and Neiva (1909) found *P. diploglossi* in *Diploglossus fasciatus* Gray and *P. tropiduri* in *Tropidurus torquatus* Wied. In 1987 there were about 59 species of saurian malaria described (Garnham and Telford 1984, Telford 1982, 1983, 1984a, b; Klein 1987). As of today, there are approximately 90 saurian malarias that are identified and described (S.R.Telford, personal communication).

The scientific community is closer to identifying the natural vectors of some saurian malaria infecting lizards, especially with the work of Klein et al. (1987) who had success with *Cx. erraticus* mosquito transmitting the *Plasmodium* parasite by bite to *A. carolinensis* lizard. Prior to this, Ayala and Lee (1970) suspected *Lutzomyia vexator* Coquillett and *Lutzomyia stewarti* Mangaberia and Galindo as possible vectors of saurian malaria to lizards. Jordan (1964) gave *Aedes canadensis* Theobald, *A. dupreei* Coquillett, *A. infirmatus* Dyar and Knab, *A. atlanticus* Dyar and Knab, *A. triseriatus* Say, *Mansonia perturbans* Walker, *Anopheles crucians, A. punctipennis* Say, *Culex nigripalpus* Theobald, *Cx. territans* Walker, *Cx. restuans* Theobald, *Cx. quinquefasciatus* Say, *Psorophora confinnis* Lunch Arribalzaga, *P. varipes* Colquillett, *P. ciliata* Wiedemann, and *P. ferox* Humbolt the opportunity to feed on infected *Sceloporus* and *Anolis* lizards, and all failed to produce results except for three of the *Culex* spp. that produced oocysts on the mid-gut. Jordan indicated that this was the first step in detecting that mosquitoes may be the true invertebrate host in transmitting *Plasmodium floridense.*

Plasmodium mexicanum Thompson and Huff was reported by Ayala (1970b) and Ayala and Lee (1970) to have gone through the sporogonic development in *L. vexator* and *L. stewarti*. However, the transmission was via artificial inoculation of sporozoites found in the hemocoel and salivary glands from wild-trapped female flies that had previously fed on *Sceloporus occidentalis* Baird and Girard in the laboratory. What this research showed was that the sand flies could successfully develop the malaria parasite through complete sporogony. When the sporozoites were injected into *S. occidentalis*, the lizard became infected.

Thompson (1944) and Thompson and Huff (1944a, b) found that by artificially inoculating noninfected lizards with malaria-infected blood from infected lizards, the development of the parasites *P. mexicanum* and *P. floridense* was slower than those found in mammal and avian malarias. It was several years later that ambient temperature was found to be a key part of the equation in understanding the development of the malaria parasite by inoculating noninfected *A. carolinensis* lizards with temperature regulated citrated blood (Thompson and Winder 1947).

Thompson and Winder found that when *A. carolinensis* was artificially inoculated with parasitized citrated blood and held at 20ºC, the parasitemia has an average peak of 55 days. In comparison, lizards inoculated with the same blood at the same time and held at 30ºC average 13 days. This variance in the number of days for parasitemia between the lizard groups helped to solidify that poikilotherms regulate their internal temperature by the external ambient temperature, which in turn helps to regulate the asynchronous parasite development of the malaria organism.

Goodwin (1951) observed that female *Sceloporus undulatus undulatus* lizards generally stay in the same habitat and do not venture more than 30 feet radius from that habitat. He surmised that if these lizards remained in their respective habitats, that studying the malaria infection rates in a population could be done through the course of individual infections under natural conditions.

Goodwin (1951) marked each specimen by clipping a combination of toes and performing a blood smear in the field. Goodwin found at the end of the study that infections were observed in the adult lizards each month, but the hatchlings of the present year studied did not occur until November. Goodwin also noted that more lizards were infected during the fall and winter months than during other months. The specimens that Goodwin observed in the laboratory developed natural parasitemia in about two weeks. These observations suggested that *Sc. undulatus* had a long prepatent period interaction with *P. floridense*.

Thompson and Huff (1944) described the exoerythrocytic schizogony in detail and showed that later stages were common in a variety of haemopoietic cells and in the endothelium. Thompson and Huff also found that infected lizards have exoerythrocytic schizonts present in peripheral blood and other cells including myelocytes, lymphocytes, monocytes, macrophages and thrombocytes. Thompson and Huff used *P. mexicanum* as the first saurian malaria parasite to describe the exoerythrocytic stage. It was also shown that mature schizonts can be found in tissue or organ smears from the bone marrow, spleen, lungs, kidney, heart, endothelial cells in subcutaneous tissue, and between the heart muscle fibers.

Merozoites that continue to develop with a third generation of schizogony in fixed tissue of the host are called phanerozoites. These specialized stages of malaria produce merozoites that can either invade circulating erythrocytes (red blood cells) or reinvade endothelial cells to continue more generations of schizogony in fixed tissues (Garnham 1966). Phanerozoites were found to occur in naturally infected birds with certain avian malaria (Corradetti 1955). Telford (1998) found phanerozoites of the Japanese saurian malaria parasite, *Plasmodium sasai* Telford and Ball. Telford (1996) found phanerozoites present when he performed histological examinations on 422 hearts of the Japanese lacertids, *Takydromus tachydromoides* Schlegel.

Klein et al. (1987) blood fed *Cx. erraticus* mosquitoes on *P. floridense* infected *A. carolinensis* lizards, and then by mosquito bite transmitted the malaria organism to a noninfected *A. carolinensis* lizard. This was the first successful transmission of *P. floridense* by bite of a mosquito to a lizard. Now it is important to follow the preerythrocytic stage of this saurian malaria and where it establishes itself prior to entering the red blood cells to complete its development in the host.

Quoting Garnham (1966) "*P. floridense* rarely multiplies sufficiently and extensively in the lizard's blood to produce symptoms, and the anaemia found in experimental infections is due more to frequent bleedings than to the disease." It is presumed that most lizards die after high parasitemia, especially after relapses, and this may be the case with *A. carolinensis*, but this has not been documented as of this time (Huff and Marchbank 1953).

This research study will concentrate on four objectives. (1) Trapping wild *Cx. erraticus* mosquitoes and bringing them into the laboratory and establishing a large and

stable population through artificial nutrition. (2) Evaluate whether *Cx. erraticus* has a preference in selecting *A. carolinensis* lizard infected with *P. floridense* malaria over noninfected lizards. (3) Allow *Cx. erraticus* mosquitoes to become infected with *P. floridense* by feeding on infected *A. carolinensis* lizards. (4) Allow the infected mosquitoes to feed on noninfective lizards and track the post blood feeding development of phanerozoites of the *Plasmodium floridense* (Eucoccidiorida: Plasmodidae) in the tissues of euthanized *A. carolinensis* (Squamata: Iguanidae) green anole.

CHAPTER 2
LABORATORY REARING OF *Culex erraticus*

Introduction

Culex (Melanoconion) erraticus Dyar and Knab is one of approximately 250 species of mosquitoes in the subgenus *Melanoconion* (Pecor et al. 1992, Peyton and Harbach 1991). *Culex erraticus* is found in many parts of the world, including the southeastern United States especially in all areas of Florida (Darsie and Ward 2005, Reinert 2001).

In 1972, several species in the subgenus *Melanoconion* were incriminated as possible vectors of arboviruses. Adames and Galindo (1972) believed it was important that more species of *Melanoconion* mosquitoes should be colonized so experimental transmission work can be perform with these possible vectors. Adames and Galindo (1972) only found published literature on three species of *Melanoconion* mosquitoes that were successfully colonized under laboratory conditions at that time. Takahashi (1968) colonized *Culex portesi* Senevet and Abonnenc at the Trinidad Regional Virus Laboratory. Hair (1968) colonized *Cx. cedecci* Stone and Hair at the Communicable Disease Center and *Cx. peccator* Dyar and Knab was colonized by Chapman and Barr (1969) at Lake Charles, Louisiana. However, the previous three *Melanoconion* mosquitoes did not have population strengths that allowed them to be used in the arbovirus transmission experiments. Very few *Melanoconion* mosquitoes have been successfully colonized, of those that have significant laboratory bionomic information on rearing techniques has been gained (Chadee and Tikasingh 1985, Hair 1968). Some

species of *Melanoconion* have not been reared successfully in the laboratory, but they have been reared successfully in the field.

In 1948, a new species of *Melanoconion* was collected from limestone solution holes in the Florida Keys and was identified as *Cx. mulrennani* (Basham 1948). Rearing the larva in the laboratory was unsuccessful, but rearing was accomplished in the field by placing rearing containers over the lime rock holes, allowing the adults to emerge in their natural habitat. Sampling of the larvae from the holes determined that the fourth instar ranged from one to two days and the pupa lasted approximately 14 to 18 hours. It was also noted that many larvae were acquired from the lime rock habitat, but collection of adults in the field other than rearing by covering the holes, has been rare.

Hair (1968) stated, "at the present state of our knowledge, the adult females of this subgenus (*Melanoconion*) cannot be identified with certainty; the specific identification being dependent upon characteristics of the larvae and male terminalia. Therefore, precise knowledge of vector relationships is difficult to obtain".

Usually mosquito species in a particular genus share similar life cycle strategies, including preoviposition time period, egg types (laid singly or as rafts), places of oviposition, larval, pupal and adult development. However, in the subgenus *Melanoconion* within the small number of species successfully reared in laboratory conditions, a broad spectrum of variations in these life cycle strategies are found, making adapting rearing techniques difficult from one species to the next (Adames and Galindo 1972, Chadee and Tiaksingh1985, Chapman and Barr 1969, Davies and Martinez 1970, Dziem and Cupp 1983, Hair 1968, Takahashi 1968a, b).

Larval diets are important for the survival and fitness of the larva to progress through its stages and to survive as a pupa while going through the necessary physiological and morphological changes in order to eclose as an adult (Larsen and Bodenstein 1959). The most common larval nutrients used in rearing various species of mosquitoes are Tetramin® Tropical Flakes Fish Food, hog chow pellets, liver powder, brewers yeast, laboratory chow, pulverized guinea pig chow, daphnia, ground mosquito larvae, powdered instant milk, dried blood meal, blood fibrin, yeast extract and Purina Dog Chow (Adames and Galindo 1972, Bradshaw and Lounibos 1972, Chadee and Tikasingh 1985, Evans and Brust 1972, Fish and Hall 1978, Istock et al. 1975, Lang 1978, Lillie et al. 1980, O'Meara et al. 1981, Smith and Brust 1971). The nutrition for the adults is just as important as that for the larvae because the blood meal sequestered by mosquitoes can affect egg maturation (Clements 1963, Lavoipierre 1961, Roy 1936, Shelton 1972).

Developmental times of most mosquito larvae in the subgenus *Culex* compared to mosquitoes in the subgenus *Melanoconion* is reported to be considerably less (Takahashi 1968a). *Culex inflictus* Theobald and *Culex nigripalpus* Theobald larvae averages seven days; *Culex (Eubonnea) amazonensis* Lutz larval development averages nine days.

In the subgenus *Melanoconion, Culex caudelli* Dyar and Knab, *Culex pilosus* Dyar and Knab, *Culex alogistus* Dyar, *Culex hesitator* Dyar and Knab, *Culex aikenii* Aiken and Rowland, and *Culex vexillifer* Komp oviposited single eggs on moist surfaces or on surfaces just above the water line instead of egg rafts (Adames and Galindo 1972, Chadee and Tikasingh 1985). Chadee and Tikasingh (1985) showed that *Cx. caudelli* deposited single eggs on the surface of the water and on the moist surface of inverted clay flower

pots. Hair (1968) reported that *Cx. pilosus* preferred soft, moist mud on which to lay its single eggs. *Culex portesi* prefer to lay their egg rafts on the water surface, while *Cx. peccator* prefer to lay their egg rafts on the water surface and above the water line on moist paper towels (Chapman and Barr 1969, Osgood 1971, Takahashi 1968a).

Takahashi (1968a) showed that *Cx. portesi* had maximum egg production one week following the blood meal, with the shape of the egg rafts being elongate instead of subcircular as found in many species of the subgenus *Culex*. Takahaski also found that the mean number of eggs in an egg raft for *Cx. portesi* was 56 and the development time averaged approximately two days. Takahashi reported that the mechanical aeration of the rainwater used to rear the larvae caused a high mortality soon after the larvae hatched from the eggs, so the aeration was removed. Rearing was accomplished in an outdoor insectary without artificial lighting. The diet regime for the larvae consisted of two grams of powdered brewers yeast. The life cycle of *Cx. portesi* was reported as one to five days for egg development, 14 days larval development, three days for pupa, and no data is present for the life span of the adults. Takahashi observed that as the number of egg rafts increased under the clay flowerpots, the more often egg rafts were often to be oviposited. This is clearly an indication of support for the role of ovipositional cues. It took several years to successfully colonize 25 generations of *Cx. portesi* with evidence of large numbers.

Hair (1968) began rearing *Cx. cedecei* in 1966 in the laboratory and maintained the colony for nine months with only six generations. Earlier, Hair reared *Cx. pilosus* for three generations before losing the colony due to the failure of a suitable larval diet. Hair compared the developmental rates of *Cx. cedecei* to *Cx. pilosus* and found the following.

Culex cedecei adult female was five to 10 days old before taking the first blood meal compared to one day for *Cx. pilosus*. *Culex cedecei* and *Cx. pilosus* adult females were both two to five days old before mating. The preovipositional incubation time for *Cx. cedecei* averaged 30 days and *Cx. pilosus* averaged 21 days. The egg raft *Cx. cedecei* was oval in shape, was not pointed at the apex, and averaged 85 eggs compared to *Cx. pilosus* having a long narrow raft with an average of 75 eggs. Egg development was the same for both species, but it was observed that the eggs of *Cx. pilosus* could withstand a short desiccation period of two to three days. This desiccation period was validated by King et al. (1960) during another laboratory rearing procedure for this mosquito species. The larval development of *Cx. cedecei* was 11 to 20 days with an average of 15 days in comparison to nine to15 days with an average of 11 for *Cx. pilosus*. Both species of mosquitoes were fed a larval diet of yeast mixed with dry dog food and water augmented with aurcomycin (tetracycline) in the water. The pupal duration for both species averaged three days. However, the average life span for the adult female *Cx. cedecei* was 90 days, and 75 days for *Cx. pilosus*. Adult female *Cx. cedecei* were initially exposed to hay infusions as oviposition sites, but it was discovered that beakers of tap water (nonchlorinated) with exuviae from previous pupa were just as effective as oviposition attractants.

Many of the *Melanoconion* mosquitoes, specifically *Cx. erraticus*, are considered to be the most common but least studied mosquitoes. They are found in many grassy, shallow margin fresh water aquatic habitats. Therefore, identifying the length of time that the egg, larval, pupal and adults remain in each stage is important for understanding their life cycle and why some species of the *Melanoconion* are considered by some researchers

to be mostly a nuisance to humans, and while others incriminate them as excellent vectors of viruses, protozoans and filarial worms.

The egg, larva, pupa and adult stages of *Cx. erraticus* have been previously identified and published as being colonized (Nayar et al. 1981). However, there is no published information on the materials and methods, or the number of eggs, larva, pupa and adults produced in the life cycle, which is important for understanding this mosquito's role in the habitat as well as the possibility of it being a vector of disease organisms to man, animals and reptiles.

The first objective of this study is to determine the length of each stage in the life cycle of *Cx. erraticus* in a laboratory controlled setting. Determining the length of each stage aids in better understanding of the behaviors for each stage in the life cycle as well as the feeding activity of the larval and adult stages of this mosquito.

The second objective is to establish a large and stable colony of *Cx. erraticus* adults, specifically females, that will be required (n=160) for olfaction trials and a large number malaria vector competent. Mosquitoes are also required to examine the salivary glands after feeding on malaria infected *Anolis carolinensis* Voight lizards.

Materials and Methods

The rearing procedure used in this study for colonizing *Cx. erraticus* is based on a prior established protocol (Klein 1985) as well as attempts on related species found in the literature. The Institutional Animal Care and Use Committees (IACUC) now regulate the use of animals in laboratory-controlled environments, requiring several modifications to Klein's protocol before this research was approved by IACUC.

Klein (1985) fed his mosquitoes on baby chickens (chicks) to promote egg development. IACUC prohibited baby chicks for feeding purposes during this study so

bovine (cow) blood treated with sodium citrate to reduce clotting and antibiotics to minimize spoilage by bacteria was used. Also, IACUC would not approve the use of host (lizard) baited traps as used by Klein in 1985 for collecting mosquitoes in the wild, so modified CDC light traps baited with CO_2 were initially used in this study with the addition of lizard extract during the actual trapping study.

Modified CDC Light Trap

A Center for Disease Control (CDC) light trap (John W. Hock Company, model 512, Gainesville, FL) was modified using four blue LED (470 nm ± 50 nm, 800 mcd, 22º [Panasonic,® Digikey Corp., Thief River Falls, MN]) and one green LED (567 nm ± 50 nm, 2400 mcd, 8º [Toshiba Tosbright,® Martech Optoelectronics, Latham, NY]). The trap was powered by four alkaline D cell batteries at 6.4 ± 0.4 volts and, to insure each LED received the same amount of power four 180 ohm resistors were soldered in series. Carbon dioxide squat #20 cylinders equipped with a two-stage regulator (Victor Equipment Company, model VTS 453B-320, Denton, TX) and a flow meter (Gilmont Instruments, no. 12, Great Neck, NY) to maintain 250 ml/min gas flow. The carbon dioxide was transported through a 1 m piece of tygon tubing with a ¼ inch inside diameter attached to the top edge of the trap with duct tape. A catch basket made of screen netting was attached to the bottom of the trap to collect the live mosquitoes as they were vacuumed in by the fan motor. A 10% sugar solution with a wick made of filter paper was suspended in the trap to keep the mosquitoes alive.

Lizard Extract

Lizard extract (LE) as an attractant was made from colony deceased *Anolis carolinensis* lizards soaked for 24 hours in 100 ml of filtered hexane. The 100 ml of

lizard wash was concentrated to 10 ml with 5 ml being placed in a 5 ml Accuform Manufacturing microvial graduated screw thread vial with open-top closure with an outside diameter of 21 mm, length of 62 mm, and GPI thread size of 20-400. A hole 0.75 cm was cut into the top of the lid to accommodate an Interflo polyethylene pellet (Hydrophobic Rod Part #p375-3, Formulation #F/N: 35-162-4 from Interflo Technologies, Brooklyn, NY) measuring 1 cm diameter by 1 cm in height to regulate attractant output in CDC traps. This 5 ml graduated vial and polyethylene pellet dispensed the LE at a rate of 0.03 ml/hr for a total of 4.5 ml during a 12 hour trapping regime.

***Culex erraticus* Colony**

The *Cx. erraticus* trapped in the modified CDC light traps were brought into the laboratory, identified and placed in a holding cage. A 100 ml vial containing a 10% sugar solution with a wick made from filter paper was placed in each cage to provide hydration and energy source for the general population of wild mosquitoes as well as future generations of colonized mosquitoes. A container of water with several Styrofoam floats for resting purposes was provided in each wild and colony reared mosquito cage for ovipositing purposes. Bovine (cow) blood (3.8 liters) was treated with sodium citrate (12 grams) and antibiotics were used to blood feed the wild adult mosquitoes and the colony reared adult mosquitoes for egg development. A sterile four-layer gauze pad soaked in bovine blood was placed on the ovipositing cage containing adult *Cx. erraticus* mosquitoes for feeding. Adult mosquito cages were placed in a Percival Growth Chamber at 27ºC and 80% relative humidity with 16:8 light dark photo period.

Both wild and colony cages were checked daily for *Cx. erraticus* egg rafts. The egg rafts were removed and placed in a petri dish containing deionized water and

examined and counted under a dissecting microscope. After being counted, the egg rafts were put in enamel larval rearing pans (30 x 18 x 6 cm) with deionized water. Four mosquito larval diets were evaluated during this study. Tetramin® Tropical Flakes Fish Food (FF), Tetramin® Tropical Flakes Fish Food combined with brewers yeast (FFBY) in a 1g:1g ratio, Tetramin® Tropical Flakes Fish Food combined with powdered milk (FFPM) 1g:1g ration, and Tetramin® Tropical Flakes Fish Food combined with powdered milk and brewers yeast (FFPMBY) in a 1g:1g:1g ratio. An equal amount of 2 mg larval diet was placed in each larval rearing pan every day. Daily counting was performed on the number of live larvae, larvae that transform into live pupae, pupae into adults as well as how many adults were male and female. The length of time in days for each life cycle completion was measured and analyzed.

Experimental Colonies

Generation adult males (n=150) and females (n=106) reared on Tetramin® Tropical Flakes Fish Food (FF) as larvae were placed in rearing cages as described previously. Generation two started with 76 males and 53 females. Generation three started with six males and five females.

Tetramin® Tropical Flakes Fish Food combined with powdered milk started generation one with 66 males and 44 females. Generation two started with 10 males and 6 females. This larval diet did not produce a third generation.

Tetramin® Tropical Flakes Fish Food combined with brewers yeast started generation one with 62 males and 37 females. Generation two had 204 males and 149 females. Generation three started with 76 males and 57 females.

Tetramin® Tropical Flakes Fish Food combined with powdered milk and brewers yeast started generation one with 84 males and 57 females. Generation two started with 80 males and 76 females. Generation three started with 19 males and 15 females.

Statistical Analysis

A general linear model (GLM) for analysis of variance (SAS Institute, 2001) comparing the means of the four different larval diet treatments, the generations that occurred and comparison of the treatments to the generations for the number of eggs to larvae, eggs to pupae, eggs to adults, eggs to males, eggs to females, larvae to pupae, larvae to adults, larvae to males, larvae to females, pupae to adults, pupae to males, and pupae to females. Significance was set at 95% confidence limits (≤ 0.05). A 1-tailed *t*-test ($\alpha = 0.05$) was used for comparison between the four diet treatments and the generations.

Results

Summary of Life Stages Data

Table 2-1 shows the data for the colony rearing process during this study. The summary of data for the mean percent values shown in the graphs of this chapter is found in Appendix A.

Colony Egg Description

Culex erraticus eggs were deposited in rafts with a curved base for flotation that had double or triple zigzagged rows (Figure 2-1). There is a very small inconspicuous corolla on the anterior end that has an elevated floor to the egg. The anterior end of each egg also has an area that is sclerotized and translucent. Each egg is cylindrical with the ventral surface flattened and the dorsal surface tapering, creating a sharp pointed apice

turned outward from the major axis at the posterior end (Figure 2-2) (Mattingly 1970, 1976).

Table 2-1. Summary of data for the life stages of *Culex erraticus* mosquito colony

Diet/Gen	No. of Egg Rafts	No. of Eggs	Eggs/Raft Mean	No. of Larvae	No. of Pupae	No. of Adults	No. of Males	No. of Females
FF1	20	1938	97	1734	801	256	150	106
FF2	13	1149	88	970	436	129	76	53
FF3	3	181	60	127	50	11	6	5
FFBY1	16	1009	63	950	326	104	62	37
FFBY2	4	1508	377	1383	878	353	204	149
FFBY3	12	462	39	445	303	135	78	57
FFPM1	19	1328	70	1227	533	110	66	44
FFPM2	5	233	47	199	105	16	10	6
FFPMBY1	18	1521	85	1374	572	141	84	57
FFPMBY2	13	894	69	794	518	156	80	76
FFPMBY3	2	136	68	124	82	34	19	15
Totals:	**125**	**10359**	**97**	**9327**	**4604**	**1445**	**835**	**605**

Figure 2-1. *Culex erraticus* egg raft showing two and three uneven rows. Eggs darken in color within several hours after being oviposited. Egg raft broke apart when photo was being taken.

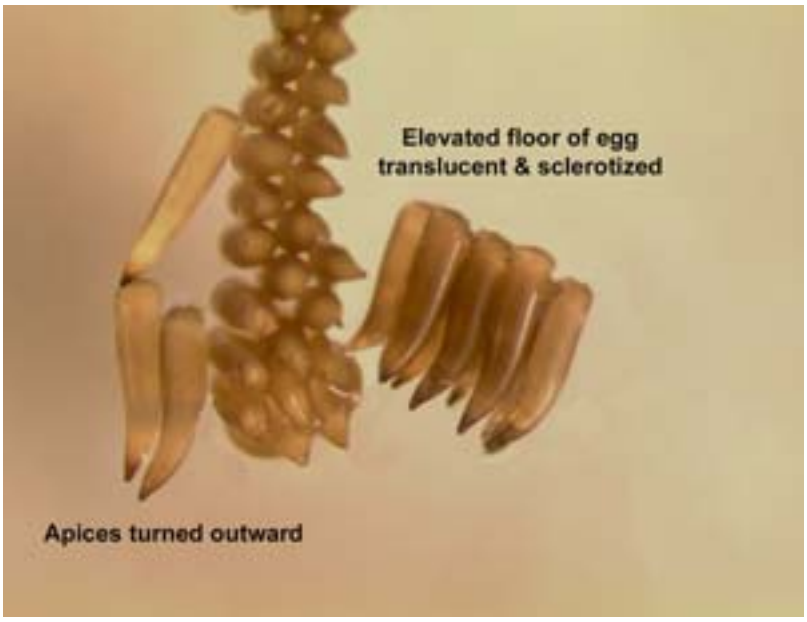


Figure 2-2. *Culex erraticus* eggs showing the sharp point of the apex curving outward away from the major axis of the raft. The anterior part of the egg has a circumference that is translucent and sclerotized.

Tetramin® Tropical Flakes Fish Food Colony Produced Eggs

A comparison of the mean number of *Cx. erraticus* eggs oviposited from the fish food generation one mosquitoes to the mean number of eggs produced in generation two and generation three resulted in a significant difference ($F=2.60$; $df=2$; $p=0.048$) (Table 2-2). Generation one produced 20 egg rafts for a total number of 1,938 eggs averaging 96.9 eggs per raft. Generation two mosquitoes ovipositioned a total of 1,149 eggs in 13 egg rafts averaging 88.4 eggs per raft. The total number of 181eggs produced by generation three adults in three rafts averaged 60.3 eggs per raft. A significant reduction in the total numbers of eggs and mean number of eggs took place in the Tetramin® Tropical Flakes Fish Food (Figure 2-3). The average development time for the eggs to hatch was 2.5 ± 1.0 days for all generations.

Table 2-2. Analysis of variance comparing the number of eggs oviposited between the three generations of *Culex erraticus* mosquitoes in colony while in the larval stage being exposed to Tetramin® Tropical Flakes Fish Food.

ANOVA Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3604.68	2	1802.34	2.60	0.048	3.28
Within Groups	22849.54	33	692.41			
Total	26454.22	35				

Tetramin® Tropical Flakes Fish Food and Brewers Yeast Colony Produced Eggs

Combining the Tetramin® Tropical Flakes Fish Food and brewers yeast (FFBY) did not produce a significant difference (F=0.57; df=2; p=0.57) in the mean number of eggs produced per raft within the three generations of *Cx. erraticus* mosquitoes (Table 2-3). The 12 egg rafts produced in generation one yielded a total number of 1,009 eggs with a mean of 84 eggs per raft.

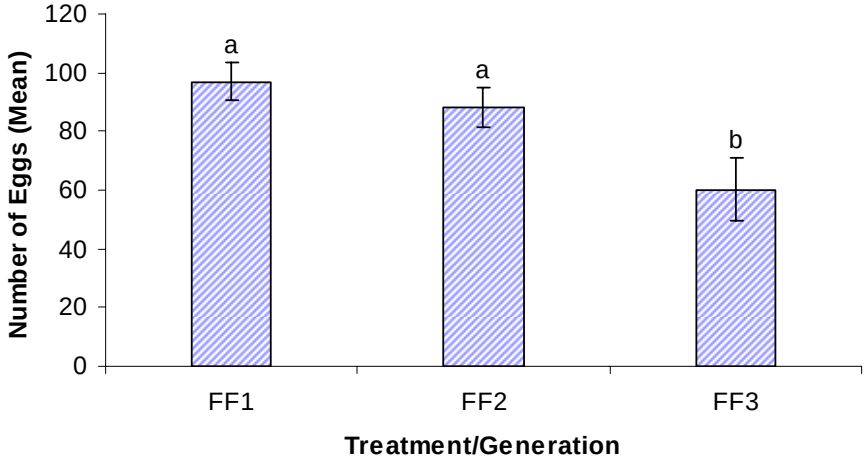


Figure 2-3. Comparison between the three generations of mean numbers of eggs per raft oviposited by *Culex erraticus* while larval stages were fed Tetramin® Tropical Flakes Fish Food only.

Generation two had an increase of 19 egg rafts, resulting in 1,508 eggs for a mean of 79 eggs per raft. The number of egg rafts in generation three decreased to five with a

total of 462 eggs giving a mean of 92.4 eggs per raft (Figure 2-4). It took 1.5 ± 1.0 day for the eggs to hatch.

Table 2-3. Analysis of variance comparing the number of oviposited eggs by *Culex erraticus* mosquitoes in three generations while exposed as larvae to Tetramin® Tropical Flakes Fish Food and brewers yeast diet.

ANOVA Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	704.21	2	352.11	0.57	0.57	3.28
Within Groups	20454.5	33	619.83			
Total	21158.8	35				

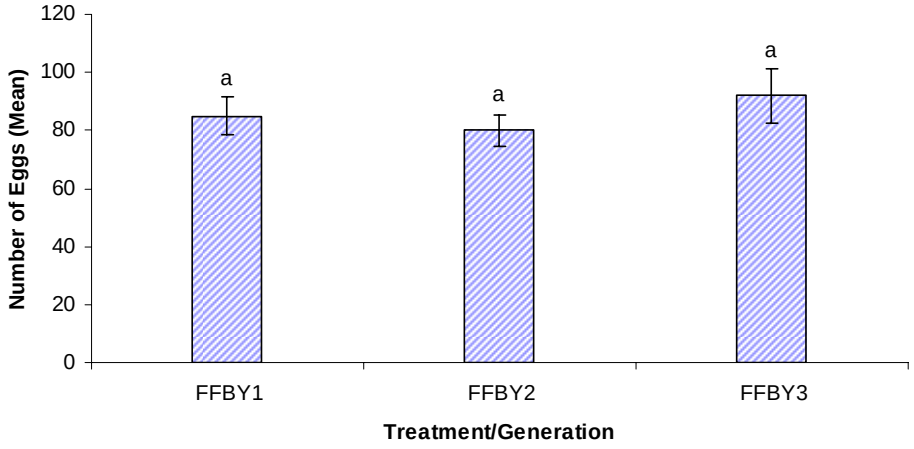


Figure 2-4. Comparison between the three generations of mean numbers of eggs oviposited by *Culex erraticus*, while larval stages were fed Tetramin® Tropical Flakes Fish Food combined with brewers yeast.

Tetramin® Tropical Flakes Fish Food and Powdered Milk Colony Produced Eggs

Combining the powdered milk with the fish food (FFPM) resulted in a significant difference (F=4.17; df=1; p=0.056) in the number of eggs per raft (Table 2-4). There were 16 egg rafts produced in generation one with a total of 1,328 eggs, resulting in a mean of 83 eggs per raft. However, generation two adults only produced four egg rafts with a total of 233 eggs, giving a mean of 58.25 eggs per raft. It is statistically correct to address the data in this treatment as approaching significance because the p-value is in a

sensitive area, being rounded up to the next number. The results in this treatment would not be numerically affected by being significant or not based on the numbers in the data (Figure 2-5). The egg development time was 2.8 ± 1.5 days.

Table 2-4. Analysis of variance of the number of eggs produced by the adult *Culex erraticus* in three generations while the larvae fed on the diet of Tetramin® Tropical Flakes Fish Food and powdered milk.

ANOVA Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1960.20	1	1960.20	4.17	0.056	4.41
Within Groups	8458.75	18	469.93			
Total	10418.95	19				

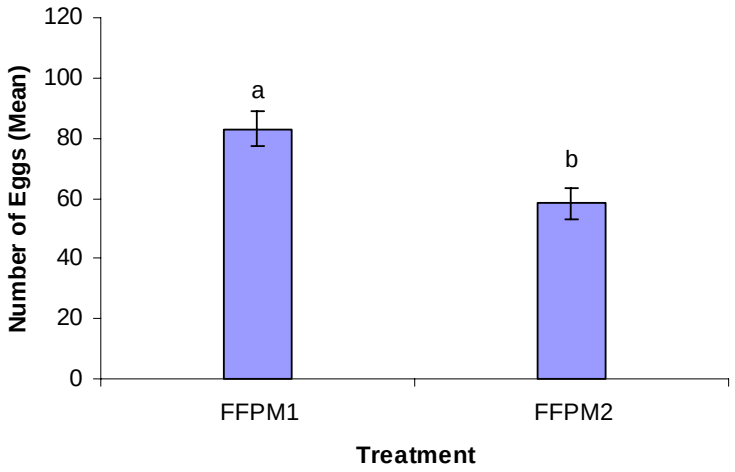


Figure 2-5. Comparison between the two generations of mean numbers of eggs oviposited by *Culex erraticus* while larva stages were fed Tetramin® Tropical Flakes Fish Food combined with powdered milk.

Tetramin® Tropical Flakes Fish Food, Powdered Milk and Brewer's Yeast Colony Produced Eggs

The Tetramin® Tropical Flakes Fish Food combined with the powdered milk and brewers yeast (FFPMBY) did not produce a significant difference (F=1.94; df=2; p=0.16) in the average number of eggs within the three generations (Table 2-5). The total numbers of eggs in generation one was 1,558 in 18 egg rafts with an average of 86.5 eggs per raft. In generation two there was a decline in the total number of eggs to 894 with a

mean of 68.7 eggs in 13 rafts. Generation three produced 136 eggs in two rafts with a mean of 68 eggs per raft. Comparing generation one, two and three showed that the mean number of eggs per raft decreased by 18.7 eggs (Figure 2-6). The total number of eggs also declined significantly. The time it took the eggs to hatch was 2.2 days ± 1.3 days.

Table 2-5. Analysis of variance comparing the number of eggs between the three generations in the Tetramin® Tropical Flakes Fish Food combined with powdered milk and brewers yeast diet.

ANOVA Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2619.31	2	1309.65	1.94	0.16	3.32
Within Groups	20290.75	30	676.36			
Total	22910.06	32				

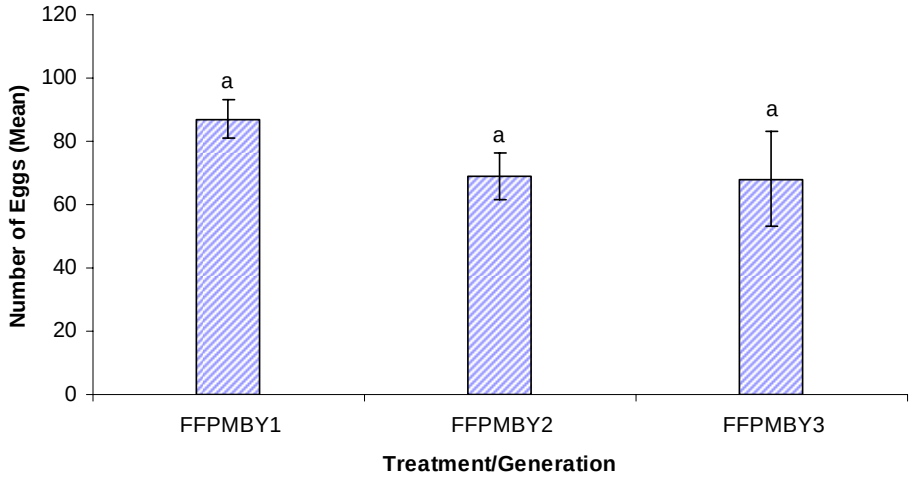


Figure 2-6. Comparison of the mean number of eggs between the three generations of Tetramin® Tropical Flakes Fish Food combined with powdered milk and brewer's yeast diet. Bars with the same letter are not statistically significant.

Comparison of Diets to Generation of Eggs

Even though there were significant differences found in three of the individual diets, overall the model did not indicate a significant difference (F=1.63; df=3; p=0.19) for the mean number of eggs (Table 2-6). However, there was a significance found in the

mean number of eggs within each generation of each diet (F=4.19; df=2; p=0.02) (Table 2-4). The FFBY was the only diet that did not show significance with a decrease as in the other three diets. The FFBY generations one and two remained stable with a slight increase in generation three. As far as the interaction between diets to generation, the model did not produce a significant difference (F=1.33; df=5; p=0.2575) (Table 2-6) (Figure 2-7).

Table 2-6. Analysis of variance comparing the mean number of eggs among the diets and generations.

ANOVA Source of Variation	SS	df	MS	F	Pr > F
Diets	3099.56	3	1033.19	1.63	0.19
Generation	5297.34	2	2648.67	4.19	0.0175
Diets per Generation	4195.61	5	839.123	1.33	0.2575

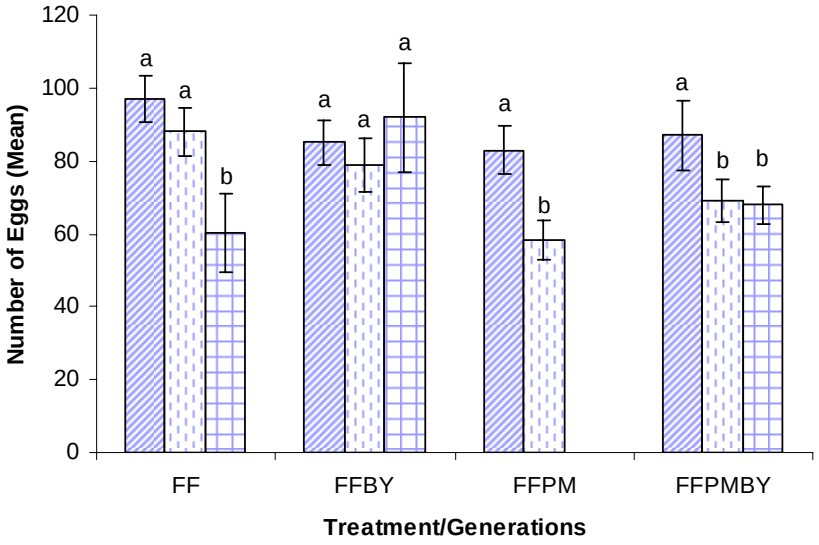


Figure 2-7. Comparison of diet to each generation of *Culex erraticus* eggs. The striped bars are generation one, dotted bars are generation two and plaid bars are generation three. Bars with the same letters are not significantly different.

Colony Larva Description

Culex erraticus larvae are green in color with the basal portion of the antennae white and the apical portion dark (Figure 2-8). The siphon usually has at least five pairs, sometimes six, of multiple hair tufts extending beyond the pectin spines (Figure 2-9). The comb plate located on the eighth abdominal segment averages 18 scales with the minimum being 11 and the maximum being 29 (Figure 2-9). The scales are arranged in a single row with part of the scales appearing to be in double rows. The C5 and C6 head hairs are long and single, but are not seen on the photographs below (Darsie and Ward 2005).

Figure 2-8. *Culex erraticus* larva is green in color with dark color on the antennae and white at the basal portion.

Eggs to Larvae Comparison of Diets

A significant difference was found in the total number of eggs that hatched into larvae among the four diets ($F= 4.05$; $df= 3, 114$; $p= 0.009$). The significance as indicated by the statistical model occurred between the mean percent of the FF diet ($\bar{x}=85$) and the FFBY diet ($\bar{x}=92$) (Figure 2-10).

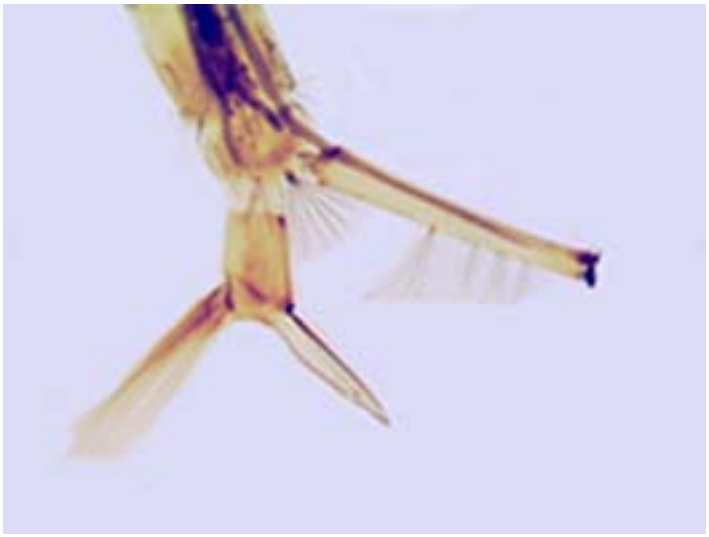


Figure 2-9. Siphon with five to six pairs of tufts.

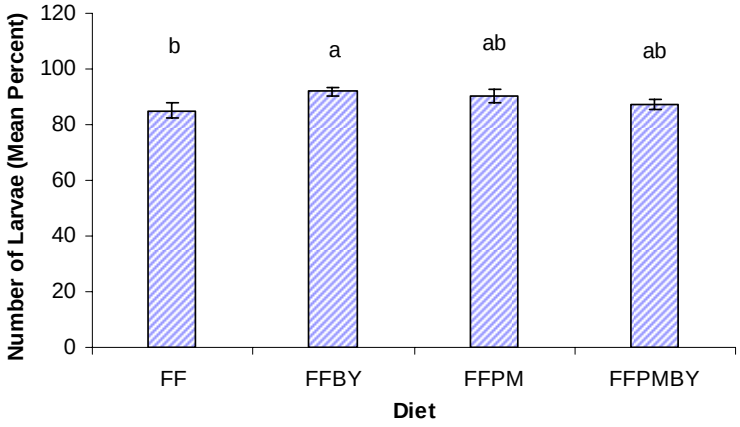


Figure 2-10. The number of eggs that developed into larvae in the four larval diets Tetramin® Tropical Flakes Fish Food, Tetramin® Tropical Flakes Fish Food and brewers yeast, Tetramin® Tropical Flakes Fish Food combined with powdered milk, and Tetramin® Tropical Flakes Fish Food combined with powdered milk and brewer's yeast. Diets with the same letters are not significantly different. The mean percent of larvae that developed into live larvae for Tetramin® Tropical Flakes Fish Food = 85 and Tetramin® Tropical Flakes Fish Food and brewers yeast =92.

Eggs to Larvae Comparison of Generations

When comparing the mean percent of the three generations of eggs developing into larvae the statistical model found no significant difference ($F= 1.02$; $df= 2, 114$; $p= 0.36$) (Fig. 2-11). Although the total number of eggs in generations one (n=5850) and two

(n=3784) were greater than those in generation three (n=779), the mean percent number of eggs that hatched into larvae were not significantly different (Figure 2-11).

Eggs to Larvae Comparison of Diets to Generations

No significant difference (F= 1.05; df= 5, 114; p= 0.39) was found comparing diets to the generations for the mean percent of eggs developing into larvae (Fig. 2-12). However, there was a difference in the FF diet with the mean percent of eggs developing into larvae between generations one and three.

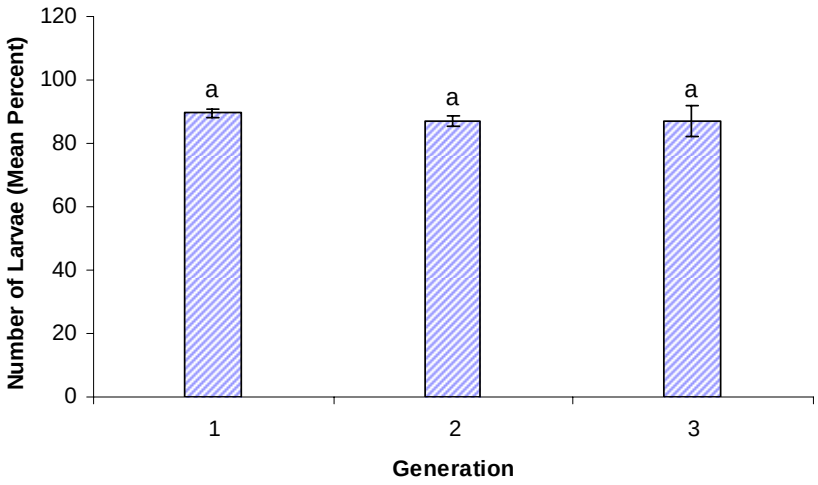


Figure 2-11. All three laboratory generations have the same letter showing no significant difference for the mean percent of eggs developing into live larvae.

Colony Pupa

The *Culex erraticus* pupa is green in color with broad, transparent paddles at the end of the abdomen, which are used for locomotion. Figure 2-13 shows trumpets in the downward position when pupa is below the water. Figure 2-14 shows trumpets in the up position when making contact with water surface for oxygen consumption.

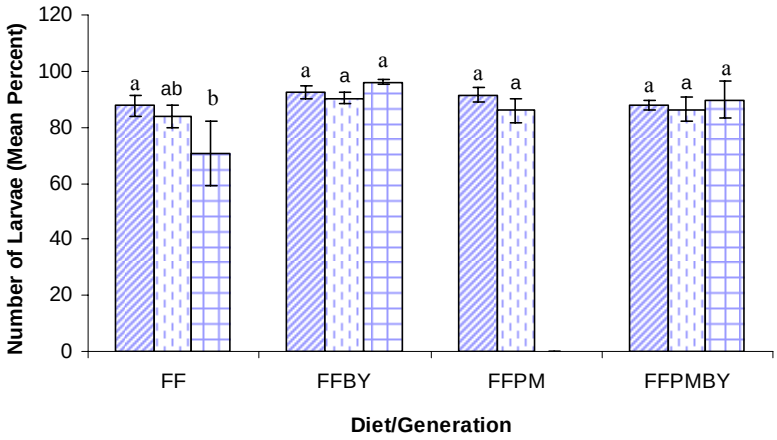


Figure 2-12. Comparison of eggs developing into larvae with Tetramin® Tropical Flakes Fish Food. There was a significant decrease in the mean percent of eggs that developed into larvae between generations one to three. Bars with the same letters are not statistically different.

Figure 2-13. *Culex erraticus* pupa has relaxed abdomen and trumpets in downward position.

Eggs to Pupae Comparison of Treatments

Significance was found in the number of eggs that developed into pupae between the four diet regimes (F= 4.94, df= 3, 114; p=0.003). The FF had a lower mean percent (n=39.2) of pupa that developed into larvae than the FFBY (n=48.7) and the FFPMBY

(n=46.8). However, the two diets with brewers yeast FFBY and the FFPMBY are not significantly different (Fig. 2-15).

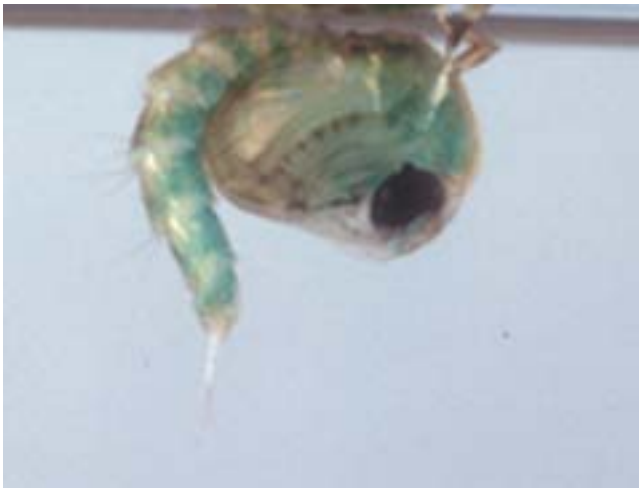


Figure 2-14. Culex erraticus pupa is curled when trumpets are in contact with surface for oxygen consumption.

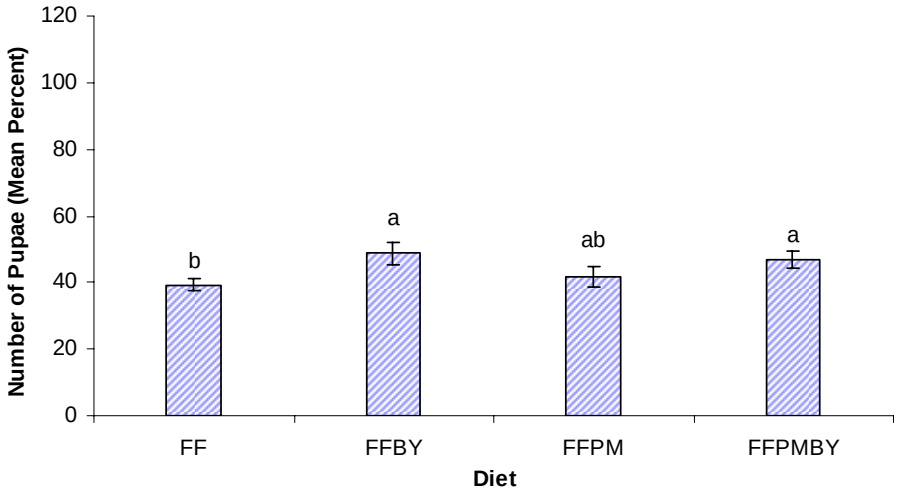


Figure 2-15. The number of eggs developing into pupae show a significant difference between diets Tetramin® Tropical Flakes Fish Food, Tetramin® Tropical Flakes Fish Food and brewers yeast and between diet Tetramin® Tropical Flakes Fish Food and Tetramin® Tropical Flakes Fish Food combined with powdered milk and brewers yeast.

Eggs to Pupae Comparison of Generations

A significant difference was found ($F=7.47$; $df= 2$; $p=0.0009$) in the mean percent number of eggs that developed into larvae. Generation one had a mean percent of 38.9

pupae compared to generation two producing a mean percent of 50.1 and generation three with a mean percent of 51.8. Generation one had a total of 5,833 eggs compared to 3,784 for generation two and 779 eggs for generation three. Even though the number of eggs declined in the three generations there were significantly more pupa development in generation two and three (Figure 2-16).

Eggs to Pupae Comparison of Diets to Generations

In the overall model comparing diets to generations, there was a significant difference ($F=4.64$; $df=5$; $p=0.0007$) found. FF did not produce a significant difference among the three decreasing generations. FFBY produced a significant difference among generation one and both generations two and three, but no difference was found in the number of eggs that made it to the pupa stage between generations two and three. FFPM did not produce a significant difference in generations one or two and failed to produce a third generation (Figure 2-17).

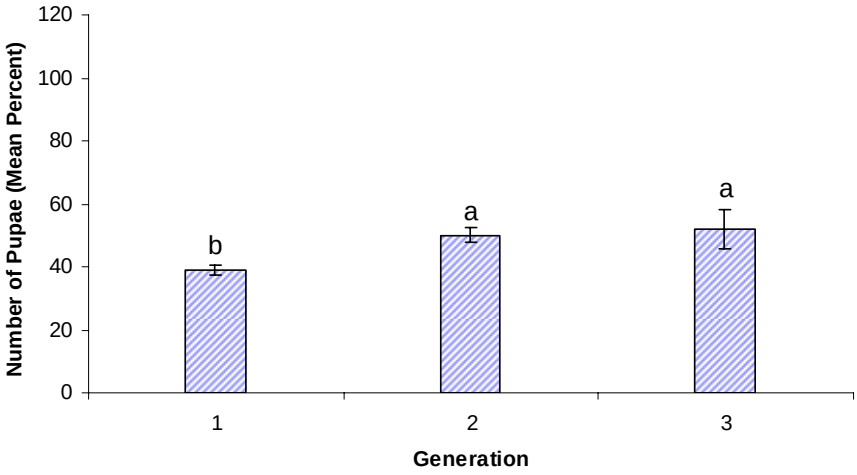


Figure 2-16. Comparison of the three generations of *Culex erraticus* eggs that successfully developed into the pupa stage. Bars with the same letter are not statistically significant.

Eggs to Pupae Comparison of Diets to Generations

In the overall model comparing diets to generations, there was a significant difference (F=4.64; df=5; p=0.0007) found. FF did not produce a significant difference among the three decreasing generations. FFBY produced a significant difference among generation one and both generations two and three, but no difference was found in the number of eggs that made it to the pupa stage between generations two and three. FFPM did not produce a significant difference in generations one or two and failed to produce a third generation (Figure 2-17).

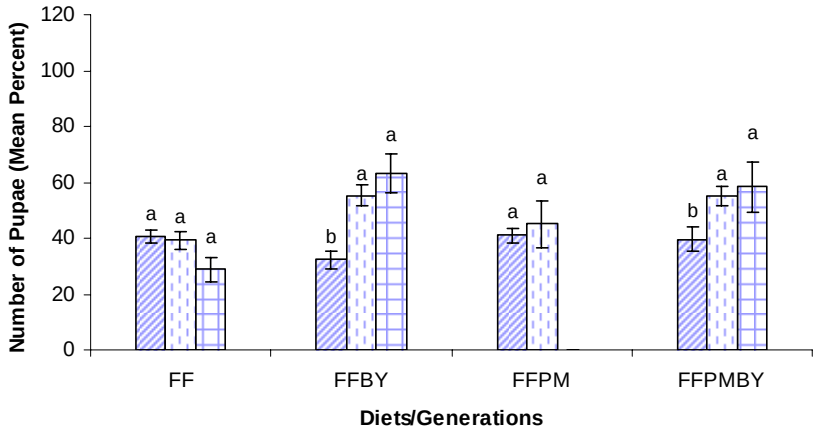


Figure 2-17. Comparison of treatments to generations for the mean percent of *Culex erraticus* eggs that developed into the pupa stage. Bars with the same letter are not statistically different.

Colony Adult

The female *Cx. erraticus* reared in colony is a small, dark brown Culicidae mosquito with pale basal scales on each of the eight abdominal segments (Figure 2-18). The mid-dorsal acrostichal setae are absent and the occiput (margin of eye) has broad, flat scales (Figure 2-19). The mesoepimeron is located near the back and under the wings on each side of the thorax. The *Cx. erraticus* mesoepimeron usually has a patch of six or more scales present (Figure 2-20) (Darsie and Ward 2005).

Figure 2-18. Female *Culex erraticus* colony reared. Pale basal scales are found on each of the eight abdominal segments.

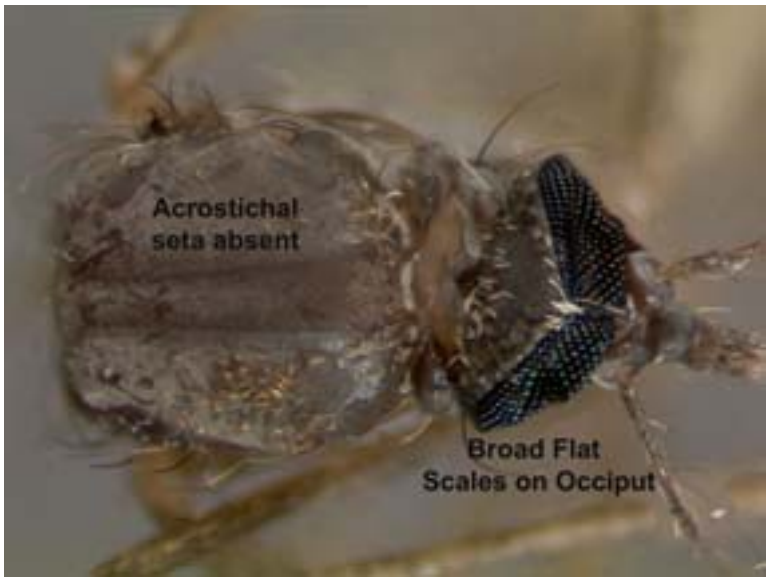


Figure 2-19. *Culex erraticus* does not have mid-dorsal acrostichal seta located in the middle top of the thorax. The broad, flat scales are on the interior margin of the eye.

Eggs to Adults Comparison of Treatments

Comparison among the four diets showed significance (F=9.97; df=3; p=0.0001) in the mean percent of eggs that developed to the adult stage. The diet producing the least mean percent ($\bar{x}$ =7.9) of adults was FFPM. Diets FF had a mean percent of 12.1 eggs develop into adults and FFPMBY had a mean percent of 13.8 adults. FFBY produced a mean percent of 19.6 adults (Figure 2-21).

Figure 2-20. Six or more pale broad scales are located on the mesoepimeron of *Culex erraticus*.

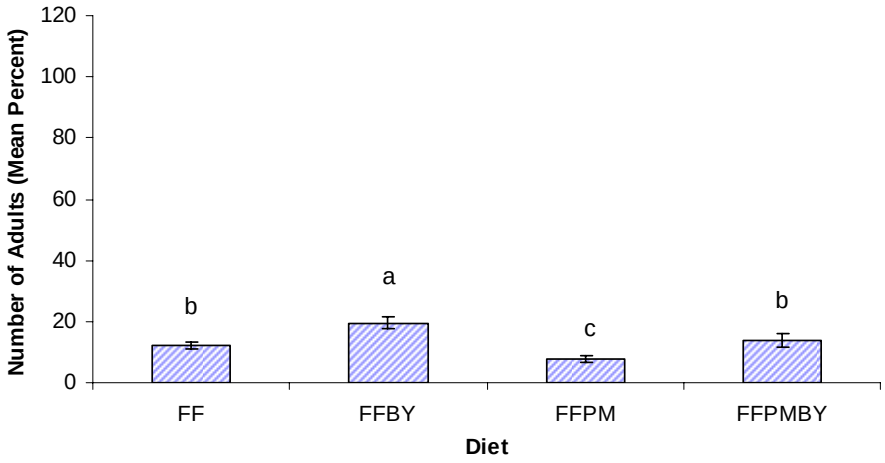


Figure 2-21. Comparison of diets show Tetramin® Tropical Flakes Fish Food with brewer's yeast produced a higher percent of adults from eggs than the other three diets. Bars with the same letter are not statistically significant.

Eggs to Adults Comparison of Generations

The number of eggs developing to the adult stage in generation two ($\bar{x}$=17.3) and three ($\bar{x}$=20.3) compared to generation one ($\bar{x}$=10.6) resulted in a significant difference (F=5.72; df=2; p=0.004). There was no significant difference found between generations two and three. Although treatment FFPM failed to produce a third generation of *Cx.*

erraticus, the mean percent of adults produced in generation three were higher than those produced in generations one and two (Figure 2-22).

Eggs to Adults Comparison of Diets to Generations

The number of eggs developing into adults when diets were compared to the generations was significantly different (F=5.92; df=5; p=.0001). The mean percent of eggs in the FF diet that developed into adults decreased from 13.6 percent in generation one to 6.3 percent in generation three. There was an increase in the FFBY from 10.3 percent in generation one to 27.8 percent in generation three.

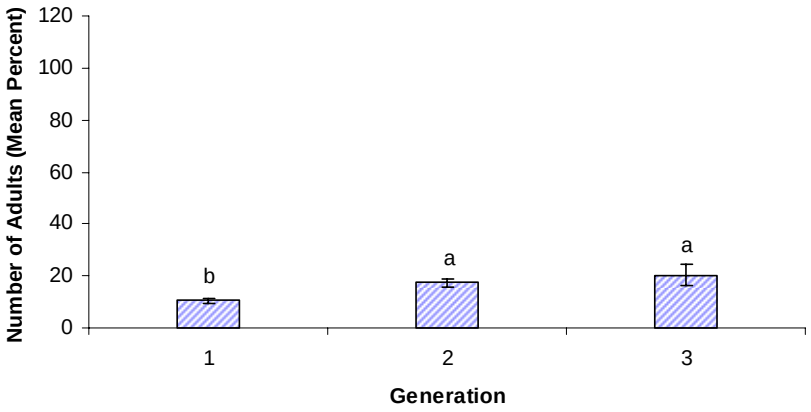


Figure 2-22. Comparison of the three generations for the number of eggs that developed to the adult stage. Bars with the same letters are not statistically significant.

FFPM produced a mean percent of 8.1 adult *Cx. erraticus* in generation one, a mean percent of 7.3 adults in generation two and failed to produce a third generation. FFPMBY produced a mean percent of 9.6 adult mosquitoes in generation one, 18.2 mean percent in generation two and a mean percent of 22.5 adults in generation three (Figure 2-23).

Eggs to Males Comparison of Diets

Comparing diets the mean percent of adult males developing from eggs for these generations resulted in a significant difference (F=7.62; df=3; p=0.0001).

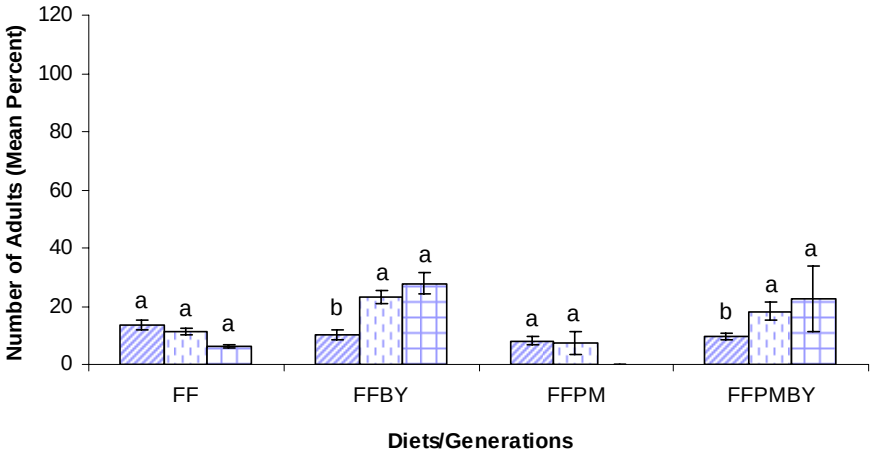


Figure 2-23. Comparison of diets for the number of eggs developing into adults in each generation. The Tetramin® Tropical Flakes Fish Food combined with powdered milk did not produce a third generation. The striped bars are generation one, dotted bars represent generation two and plaid bars show generation three.

The mean percent of FFBY eggs developing into adult males was 11.2. FF and FFPMBY diets produced a mean percent of 7.4 and 7.1 adults, respectively. The FFPM diet had a mean percent of 4.8 adults that developed from eggs (Figure 2-24).

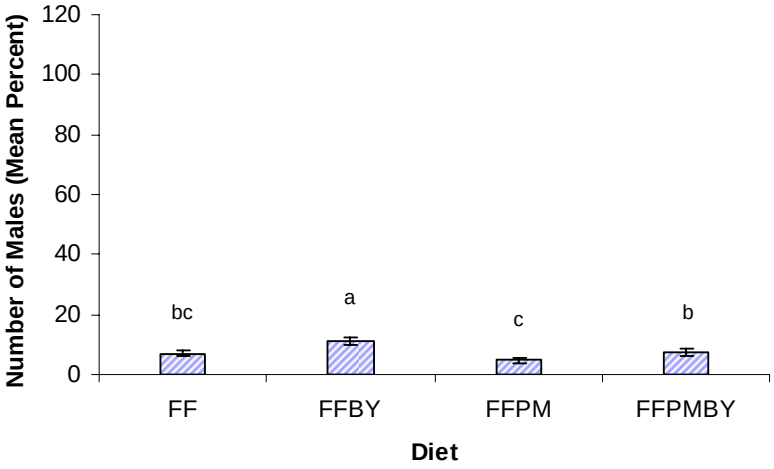


Figure 2-24. The comparison of *Culex erraticus* eggs developing into adult males in each diet. Bars with same letters are not statistically different.

Eggs to Males Comparison of Generations

A significant difference (F=3.90; df=2; p=0.023) was found in the comparison of eggs in each generation that developed into males. The mean percent of males to develop in generation one was 6.2. Generation two produced a mean percent of 9.6 adult males and generation three had a mean percent of 11.4 adult males (Figure 2-25).

Eggs to Males Comparison of Diets to Generations

When comparing the interaction of the four diets to the three generations a significant difference was found (F=4.90; df=5; p=0.0004).

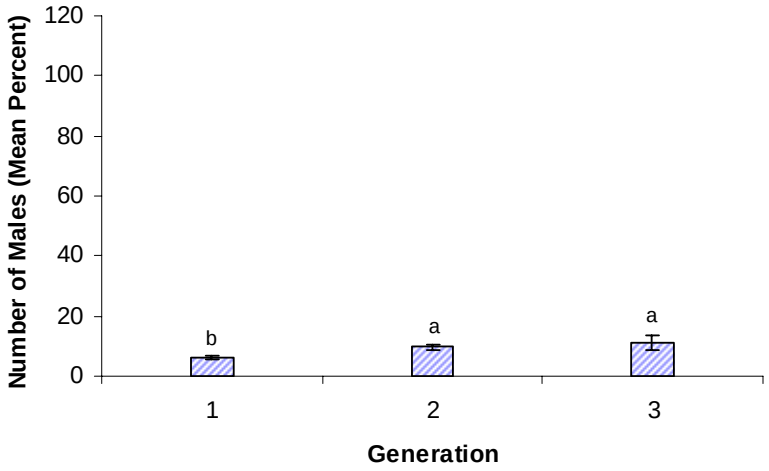


Figure 2-25. Comparison of the three generations of eggs developing into adult males. The bars with the same letter are not statistically different.

However, the FF diet did not produce a significant difference in the mean percent of adult males between generation one with 8.0, generation two had a mean percent of 6.5 adult males and generation three produced a mean percent of 3.4 adult males. The FFBY diet experienced an increased in the mean percent of 5.9 males produced in generation one, generation two had a mean percent of 13.2 adult males and generation three had a mean percent of 15.7 adult males. Generation one and two in the FFPM diet had mean percent of 4.9 and 4.6 males, respectively. FFPMBY generation one had a mean percent

of 5.6 adult males, generation two produced a mean percent of 9.0 males and generation three had a mean percent of 12.5 adult male mosquitoes. Diets FFBY and FFPMBY experienced the highest increase in eggs developing to males in all three generations (Figure 2-26).

Eggs to Females Comparison of Diets

The number of eggs developing into adult female mosquitoes in the four diets resulted in a significant difference (F=8.8; df=3; p=0.0001). The FFBY produced the highest mean percent of adult female mosquitoes being 8.3. However, there was not a significant difference found between diet FF which produced a mean percent of 4.9 adult female mosquitoes and treatment FFPM which produced a mean percent of 3.1 adult female mosquitoes (Figure 2-27).

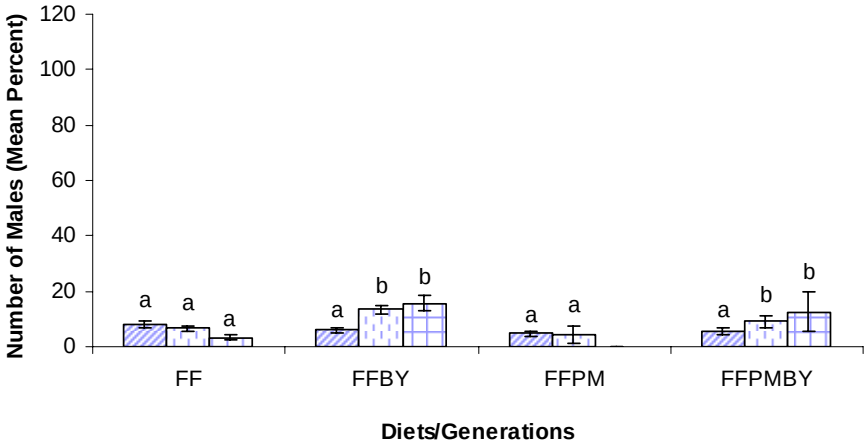


Figure 2-26. Comparison of the number of eggs developing into males between the three generations in the four diets. The striped bars represent generation one, dotted bars represent generation two, the checkered bars represent generation three.

Eggs to Females Comparison of Generations

Comparing the number of adult female mosquitoes the developed from eggs within the three generations resulted in a significant difference (F=6.6; df=2; p=0.002).

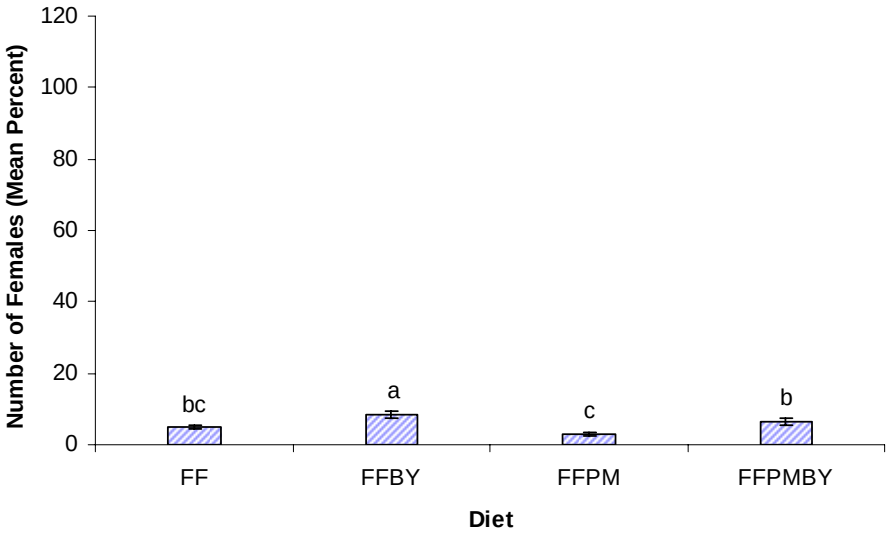


Figure 2-27. Comparison of adult females developing from eggs in four different diets. Bars with same letters are not statistically significant.

Generation one produced a mean percent of 4.3 adult female mosquitoes. Generations one and two produced a mean percent of 7.7 and 8.9 adult mosquitoes respectively (Figure 2-28).

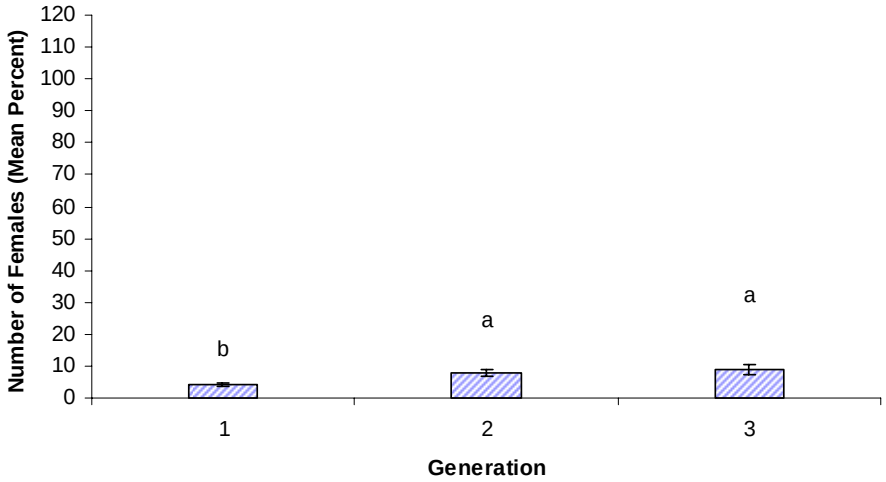


Figure 2-28. Comparison of generations for the number of eggs developing into female mosquitoes. Bars with the same letter are not significantly different.

Eggs to Females Comparison of Diets to Generations

Comparison of diets to the generations resulted in a significant difference in the number of eggs that developed into adult female mosquitoes ($F=5.6$; $df=5$; $p=0.0001$).

The FF diet did not produce a significant difference within the three generations for the mean percent of adult female mosquitoes. The FF generation one produced a mean percent of 5.5 female mosquitoes, generation two had a mean percent of 4.5 and generation three produced a mean percent of 2.8 adult female mosquitoes. Diet FFPM produced similar mean percents for adult females in generation one and two being 3.3 and 2.6 respectively. Diets FFBY and FFPMBY produced the same mean percent of adult female mosquitoes for generation one being 3.9. In generation two, both diets produced similar mean percents of 9.9 for the FFBY and 9.2 for the FFPMBY diet. However, FFBY produced a mean percent of 12.0 females compared to 10.1 for the FFPMBY diet (Figure 2-29).

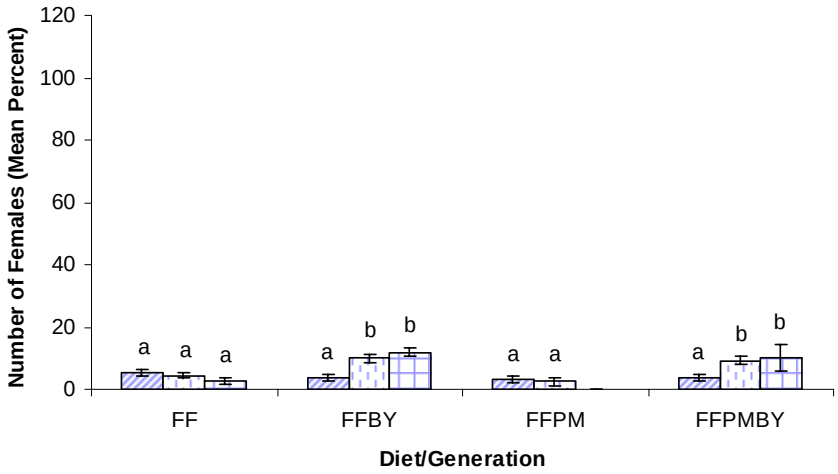


Figure 2-29. An increase in the mean percent of adult females developing from eggs took place in both Tetramin®Tropical Fish Food with brewers yeast and Tetramin®Tropical Fish Food combined with powdered milk and brewers yeast. Tetramin®Tropical Fish Food and Tetramin®Tropical Fish Food with powdered milk were not statistically different within the generations. Bars with the same letters are not statistically different.

Larvae to Pupae Comparison of Diets

Comparison of the four diets showed a significant difference ($F=2.61$; $df=3$; $p=0.055$) in the mean percent of larvae that developed into pupae. SAS showed a

difference at the 95% confidence limits between diets FFPMBY and FF, which may or may not be statistically different (Table 2-7). Figure 2.30 shows the significance between diets FF and FFPM, which has the same mean percent of pupae but different standard of errors. Figure 2-31 shows the letters on the bars differently to reflect the significant difference. The p-value in table 2-6 for the treatment is 0.055 which can be considered approaching significant because the rounding off of 0.055 to 0.06. Diet FFBY had a mean percent of 54.2 larvae develop into pupae and diet FF had a mean percent of 46.6 larvae develop into pupae. Diet FFBY showed a mean percent of 52.9 pupae and FFPM showed a mean percent of 46.6 pupae. The interpretation of the statistical model by SAS gave a statistical significance between FFPMBY ($\bar{x}$ =53.6) and FF ($\bar{x}$ =46.6) but not between FFPMBY ($\bar{x}$ =53.6) and FFPM ($\bar{x}$ =46.6) which has the same mean percent as diet FF (Table 2-8).

Table 2-7. Analysis of variance comparing the treatments, generations and the interaction of the treatments to generation for the mean percent of larvae that develops into pupae.

ANOVA Source of Variation	SS	df	MS	F	Pr > F
Treatment	1521.95	3	507.318	2.61	0.055
Generation	4324.87	2	2162.43	11.12	0.0001
Treatment * Generation	4101.87	5	820.375	4.22	0.0015

Table 2-8. Treatments with the mean percent ± SEM for development of larvae to pupae.

Treatment	Mean Percent ± SEM
FF	46.6 ± 1.7
FFBY	52.9 ± 3.2
FFPM	46.6 ± 2.9
FFPMBY	53.6 ± 3.2

Figure 2.30 shows the significance between diets FF and FFPM which has the same mean percent of pupae but different standard of errors. Figure 2-31 shows the letters on

the bars differently to reflect the significant difference. The p-value in table 2-6 for the treatment is 0.055 which can be considered approaching significant because the rounding off of 0.055 to 0.06.

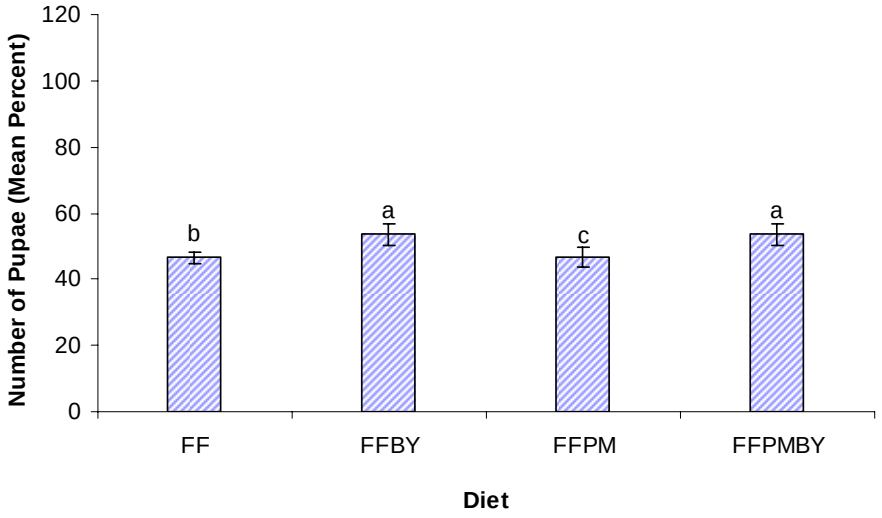


Figure 2-30. Larvae to pupae comparison of diets Tetramin® Tropical Flakes Fish Food is significant to Tetramin® Tropical Flakes Fish Food with powdered milk which has the same mean of 46.6 but standard error for Tetramin® Tropical Flakes Fish Food is 1.7 and Tetramin® Tropical Flakes Fish Food with powdered milk is 3.2. See figure 2-30 for second interpretation.

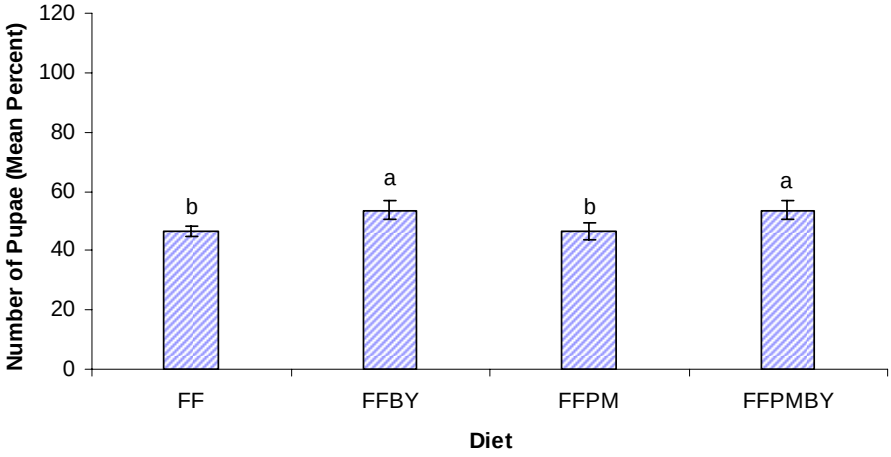


Figure 2-31. Larvae to pupae second interpretation of the results showing a significant difference between the four larval diets.

Larvae to Pupae Comparison of Generations

The generation comparison of larvae that developed into pupae was found statistically significant (F=11.12; df=2; p=<.0001). Generation one had a mean percent of 43.8 larvae that developed into pupae and was significant to generation two and three. Generations two and three had mean percents of 57.4 and 58.1, respectively (Figure 2-32).

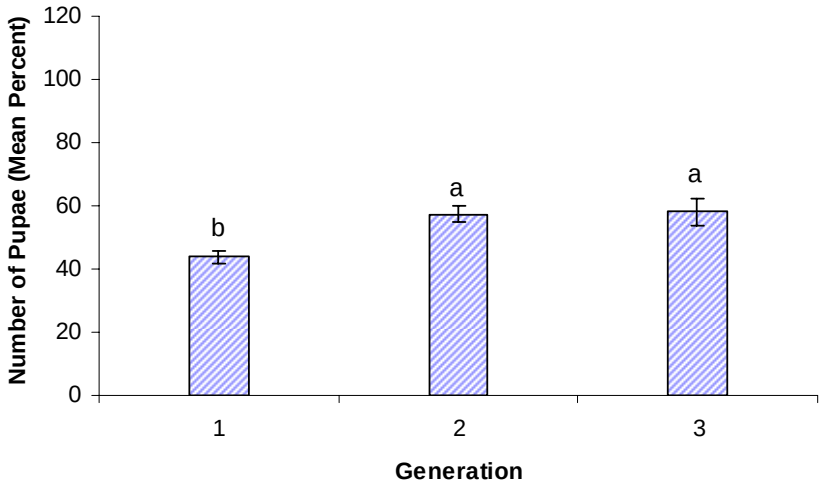


Figure 2-32. The mean percent of larvae developing into pupae in the three generations. Bars with the same letters are not statistically different.

Larvae to Pupae Comparison of Diets to Generations

The number of larvae that developed into pupae in each of the four diets through each of the three generations showed a significant difference (F=4.22; df=5; p=0.002). No significance was found within the generations of the diets FF and FFPM. Diet FF generation F_1 had a mean percent of 47.3 which decreased to 41.1 mean percent of pupae in the F_3 generation. Diet FFPM had an increase in the mean percent from 45.3 in the F_1 generation to a mean percent of 52.3 in the F_2 generation and failed to produce a third generation of pupae. Diet FFPMBY produced a mean percent of 44.8 larvae that developed to pupae in the F_1 generation then increased the mean percent in both F_2 and F_3

generations to 64.2 and 64.6, respectively. Diet FFBY showed a mean percent of 34.7 in the F_1 generation then increased the mean percent of larvae developing to pupae to 61.1 in the F_2 generation and 65.7 in the F_3 generation (Figure 2-33).

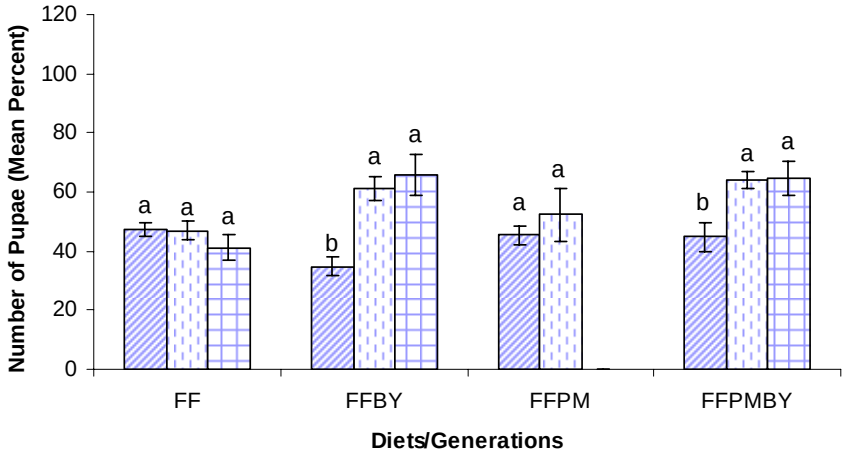


Figure 2-33. The mean percent of larvae developing into pupae were not statistically different in diets Tetramin® Tropical Flakes Fish Food and Tetramin® Tropical Flakes Fish Food with powdered milk. Treatments Tetramin® Tropical Flakes Fish Food with brewers yeast and Tetramin® Tropical Flakes Fish Food combined with powdered milk and brewers yeast showed significance within the generations. Bars with the same letters are not statistically significant.

Larvae to Adults Comparison of Diets

The number of larvae developing to the adult stage was found to be statistically different ($F=7.96$; $df=3$; $p=<.0001$). The diet FFPM produced a mean percent of 8.7 larvae that developed into adults which was the lowest of the four diets. Diet FFPMBY resulted in a mean percent of 16.2 and diet FFBY resulted in a mean percent of 21.4 larvae that developed into pupae (Figure 2-34).

Larvae to Adult Comparison of Generations

A statistical difference ($F=6.61$; $df=2$; $p=0.002$) was found between the three generations. The F_1 generation produced a mean percent of 11.8 larvae that developed

into adults. The F_2 and F_3 generations had mean percents of 20.2 and 22.0 respectively (Figure 2-35).

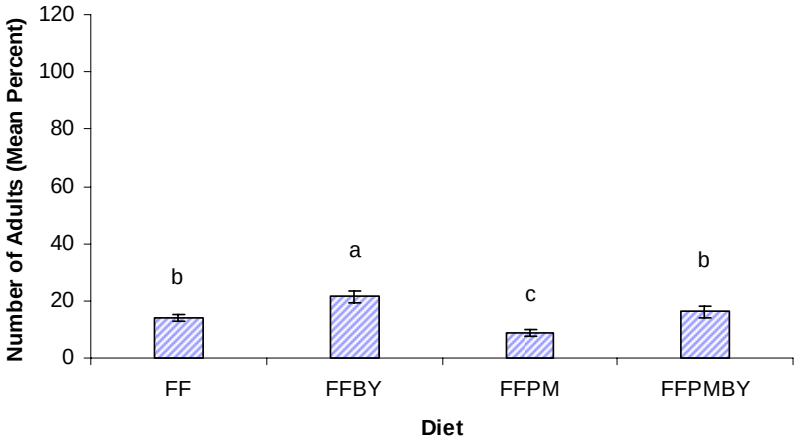


Figure 2-34. Diet Tetramin® Tropical Flakes Fish Food with powdered milk produced the lowest number of larvae that developed into pupae. Tetramin® Tropical Flakes Fish Food with brewers yeast had the highest mean percent of larvae to develop into pupae. Bars with the same letter are not statistically different.

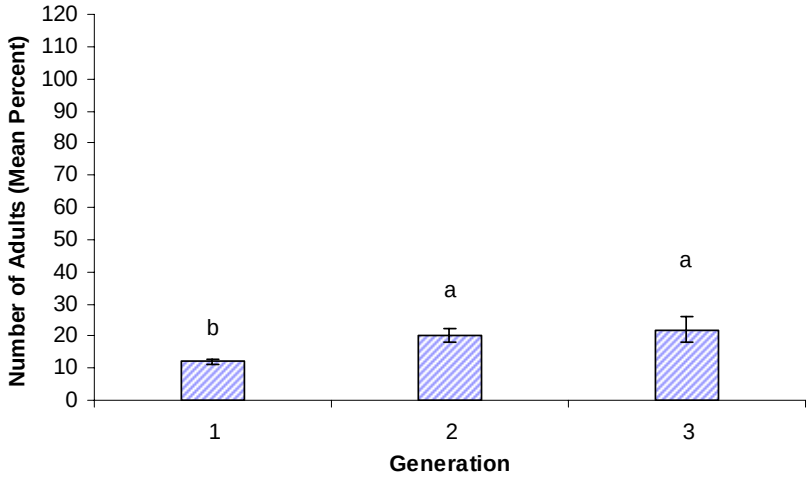


Figure 2-35. The comparison of the F_1 generation to both F_2 and F_3 generations for the number of larvae that developed into adults. Bars with the same letter are not statistically different.

Larvae to Adult Comparison of Diets to Generations

Comparison of diets to generations resulted in a significant difference (F=5.08; df=5; p=0.0003). The number of larvae that developed into adults in the FF diet decreased from 15.3 mean percent in the F_1 generation to a mean percent of 9.2 in the F_3 generation with no significance being reported within these generations. Diet FFPM was not significant in the two generations of larvae that developed into pupae. The F_1 generation had a mean percent of 8.8 and the F_2 generation had a mean percent of 8.2 larvae developing into pupae. Diets FFBY and FFPMBY both showed significant increases between the F_1 and F_2 generations with mean percents of 11.2 and 10.9 larvae developing into pupae. However, both diets doubled their mean percents with FFBY producing a mean percent of 25.8 in the F_2 generation and FFPMBY produced a mean percent of 22.2 in its F_2 generation. Both FFBY and FFPMBY had a final increase in the mean percent of the F_3 generations of 28.8 and 24.3 respectively (Figure 2-36).

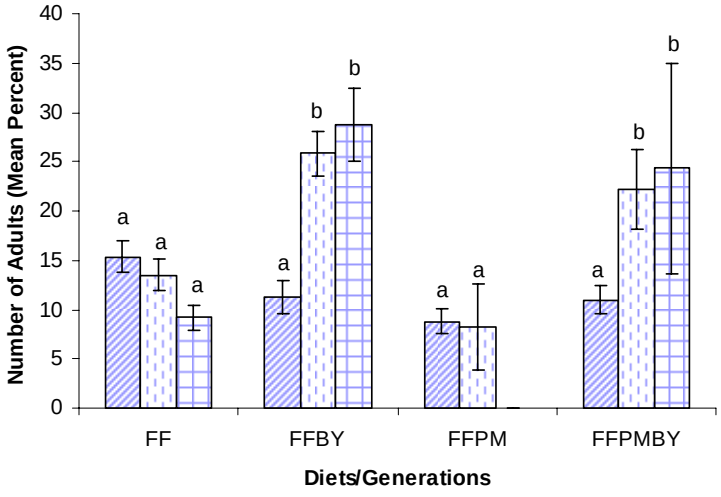


Figure 2-36. Comparison of larvae to adults in the Tetramin® Tropical Flakes Fish Food which showed a decrease in the three generations with no statistical significance found within the generations of both Tetramin® Tropical Flakes Fish Food and Tetramin® Tropical Flakes Fish Food with powdered milk. Stripped bars are F_1 generation, lined bars are F_2 generation and plaid bars are F_3 generation. Bars with the same letters are not statistically significant.

Larvae to Males Comparison of Diets

A significant difference was found (F=5.75; df=3; p=0.001) in the number of males that developed from larvae within the four different diets. No significance was found between the diets FF that had a mean percent of 8.6 and FFPMBY having a mean percent of 8.5. The FFPM diet had the lowest mean percent of 5.3 larvae developing into males. However, diet FFBY produced a mean percent of 12.1 adult males that developed from larvae (Figure 2-37).

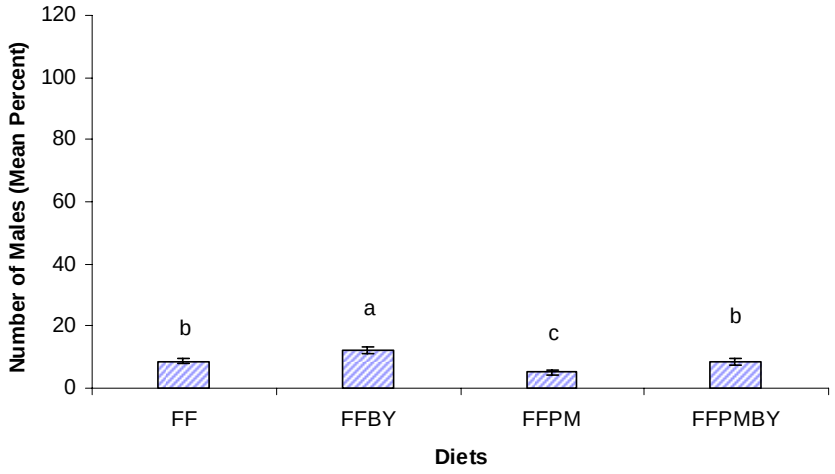


Figure 2-37. The mean percent of adult males that developed from larvae in the four diets. Bars with the same letters are not statistically significant.

Larvae to Males Comparison of Generations

The number of larvae that developed into males within the three generations showed a significant difference (F=4.67; df=2; p=0.001). The significance appeared between the F_1 generation having a mean percent of 7.0 males and the mean percent of generations F_2 and F_3. No significance was found between the F_2 and F_3 generations because their mean percents were very similar in being 11.1 and 12.5, respectively (Figure 2-38).

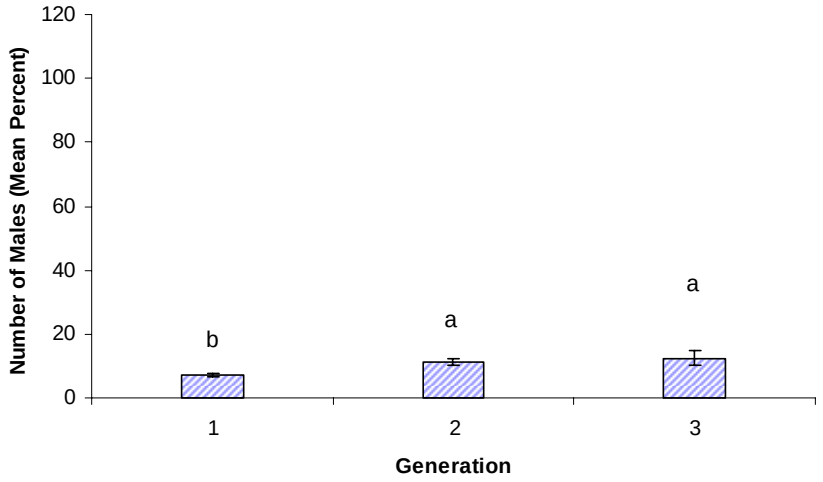


Figure 2-38. The F_1 generation is significant to F_2 and F_3 generations in the mean percent of males produced from larvae. Bars with the same letters are not statistically different.

Larvae to Males Comparison of Diets to Generations

A significant difference (F=4.20; df=5; p=0.002) was found in the number of larvae that developed into males in the four diets within each of the three generations. The FF and the FFPM diets did not produce a significant difference in the mean percent of males within the three generations. The FF treatment showed a decrease in the mean percent of males ($\bar{x}$=9.3) in the F_1 generation, F_2 ($\bar{x}$=8.2) and the F_3 ($\bar{x}$=5.5) generation. The mean percent of adult males remain virtually the same in the F_1 ($\bar{x}$=5.4) and F_2 ($\bar{x}$=5.3) generations in diet FFPM with no third generation being produced. No significant difference was found between diets FFBY and the FFPMBY. Both diets FFPMBY and FFBY had similar mean percents of 6.3 and 6.4, respectively in the F_1 generation. However, diet FFBY had the largest increase of males with a mean percent of 16.3 in the F_3 generation compared to diet FFPMBY having a mean percent of 13.4 in the F_3 generation (Figure 2-39).

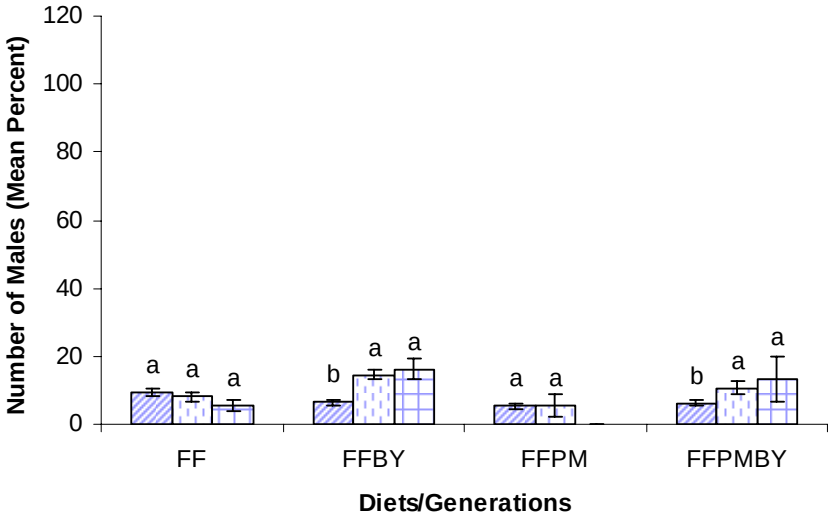


Figure 2-39. The mean percent of larvae developing into male *Culex erraticus* did not show significance between diets Tetramin® Tropical Flakes Fish Food and Tetramin® Tropical Flakes Fish Food with powdered milk. Diets Tetramin® Tropical Flakes Fish Food with brewers yeast and Tetramin® Tropical Flakes Fish Food combined with powdered milk and brewers yeast did show significance with their respective generations to the number of adult males produced from larvae. Bars with the same letters are not statistically different.

Larvae to Females Comparison of Diets

When comparing the mean percent of females developing from larvae in each of the four diets a significant difference was found ($F=6.94$; $df=3$; $p=0.0002$). Diet FFPM produced the least number of adult females with a mean percent of 3.4 in comparison with diet FF which produced a mean percent of 5.6 adult females. Therefore, no significant difference was found between FFPM and FF diets. However, significance was found between both FFPM and FF in comparison with diets FFPMBY and FFBY. FFPMBY produced a mean percent of 7.7, and FFBY produced a mean percent of 9.1 adult female mosquitoes. No significant difference was found between diets FFPMBY and FFBY (Figure 2-40).

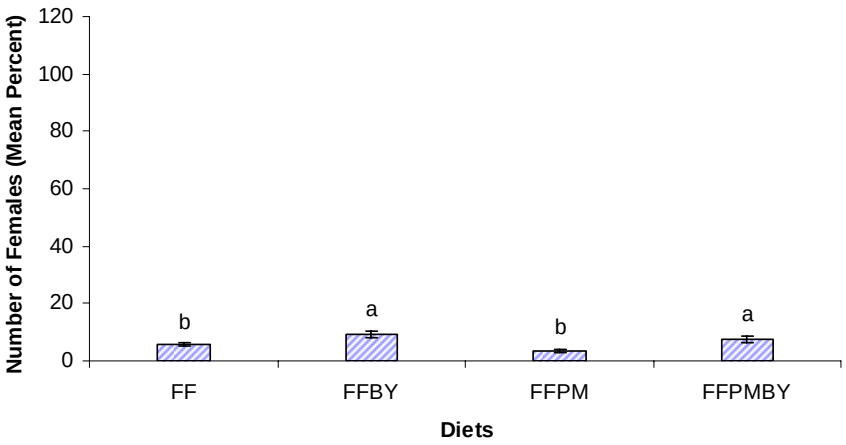


Figure 2-40. Significance is shown between both treatments Tetramin® Tropical Flakes Fish Food with brewers yeast and Tetramin® Tropical Flakes Fish Food combined with powdered milk and brewers yeast in comparison to treatments Tetramin® Tropical Flakes Fish Food and Tetramin® Tropical Flakes Fish Food with powdered milk for the number of adult female *Culex erraticus* developing from larvae. Bars with the same letters are not significantly different.

Larvae to Females Comparison of Generations

A significant difference (F=6.60; df=2; p=0.002) was found in the number of adult females produced within the three generations. The F_1 generation produced a mean percent of 4.7 adult female mosquitoes that was the least of the three generations. The significant difference was found between the F_1 generation and both F_2 and F_3 generations. Both F_2 and F_3 generations produced a mean percent of 9.1 and 9.6 adult females, respectively (Figure 2-41).

Larvae to Females Comparison of Diets to Generations

Comparison of diets within each generation produced a significant difference (F=4.56; df=5; p=0.0008). Both diets FF and FFPM did not produce significance within the generations even though a decrease was shown between the generations. The F_1 generation of diet FF had a higher mean percent of 6.0 adult females in comparison to diet FFPM having a mean percent of 3.5. The mean percent in the F_2 generations of FF

decreased to 5.3 females in comparison to 2.9 in the FFPM treatment. The mean percent in diet FF decreased in the F_3 generation to 3.8 adults and the treatment FFPM did not produce a third generation (Figure 2-42). Both diets FFBY and FFPMBY had similar mean percent for the F_1 generations of 4.3 and 4.5, respectively. Both diets increased by approximately the same mean percent in the F_2 generation to 11.2 for FFBY and 11.5 for FFPMBY. However, diet FFBY increased to a mean percent of 12.5 in the F_3 generation, and FFPMBY basically remained the same with a mean percent of 11.0 (Figure 2-42).

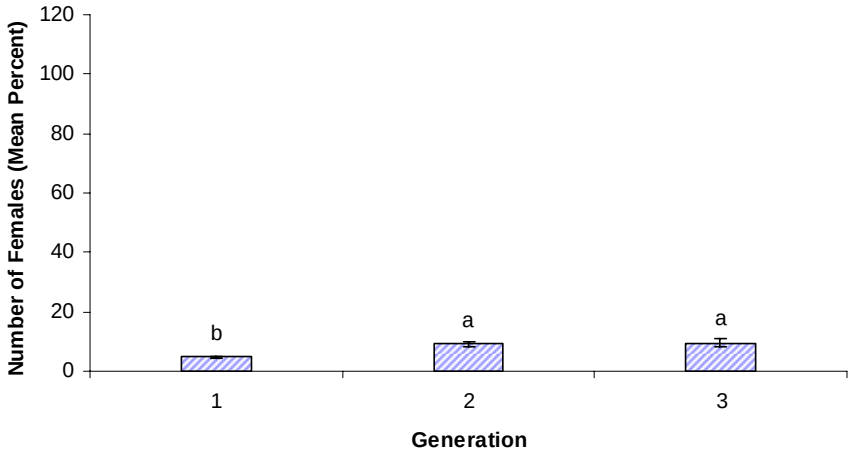


Figure 2-41. The F_1 generation produced significantly less adult females than the F_2 and the F_3 generations. Bars with the same letters are not significantly different.

Pupae to Adults Comparison of Diets

A statistical difference was found when comparing the four diets to the mean percent of pupae that developed into adults ($F=4.44$; $df=3$; $p=0.006$). The significant difference was found between diet FFPM and the other three diets. Diet FFPM had the lowest number of pupae developing into adults with a mean percent of 18.9. However, there was no significant difference found between the remaining three diets. The FF diet produced a mean percent of 30.3 compared to diet FFPMBY, which had a mean percent

of 35.2. The mean percent for diet FFBY was the highest at 39.1 pupae developing into adults (Figure 2-43).

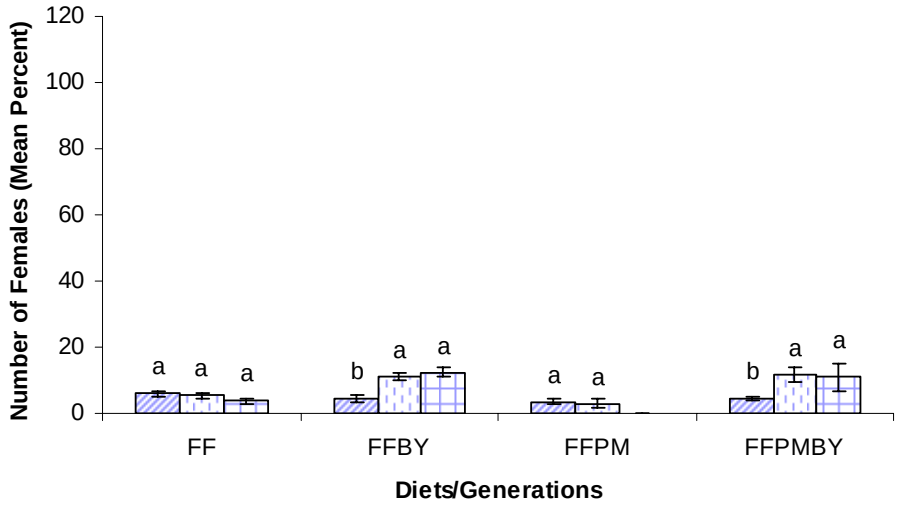


Figure 2-42. Comparison of diets within generations showed no significant difference with the generations of treatments FF and FFPM in larvae developing into adult female mosquitoes. Significance was found with the generations of FFBY and FFPMBY. Bars with the same letters within each treatment are not significantly different.

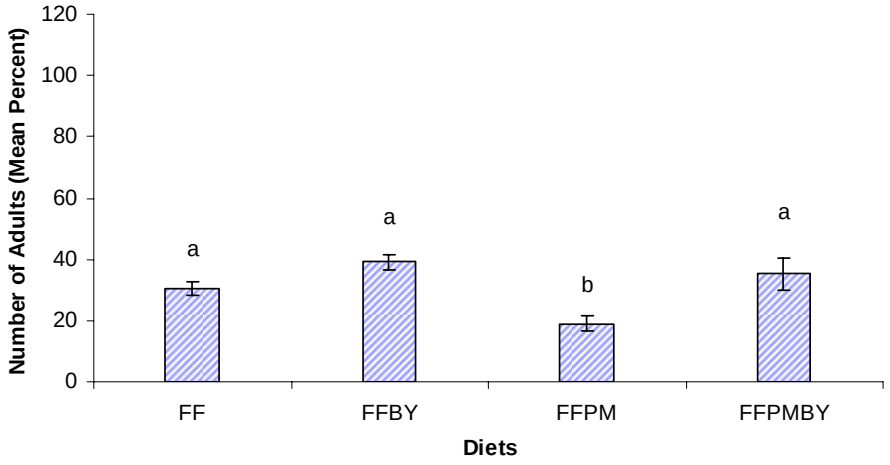


Figure 2-43. Significance was found between treatment Tetramin® Tropical Flakes Fish Food with powdered milk and the other three treatments Tetramine® Tropical Flakes Fish Food, Tetramin® Tropical Flakes Fish Food combined with powdered milk and brewer;s yeast, and Tetramin® Tropical Flakes Fish Food with brewer,s yeast in the number of pupae that developed into adults. Bars with the same letters are not statistically significant.

Pupae to Adults Comparison of Generations

When comparing the number of pupae that developed into adults between the three generations, there was no significance found (F=0.02; df=2; p=0.98). The F_1 generation produced a mean percent of 29.9 adults that developed from pupae. The F_2 generation showed a mean percent of 35.0 adults compared to the F_3 generation which produced a mean percent of 35.7 adults (Figure 2-44).

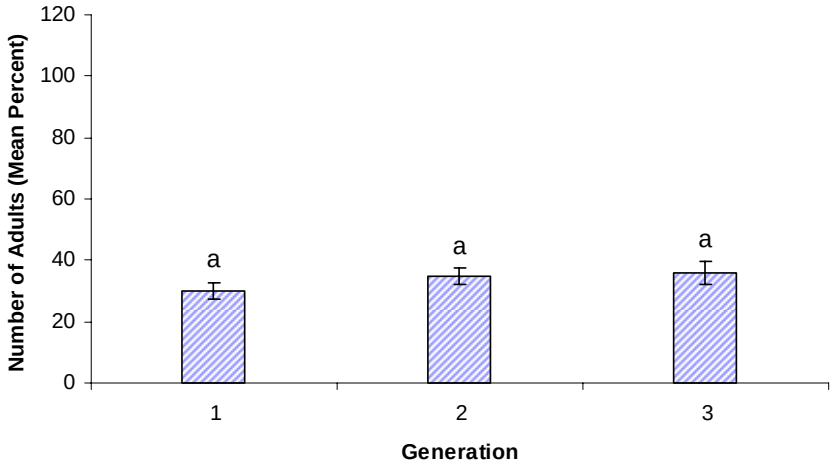


Figure 2-44. No significance was found between the generations for the mean percent of adults developing from pupae. Bars with the same letters are not significantly different.

Pupae to Adults Comparison of Diets to Generations

No significant difference was found within the three generations when compared to the four diets (F=0.78; df=5; p=0.6). The FFPM diet had the lowest reported adults in both F_1 and F_2 generations with a decrease in the mean percent from 20.3 adults to 13.6 adults, respectively. Diet FFPM failed to produce a third generation. The F_1 generations of the remaining three diets were similar with FF having a mean percent of 31.8, FFBY a mean percent of 31.3, and FFPMBY produced a mean percent of 35.2 adults (Figure 2-45). Two diets in the F_2 generation had mean percents that basically remained the same,

30.0 for FF, and 35.2 for FFPMBY. Diet FFBY showed an increase in the mean percent in the F_2 generation of 43.0 adults. The F_3 generation of the FF diet had a decrease in the mean percent of 22.4. There was a slight increase in the mean percent for both FFPMBY and FFBY being 36.6 and 43.4 respectively (Figure 2-45).

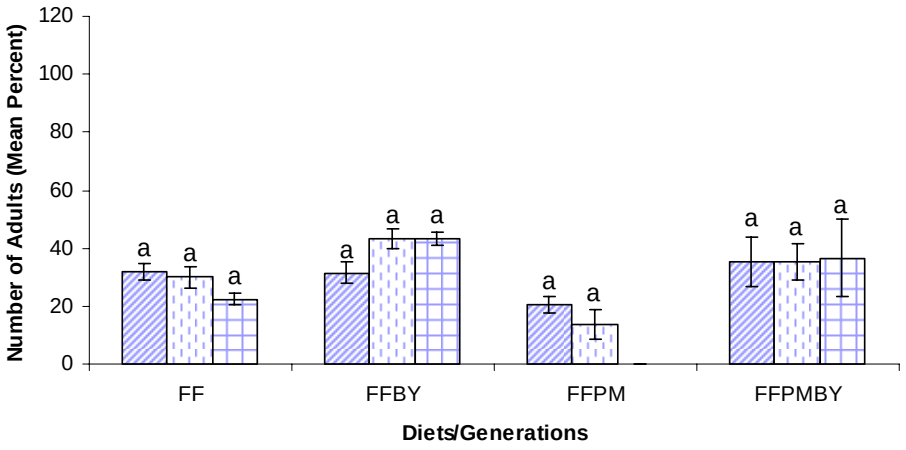


Figure 2-45. Comparison of treatments within generations does not show a significant difference in the mean number of pupae that develop into adult *Culex erraticus*. Bars with the same letters within each treatment are not significantly different.

Pupae to Males Comparison to Diets

A significant difference was determined when comparing the number of males developing from pupae among the four diets (F=2.77; df=3; p=0.045). The mean percent of adults occurring from diet FFPM was 11.4. No significance was found between diets FF which had a mean percent of 18.2 adults developing from pupae. However, diets FFPMBY and FFBY had mean percents of 19.3 and 22.3, respectively, which produced the significant difference found in the diet model (Figure 2-46).

Pupae to Males Comparison of Generations

No significant difference (F=0.05; df=2; p=0.94) was found when the comparison between generations was performed to account for the mean percent of adult male

mosquitoes that developed from pupae. The F_1 generation produced a mean percent of 17.8 males compared to the F_2 generation, which had a mean percent of 19.3, and the F_3 generation, which had a mean percent of 20.1 males that developed from pupae (Figure 2-47).

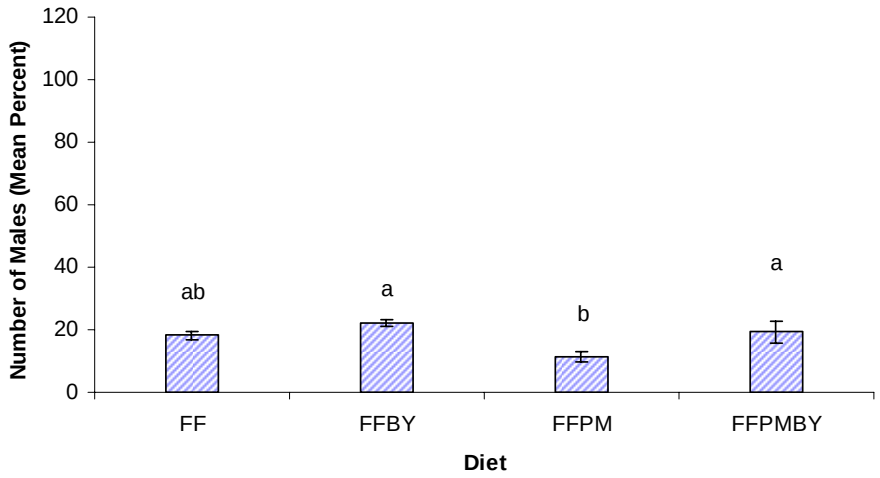


Figure 2-46. Comparison of diets for the numbers of pupae developing into adult male *Culex erraticus* mosquitoes. Bars with the same letter are not statistically significant.

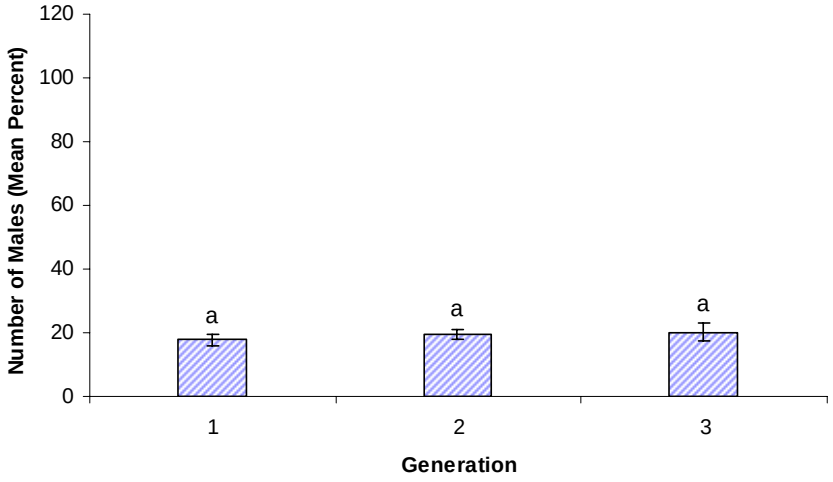


Figure 2-47. No significance was found when comparison between generations was evaluated for the mean percent of pupae that developed into adult male *Culex erraticus* mosquitoes. Bars with the same letters are not statistically different.

Pupae to Males Comparison of Treatments to Generations

Comparison among the four diets within each generation reported no significant difference (F=0.66; df=5; p=0.65). In the F_1 generation, diet FFPM produced the lowest mean percent of 12.1 males, and decreased in the F_2 generation to a mean percent of 8.7 males and this diet also failed to produce a third generation. Diets FF, FFBY and FFPMBY produced a mean percent of 19.2, 18.5 and 21.0 in the F_1 generation, respectively (Figure 2-48).

In the F_2 generation, two diets showed a decrease in the numbers of adult males with the FF diet producing a mean percent of 18.0 and the FFPMBY diet producing a mean percent of 16.8. However, the FFBY diet showed an increase in the F_2 generation with a mean percent of 24.1 (Figure 2-48).

In the F_3 generation, diet FF continued its decrease with a mean percent of 13.0 adult males. However, diet FFPMBY experienced an increase in its mean percent to 20.1 in comparison to diet FFBY, which remained at 24.3 in the F_3 generation (Figure 2-48).

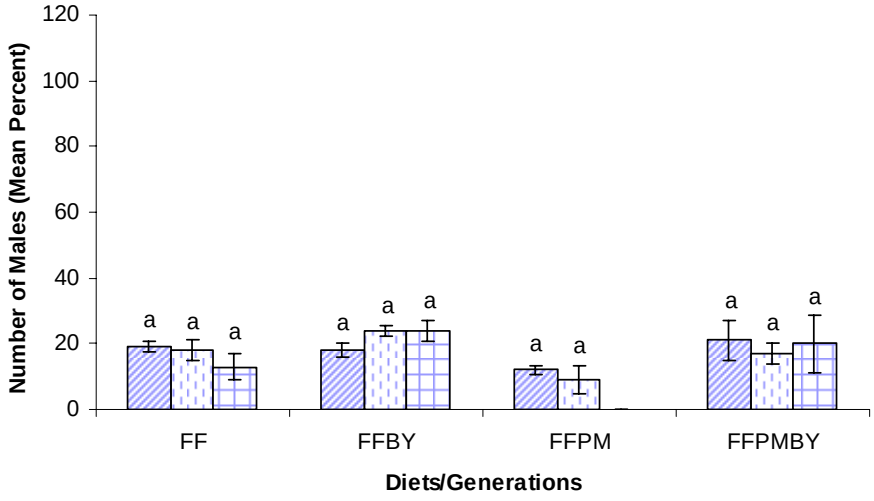


Figure 2-48. Comparison of four treatments within three generations showing the mean number of male mosquitoes developing from pupae. Bars with the same letters are not significantly different.

Pupae to Females Comparison of Diets

A significant difference (F=4.61; df=3; p=0.004) was found when the mean percent of adult females were compared among the four diets. Diet FFPM had the lowest mean percent of 7.6. The FF diet had a mean percent of 12.2, FFPMBY had a mean percent of 15.8, and diet FFBY had a mean percent of 16.3. The significance was found between diets FFBY and FFPMBY compared to diets FFPM and FF (Figure 2-49).

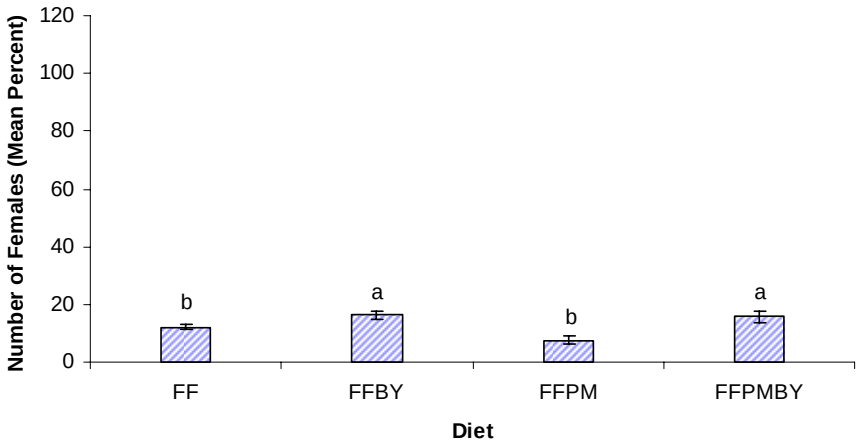


Figure 2-49. Comparison of diets and the number of adult female *Culex erraticus* mosquitoes produced showed significance between two of the diets. Bars with the same letters are not statistically significant.

Pupae to Females Comparison of Generations

The comparison between generations did not produce a significant difference (F=0.66; df=2; p=0.52) in the number of adult female mosquitoes that developed from pupae. In the F_1 generation a mean percent of 11.7 adult mosquitoes were produced in comparison to a mean percent of 15.8 in the F_2 generation and a mean percent of 15.7 in the F_3 generation (Figure 2-50).

Pupae to Females Comparison of Diets to Generations

Comparison of the three generations within each diet showed that no significant difference was found (F=1.33; df=5; p=0.26) in the number of adult female mosquitoes

emerging from pupae. Diet FFPM showed the lowest number of female mosquitoes with a mean percent of 8.3 in the F_1 generation, decreasing to a mean percent of 5.0 in the F_2 generation and failing to produce a third generation. Diets FF, FFBY and FFPM had mean percents of 12.4, 11.2 and 13.9, respectively, in the F_1 generation. Diet FF decreased the number of females it produced in the F_2 and the F_3 generations with a mean percent of 12.0 and 9.4, respectively. The FFPMBY diet had an increase in the number of females in the F_2 generation with a mean percent of 18.4 but decreased in the F_3 generation to a mean percent of 16.5. Diet FFBY showed an increase in the F_2 generation with a mean percent of 18.8 and an increase in the F_3 generation with a mean percent of 19.2 female mosquitoes.

The only significance found within generation was in the FFBY diet between the F_1 generation and the F_2 and F_3 generations (Figure 2-51).

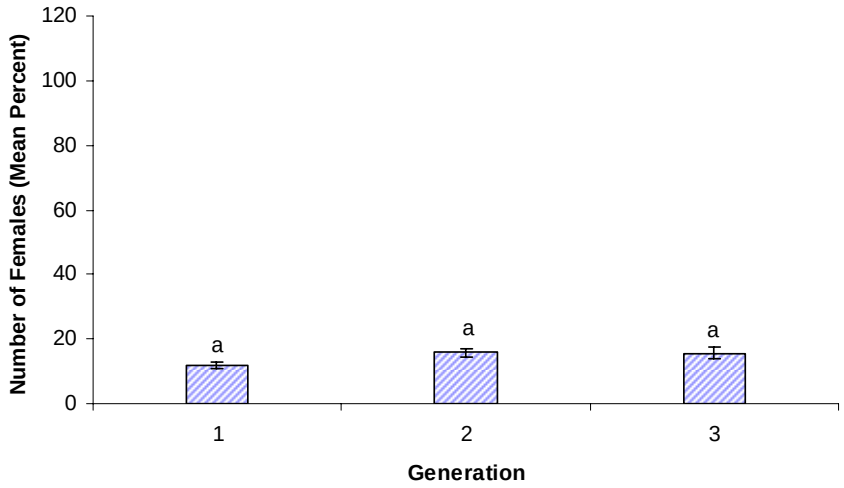


Figure 2-50. Comparison of the number of adult female mosquitoes that emerged from pupae in three generations. Bars with the same letters are not statistically different.

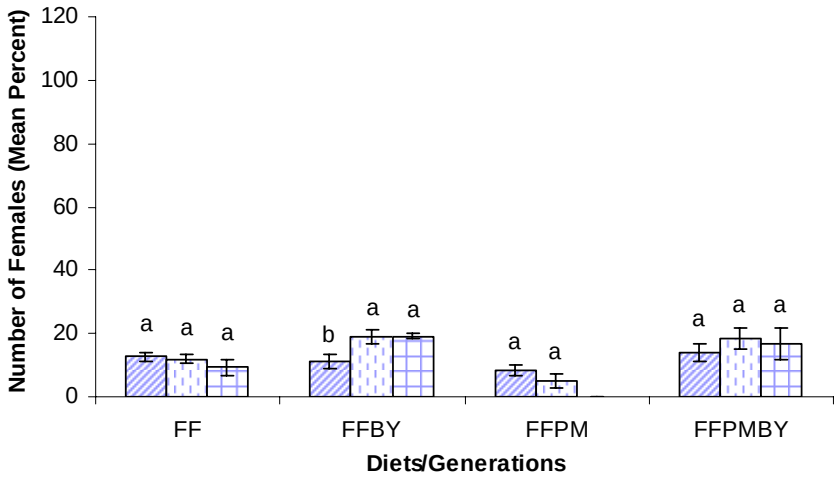


Figure 2-51. Comparison within the three generations between the four treatments did not result in a significant difference. Bars within the same treatment that have the same letters are not statistically significant.

Discussion

There are an estimated 250 species of *Melanoconion* mosquitoes that have been identified in the adult and larval stages (Pecor et al. 1992, Peyton and Harbach 1991). However, it is unknown at this time how many of these species have been successfully reared in colony. There are some references to colonization in various published works, but there are very few references that have documented the materials and methods establishing the laboratory techniques (Adames and Galindo 1972, Chadee and Tikasingh 1985, Hair 1968, Nayar et al. 1981). Establishing materials and methods for colonization of *Cx. erraticus* is important to add to published information on these common and most peculiar mosquitoes. Most of the identification of the species on record has come from wild specimens including the adults, larvae and pupae stages. It was the intent of this study to establish a large and stable colony of *Cx. erraticus* and document the length of the adult, egg, larvae and pupae stages in the life cycle.

Two factors are extremely important for the reproductive potential of most insects, the physiological and the environmental (Bellamy and Bracken 1971, Gillett 1956). With little information on the physiological needs of *Melanoconion* mosquitoes, developing a protocol for larval diet is difficult, especially when it has been reported that rearing larvae for most *Melanoconion* mosquitoes in laboratory conditions is difficult (Basham 1948).

The larval period is the most important period of a mosquito's life cycle because it is here that the initial fitness of the adult begins (Akoh et al. 1992, Briegel 2003). Mosquito larvae are similar to Lepidoptera caterpillars, eating nonstop in order to meet the demands put on the physiology to develop into an adult within a certain period. It has long been thought that most mosquito larvae need some form of animal protein in combination with plant compounds to meet these needs (Timmermann and Briegel 1996). Tetramin® Tropical Flakes Fish Food, liver powder, brewer's yeast, powdered milk, hog chow pellets, guinea pig chow, daphnia and mosquito larvae has been used in previous successful mosquito colonizing studies (Bradshaw and Lounibos 1972, Evans and Brust 1972, Fish and Hall 1978, Istock et al. 1975, Lang 1978, Lillie et al. 1980, O'Meara et al. 1981, Smith and Brust 1971, Suleman 1982). Four larval diets were used in this study based on diets used in previous published methodologies for mosquitoes in several genera including *Culex.* Two important factors were not known about the adult *Cx. erraticus* brought in from the wild.

The first unknown was the age of the mosquito, which regulates how many gonotrophic cycles it has gone through before being trapped (Abdelnur 1968, Woke et al. 1956) and second, whether the mosquito had mated before being trapped. Only two blood-fed females were trapped during this study, and no males were captured in the

CDC light traps, perhaps limiting the initial production of eggs in some of the females housed in colony.

Initially, the number of *Cx. erraticus* egg rafts laid in colony from the wild was very few even though the females were feeding on the bovine blood and had distended abdomens. The engorged females would not deposit their eggs on the surface of the water. It was thought that the sodium citrate and antibiotics used in the bovine blood was having some effect but ruled out later into the rearing process.

Klein (1985) placed his wild caught mosquitoes in an 18 x 18 x 21 cm cage with an *A. carolinensis* or *Sc. undulatus* lizard for blood feeding for 24 hours, then placed engorged mosquitoes in individual 15 ml oviposition vials half full of water with cotton sealing the top. Klein also put a leaf of duckweed in each vial but later found that the mosquito would lay its eggs on the surface of the water or sides of the culture dish, so Klein discontinued the use of plants as an oviposition substrate. Several *Melanoconion* species also display this same oviposition behavior (Chapman and Barr 1969, Osgood 1971, Takahashi 1968a). Klein would also feed his *Cx. erraticus* on baby chicks to enhance the gonotrophic cycle. However, the Institutional Animal Care and Use Committee (IACUC) prohibited the use of animals including reptiles in this study for the purpose of blood feeding mosquitoes for egg production. However, Dr. Sandra Allan (USDA, Gainesville, FL) has an approved protocol to use chickens, so I was allowed to expose approximately 300 *Cx. erraticus* to an adult chicken for approximately 1 hour and only one wild *Cx. erraticus* mosquito fed on the chicken. The feeding time took place in the afternoon in a lighted room in the insectary. The mosquito that took a partial blood meal on the chicken, failed to lay its egg raft in the rearing cage.

Following Klein's protocol, the bovine blood-fed females were placed individually in separate vials half filled with water and with screen tops with a piece of cotton soaked with 10% sugar solution placed on top of the screen. The vials were examined daily for egg rafts. The number of egg rafts did increase by 20% but not enough to establish a large and stable colony at this particular time.

The number of *Cx. erraticus* egg rafts increase significantly in the oviposition container when other rafts and/or exuviae from previous larvae and pupae were present. This ovipositioning behavior may possibly include pheromones from the previous exuviae, visual cues and ovipositional cues including the apical droplet on the apex of the eggs, as documented for various species of mosquitoes including the genus *Culex* (Aharoni and Zweig 1973, Allan and Kline 1995, Dawson et al.1989, Iltis and Zweig1962, Kramer and Mulla 1979, Laurence and Pickett 1985, Millar et al.1994). The individual oviposition vials in this study were discontinued and the open oviposition container in each cage was continued to obtain the necessary quantity of egg rafts for this study and to establish a laboratory colony. The *Cx. erraticus* eggs described in this study match previous descriptions (Mattingly 1970, 1976).

The mean number of eggs in a wild *Cx. erraticus* egg raft averaged 109 and the colony rafts averaged 81.8 eggs with development taking approximately 2.5 to 3.5 days to hatch in this study. Comparing this study to Klein (1985), he did not report the number of egg rafts, number of eggs per egg raft nor the number of egg rafts placed in the enamel rearing pans. Klein (1985) reported that he used deionized water and placed a small amount of Tetra® Fish Food in the pans daily. He did not report the amount of diet used in each pan of 100 to 150 larvae per pan. Klein also did not report the mortality of

larval, pupal and adult stages. Of the four larval diets, the Tetramin® Tropical Flakes Fish Food combined with brewers yeast (FFBY), and the Tetramin® Tropical Flakes Fish Food (FF) yielded the same number of egg rafts but different number of eggs.

The number of egg rafts produced from the three generations of the FF diet was 36 with a combined total of 3,268 eggs compared to the FFBY diet producing 36 egg rafts with a total of 2,979 eggs. At this time, there is no explanation why the FF diet produced the highest number of eggs during generation one while the FFBY diet produced its highest number of eggs during generation two. One possible explanation is that the first generation of adults in the FF diet was more fit resulting in more adult females laying egg rafts or more egg raft being produced by fewer females. Also, the FFBY diet may have produced healthier females, which provided the increase in the number of egg rafts and total number of eggs during the second generation. However, both FF and FFBY diets drastically decreased in the number of egg rafts and total number of eggs in the generation three.

The treatment Tetramin® Fish Food combined with powdered milk yielded significantly fewer rafts and total number of eggs, and the eggs took approximately four days to hatch. It appears that the powdered milk could have something in it that affects the fecundity and reproduction of *Cx. erraticus* because this diet failed to produce a third generation. Interestingly the Tetramin® Fish Food combined with powdered milk and brewer's yeast (FFPMBY) produced three less egg rafts than the FFBY and FF, however, the number of eggs produced by the FFPMBY treatment was the same as FFBY. The average hatching time of the FF, FFBY and FFPMBY eggs was 2.5 days. The mean number of egg rafts per female average 2.5 to 3.1, regardless of diet.

The larva described in this study matches that found in the literature (King and Bradley 1937). The *Cx. erraticus* larvae are green in color, which is common in other species of mosquitoes, which change in pigmentation according to the color of the environment, especially *An. albimanus* Wiedemann, *An. quadrimaculatus* Say, *Cx. quinquefasciatus* Say, *Cx. salinarius* Coquillett, *Ae. aegypti* (L) and *Ae. taeniorhynchus* Wiedemann (Benedict and Seawright 1987). A change in color at will by the larva to blend in with the environment is termed homochromy and is used for protection and is executed from a visual response of the larva's environmental background (Benedict and Seawright 1987). It is not known if this is the case with *Cx. erraticus* larvae since they were green in the white enamel pans. It is possibly a physiological occurrence that normally takes place within the larva that is genetically predisposed for survival no matter what the background. The favored environment of this species of mosquito is grassy shallow margins of ponds with various other aquatic vegetations, which may account for the blending in with the environment (Furlow and Hays 1972, Lothrop et al 1995, Lounibos and Escher 1985, Self and Sebastian 1971).

The mean life span of *Cx. erraticus* reared in the FFPM diet had the shortest life span of 14.1 days in comparison to the three other diets. The *Cx. erraticus* remained in the longest life cycle of 21.2 days and had a 93.3% egg hatch rate. No significance was found among the three generations or within the three generations among treatments for the number of eggs hatching therefore, showing that the diet medium was not significant in the hatching of the eggs. The mean life span of *Cx. erraticus* larvae is 18.6 ± 2.3 days, which is similar to the other 10 species of *Melanoconion* mosquitoes having means ranging from 17 days to 22 days (Chadee and Tikasingh 1985). The larval mortality in

all four diets were significant enough that the reduction of pupae and adults drastically affected the success of the colony.

It is important to know the percentage of eggs that developed into pupae because there is a difference in the number of larvae that develop into pupae and the number of eggs that actually make it to the pupal stage. The difference in, the number of larvae making it to the pupal stage and those that do not is known as the percent mortality.

There was a significant increase in the F_2 and F_3 generations in the number of eggs that developed into the pupal stage. The two diets with brewer's yeast produced the highest number of eggs that developed into pupae. There is a pattern thus far with treatment FF showing the same decrease in the mean number of eggs, mean number of larvae and the mean number of pupae. It appears that the properties in the FF alone may not be enough to effectively promote larval development. The FFPM diet did not show a significant difference in the number of eggs developing into pupae and this diet also failed to produce a third generation. This is important because the total number of eggs in the FFPM diet was 1,560, and 1,426 (91%) of the eggs hatched with 638 (44.7%) making it to the pupal stage, creating a larval mortality rate of 55.2%, or 778 larval deaths.

The colony adult female matched the *Cx. erraticus* trapped in the wild, as previously described (King and 1937). The number of eggs that developed into the fewest adults was found in treatment FFPM. Only 0.08% of the total number of eggs developed into adults. The larval fitness played a very important role in this diet failing to produce fit adults as well as only producing two generations. The larval fitness in the FFPM diet had all indications that the diet may not have contained the necessary nutrients

for *Cx. erraticus* to complete its life cycle. The overall production of adults in the other three treatments is low considering the FF diet had 3,268 eggs develop into 396 adults (12.1%). The FFBY diet had 20% and FFPMBY diet had 13% of its eggs develop into adults.

A significant difference was found in the mean number of larvae that developed in to pupae. It appeared that the significance favoring the FFBY and the FFPMBY diets over the FF and FFPM was because the former two diets had brewer's yeast which is rich in amino acids and chromium and has been used in many successful rearing protocols for mosquitoes (Smith and Brust 1971; Lang 1978; Bradshaw and Lounibos 1972). At this time it is unknown how the F_2 and the F_3 generation produced a higher mean of pupae than the F_1 generation even though treatment FFPM did not produce a third generation itself.

Although significance wais found within this study in the mean number of larvae, pupae and adults, there was a set pattern that repeated itself within each model pointing to larval fitness early in the rearing process. The *Cx. erraticus* larvae required more than these larval diets were providing and it appeared that the longevity of the larval life span may be a key to the success of this insect in the wild.

Larval fitness is a huge part in the reproduction strategy of insects and *Cx. erraticus* is no different. The initial number of eggs produced by the F_1 generation adults decreased in the F_2 generation by 2,000 eggs, which is an extremely large deficit and first indicator that the colony adults were not reproducing as they should. Also, the mortality of the larvae was another indication of how the colony was doing. With the decrease in

the number of larvae developing into pupae a decrease also occurred in the number of adults produced.

The mean number of males was higher than the mean number of females in each treatment and within each generation. A decrease in the number of females will inevitably bring about a decrease in the number of eggs produced.

The lack of proper larval nutrients was most likely the key explanation why the colony had a high mortality rate in the larvae and also why the fitness of the pupae was not strong enough to survive the eclosion process on the water surface. Many of the adults died during eclosion, which also caused a decrease in the colony number and in the numbers of viable eggs being deposited. Dadd and Kleinjan (1974) examined the larval nutrient requirement of *Cx. pipiens* L. and found that when mammalian serum lipoproteins are added to the larval water, the insects have faster larval development, there are significantly more adults that could more easily eclose from the pupa, and the newly emerged adults could recover faster to free themselves from the water surface and survive for longer periods of time. Klein (1985) augmented the *Cx. erraticus* larval rearing water with a liquid vitamin, but there is no data on how much is added or how effective this is in the fitness of his larva and adults.

Klein fed his adult female *Cx. erraticus* on baby chicks for 24 hours, which is important for the fecundity of the females, and for the fitness of the newly hatched larvae. The citrated bovine blood used in this study also contained antibiotics to reduce the spoilage and at the same time the antibiotics could have had an adverse affect on the mosquitoes.

One of the problems in rearing *Culex (Melanoconion) portesi* was that the adults would not mate in the laboratory environment, so the rearing took place in the wild. Mating did occur in this study as F_1 and F_2 viable eggs were produced, indicating that *Cx. portesi* was specific in its mating behavior in the wild which was not demonstrated in the laboratory. *Culex erraticus* brought in from the wild would mate under laboratory conditions and the adult female would readily feed on the bovine blood and deposit egg rafts. However, the adult fitness is questionable because of the bovine blood and living in closed conditions in the laboratory without the environmental factors. This study would have been more successful if *Cx. erraticus* had been established as a stable and flourishing colony.

Knowing the age of the mosquito for feeding purposes, and using colony reared mosquitoes for disease transmission would have validated how many days after eclosion that the males and females mated, how many days after mating did the females take the first blood meal, the length of time it took to digest the blood meal and deposit egg rafts, and how long after deposition of eggs the females waited before taking another blood meal.

The feeding habits of *Cx. erraticus* are important because it is not known if taking multiple blood meals from different hosts is a key to the fecundity of the female and to the fitness of the female and her progeny. It is important to know if feeding only on *A. carolinensis* satisfies the requirements of *Cx. erraticus* for egg development and transmission of *P. floridense.* With one in four wild *A. carolinensis* lizards in this study found to be infected with *P. floridense*, it is not known if they were only fed upon by *Cx. erraticus* and if the success of the transmission in the wild is based solely on *Cx.*

erraticus. Also, it is not known if the laboratory conditions are partly responsible for the fitness of the mosquito colony. Klein (1985) utilized partial sunlight in combination with illumination of florescent lighting to maintain his colony (personal communication).

Because of the lack of numbers and quality of *Cx. erraticus* production in colony, our colonization objective was not met. Therefore, field collected mosquitoes were used for olfaction and transmission studies.

CHAPTER 3
CULEX ERRATICUS TRAPPING STUDY

Introduction

Collection methods for sampling and studying populations of mosquitoes have become more elaborate as the quest for understanding the mosquito's ability to find its host continues (Ali et al. 1989, Bidlingmayer 1985, Bidlingmayer et al. 1985, Carestia and Horner 1968, Carestia and Savage 1967, Gladney and Turner 1970, Headlee 1932, Kline 1999, 2002; Kline and Mann 1998; Kline et al 1990a, b; Sudia and Chamberlain 1962). Light trap sampling systems for mosquitoes were first introduced in the United States because direct sampling mechanical methods were labor intensive and it was apparent that mosquitoes' attraction varied between hosts (Headlee 1928).

Female mosquitoes are known to be attracted to light and are trapped in light traps during host-seeking in order to complete ovarian development (Service 1995). Headlee (1932) developed the New Jersey light trap, increasing the trap catch rate and the variety of female mosquitoes collected. Carbon dioxide was initially suggested by Rudolphs (1922), as an agent to activate mosquitoes to find their host, and Headlee (1934) reported a 500% increase in adult female mosquitoes collected when carbon dioxide was used in combination with the New Jersey light trap. However, the New Jersey light trap was limited because it required locations close to electrical power or portable generators, thus making the availability to remote areas limited. Sudia and Chamberlain (1962) introduced the Centers for Disease Control (CDC) miniature light traps for collecting live mosquitoes.

The CDC miniature light traps are portable and operate from four 1.5-volt D cell batteries or one 6-volt battery. Burkett (1998) modified the CDC light trap with blue and green diodes as a replacement for tungsten bulbs. When the light trap was combined with cylinder carbon dioxide, he reported a significant increase in the numbers of mosquitoes trapped, especially *Cx. erraticus*.

Burkett found that female mosquitoes used visual cues when targeting the blue and green diodes that illuminated the underside of the aluminum top of the modified CDC light trap. Burkett also found that when the diodes were in a 360º position facing away from the trap, a significant increase in capture rate of *Cx. erraticus* took place compared to when the diodes faced up. It has long been suspected that haematophagous insects use visual cues, chemical cues, host odors and environmental cues to locate oviposition, mating, sugar/nectar location, over-wintering, and appetitive flight and host location (Allan et al. 1987, Laarman 1955, 1958, Service 1995)

Various compounds, including carbon dioxide, lactic acid and octenol, has been shown to be excellent attractants and are used to increase the numbers of mosquitoes captured in various types of light traps (Gillies 1980, Hall et al. 1984, Kline 1994a). In East Africa, Vale and Hall (1985) discovered an increase in the capture of tsetse flies when octenol, a volatile found in the breath of oxen, was used. Other studies have shown octenol combined with carbon dioxide as a successful attractant (Kline et al. 1991b; Kline 1994b). However, in this study the ability of *Cx. erraticus* to locate the *Anolis carolinensis* lizard by expelled carbon dioxide and/or body odors is undetermined.

Sceloporus occidentalis, a common fence lizard, weighs approximately 15.76 grams and produces about 1 ml of CO_2/hour (Hopkins 2004 personal communication)

compared to 250 ml of CO_2/minute in the CDC light traps. Dr. Hopkins further explained that if this lizard ate a meal weighing 4% of its body mass, which is a sizeable consumption, the lizard would increase its metabolism briefly to about 3.5 ml of CO_2/hour. These figures are a fraction of the amount of CO_2 used in the CDC modified light traps, which range from 250 ml/min to 500 ml/min. Also, the amount of environmental CO_2 plays an important role in the mosquito's ability to locate the lizard, therefore, it is speculated that the *A. carolinensis* may produce an attractant through surface glandular production through the skin similar to other attractants found in mammals, birds and reptiles (Kline et al. 1990a).

One objective of this study was to determine if the *A. carolinensis* lizard has attractive constituents and compounds on the surface of its skin, thus the washing of lizards that died in colony with filtered hexane was important in this study to establish if these compounds could act synergistically with CO_2, and/or blue and green LED lights to attract *Cx. erraticus.*

The second objective was to modify CDC trapping systems to maximize *Cx. erraticus* catch rates. In this study, CDC light traps were modified with blue and green LED diodes facing out combined with 250 ml/min of cylinder carbon dioxide (CO_2) and lizard extract (LE) collected from deceased colony *A. carolinensis* lizards that were washed in filtered hexane to evaluate the catch rate of *Cx. erraticus* mosquitoes.

The third objective was to sample different aquatic habitats to help establish where *Cx. erraticus* was located and to select a location for a 30-day randomized field evaluation of lizard extract (LE) to take place.

Materials and Methods

Center for Disease Control Light Trap Model 512

A Center for Disease Control (CDC) light trap (John W. Hock Company, model 512, Gainesville, FL) was modified using four blue LED (470 nm ± 50 nm, 800 mcd, 22º [Panasonic,® Digikey Corp., Thief River Falls, MN]) and one green LED (567 nm ± 50 nm, 2400 mcd, 8º [Toshiba Tosbright,® Martech Optoelectronics, Latham, NY]). The trap was powered by four alkaline D-cell batteries at 6.4 ± 0.4 volts. To insure each LED received the same amount of power, four 0.25 watt 180 ohm resistors were soldered in series with the diodes. Carbon dioxide #20 cylinders were equipped with a two-stage regulator (Victor Equipment Company, model VTS 453B-320, Denton, TX) and a flow meter (Gilmont Instruments, no. 12, Great Neck, NY) to maintain 250 ml/min gas flow. The carbon dioxide was transported through a 1 m length of tygon tubing with a ¼-inch inside diameter attached to the top edge of the trap with duct tape. Lizard extract (LE) was collected from colony deceased *Anolis carolinensis* lizards washed for 24 hours in 100 ml of filtered hexane. This extract was then concentrated under nitrogen to 10 ml. Dispensing the LE in the field traps was accomplished by placing 5 ml of concentrated LE in a 5 ml graduated micro-vial (Accuform Manufacturing, Fisher Scientific, O.D. 21 mm, Length 62 mm, GPI thread size 20-400, Kimble No. 60700-5, Catalog No. 06-100F) with a wick (1 cm x 1 cm, Hydrophobic Rods, Interflo polyethylene Pellet, Part #p375-3, Formulation No. F/N: 35-162-4, Interflo Technologies, Brooklyn, NY) dispensing the LE at a rate of approximately 0.05 ml/hr. A catch basket made of screen netting was attached to the bottom of the trap to collect live mosquitoes and other insects. A 10% sugar solution with a wick made of blotter paper (Domtar #80054600, 19x24, 17 pts.

148m, Unisource, Orlando, FL) was suspended in the trap to keep the mosquitoes from desiccating. The modified CDC light trap was run at each trapping site for one week from 5:00 pm until 8:00 am with daily temperatures being recorded by a four-lead Hobo® data logging device (Outdoor/Industrial 4-Channel External logger, Onset Computer Corporation). The trapping site with the highest population of *Cx. erraticus* was selected as the habitat where the 30-day randomized trapping study of LE would take place.

Lizard Extract

A crude lizard extract used in field and laboratory experiments was obtained from deceased *Anolis carolinensis* lizards that died in colony. Dead lizards were washed in 100 ml of hexane for 24 hours to obtain lizard extract (LE). The lizards were removed from the hexane and incinerated as per protocol and the 100 ml of lizard extract was concentrated under nitrogen to 10 ml. The concentrated lizard extract was used in the olfactometer to determine attractant activity and to establish the optimum dosage. Lizard extract was also evaluated by gas chromatography to determine presence of compounds in the crude extract.

30-Day Randomized Trapping Study

Three modified CDC light traps wired the same as in the previous trapping studies were used in combination with cylinder carbon dioxide (250 ml/min) and lizard extract. The light traps were randomly situated at least 300 feet apart to minimize interference and competition from each other at three different sites in the habitat. One trap had four blue and one green LED, cylinder carbon dioxide at a flow rate of 250 ml/min and lizard extract. The second trap had four blue and one green LED, cylinder carbon dioxide at a flow rate of 250 ml/min and 5 ml of filtered hexane (control). The third trap had four blue and one green LED and cylinder carbon dioxide flow rate of 250 ml/min. The traps

were run for three days at each site from 5:00 pm until 8:00 am, and then randomly rotated every fourth day until the end of the 30-day regime. Temperature data was collected by a four-lead Hobo® data logger recording device for ambient temperature, wet bulb, ground temperature and pond water temperature. The data was downloaded by the boxcar® program. The daily trapped mosquitoes were taken to the laboratory, identified, counted, and the live *Cx. erraticus* were placed in colony cages for nutrition rearing studies.

Statistical Analysis

For each of the six trapping sites An ANOVA General Linear Model (GLM) was used to compare the number of species and the number of mosquitoes in each species.

In the 30-day trapping study, the traps were manually randomized by arbitrarily selecting one of the three treatments, CDC light trap with CO_2, CDC light trap with CO_2 and hexane or CDC light trap with CO_2 and lizard extract. Each of the three sites was visited by each of the three treatments 10 times during the 30 day study. Each treatment remained at each site for three days then randomly rotated on the fourth day. Each trap was emptied daily with mosquitoes being counted and identified. A 3-way ANOVA (SAS Institute, 2002) was used for evaluating the site, treatment and number of mosquitoes trapped at each site.

Blues Creek Subdivision Alachua County, Florida

The Blues Creek study site (Figure 3-1) is located behind the clubhouse in Blues Creek Subdivision in a wooded area where *Cx. erraticus* was previously identified by Dr. Daniel Klein (personal communication). This site includes leaves thickly covering a

stagnant water surface prohibiting the growth of aquatic plants and allowing the thick growth of trees and small shrubs as shown in Figure 3-2.

Figure 3-1. Blues Creek Subdivision behind clubhouse.

Figure 3-2. Blues Creek Subdivision pond water behind clubhouse.

James Park Property Trapping Study, Marion County, Florida

The James Park site in Marion County, Florida, was selected as the second location. This study site was conducted around a three acre shallow natural pond with grassy margins and trees over hanging the pond's edge, enclosing and separating the aquatic habitat from other ecosystems in the area (Figure 3-3). This habitat has dense hardwood and cypress swamp forest that has fluctuating water levels when it rains (Minno et al. 2005). The water level of this pond is normally shallow throughout the

year, but during heavy rains the water level rises significantly above ground surface. The natural pond gradually slopes toward the center to an approximate depth of three feet. Cypress trees are the dominant species of trees interspersed with water oaks and several magnolia trees. Tall grasses are dispersed throughout the pond in dense areas as well as surrounding a large alligator mound. Aquatic vegetation of various types, including small and large duckweed, is dispersed evenly throughout the pond.

Figure 3-3. Modified CDC light trap on James Park property.

County Road 2082, Alachua County, Florida

The county road 2082 site was selected as the third location. This study site is located in an isolated small forest area that runs parallel along county road 2082 east of Gainesville, Florida (Figure 3-4). The peripheral habitat is a shallowly flooded area similar to bottomland hardwood swamp with certain areas that are exceptionally low and prone to flooding during heavy rains (Minno et al. 2005). This low area appears to be a man made drainage ditch with mostly void of direct sunlight and aquatic vegetation, and is adjacent to a vacant open field with high grasses and with poor drainage and

percolation of the soil. The standing water is muddy with a strong stagnant odor (Figure 3-5), and it is evident that open artificial containers have accumulated over time from passing motorists. Fallen trees and limbs lie decaying in the water and are damming the water in several areas. *Culex erraticus* was previously identified from this area in earlier trappings by personnel from the Center for Medical and Veterinary Entomology (CMAVE), USDA, Gainesville, Florida.

Figure 3-4. CDC light trap in forest area along county road 2082.

Figure 3-5. Stagnant water in forest area along county road 2082.

Hatchet Creek Trapping Study, Alachua County, Florida

The Hatchet Creek site was selected as the fourth location. Hatchet Creek on state road 26 is a natural free-flowing creek that empties into Newman's Lake in eastern Alachua County, Florida. The trap was set up along the creek bank, which was shaded with large trees and other small shrub-like plants (Figure 3-6). The surrounding habitat is similar to a tropical hardwood hammock because of the diversity of shrubs, plants and trees are in high numbers (Minno et al. 2005). The thick canopy of trees overhanging the creek blocks most of the sun, thereby decreasing the ambient and water temperature significantly. The creek had minimal amounts of water lettuce and was densely covered with large and small duckweed (Figure 3-7). The dense matting of duckweed reduced the flow of the creek significantly, and the numbers of artificial containers located under the bridge were numerous. This site was chosen because Klein et al. (1987) collected *Cx. erraticus* using *A. carolinensis* lizard baited traps along this creek.

Figure 3-6. Modified CDC light trap along Hatchet Creek.

Figure 3-7. A dense infestation of aquatic vegetation covering Hatchet Creek.

University of Florida Equine Teaching Center, Alachua County, Florida

The University of Florida Equine Teaching Center was selected as the fifth location. This study site is about 0 acres with free standing pond that is densely covered with water hyacinth, water lettuce, and large and small duckweed (Figure 3-8). Earlier trapping studies conducted by other researchers found *Cx. erraticus* in high numbers during different times throughout the summer. This aquatic habitat has minimal trees, decreasing the amount of shade on all areas of the habitat. The topography of the pond varies in many areas causing depth variance throughout and around the pond. The pond is located in the middle of several horse pastures where equine studies are conducted. Other trapping studies are being conducted simultaneously around the compound, but none were being conducted at the time of this trapping study.

Wade Smith Property, Marion County, Florida

The Wade Smith property site was selected as the sixth location for this study. This site is about five acres with a free standing pond with grass and other aquatic vegetation scattered throughout the pond. This habitat has shallow grassy margins with

overhanging trees allowing favorable amounts of sunlight around the pond, yet creating microhabitats ideal for many vertebrates and invertebrates (Figure 3-9). The surrounding habitat is a pine savanna, which is related to the flatwoods with a dense covering of mixed grasses and wildflowers (Minno et al. 2005). The study site is situated in the middle of a pine savanna with sporadic placement of hardwood trees encompassing the habitat. The owner stated that the pond usually remains around four to six feet deep but may reach a depth of 10 feet during heavy rainfall with moderate drainage. The edges of the pond have high vegetative growth with various tall grasses extending into the pond. The shallow areas in the middle of the pond have several alligator mounds with grasses surrounding them, and the swimming trails in the tall grasses made by the alligators are obvious from personal observation.

Figure 3-8. University of Florida Equine Teaching Center Trap.

Figure 3-9. Free standing five acre pond Wade Smith property.

Results

The six mosquito trapping study sites were evaluated using modified CDC light traps combined with CO_2 and lizard extract (LE). The purpose of these studies was to evaluate the performance of the LE under field conditions in various trapping sites in Marion and Alachua counties, Florida. The selected six habitats were important for identifying the target mosquito *Cx. erraticus*, in its ideal habitat, but the main focus of the study was to evaluate the lizard extract as an attractant by the target mosquito.

Cx. erraticus prefers aquatic habitats associated with aquatic vegetation such as water lettuce, water hyacinth, *Lemna*, large and small duckweed, and various aquatic grasses (Bradley 1932, King et al.1960, Smith and Enns 1967).

Blues Creek Subdivision

Using the modified CDC light traps with LE and CO_2, 1.2% of total trapped mosquitoes (n=972) at Blues Creek were *Cx. erraticus* (n=12) (Table 3-1). Although a significant difference ($p=<0.0001$; $df=9$; $F=34.00$) was found for the number of trapped mosquitoes of each species (Table 3-2), no significant difference was demonstrated for

the number of mosquitoes trapped each day. Figure 3-10 shows the daily mean number of 10 species of adult mosquitoes trapped from June 4, 2001 through June 10, 2001.

Anopheles crucians had the highest number of mosquitoes of the 10 species trapped at this location. The ambient temperature for the seven trapping days averaged 25.8ºC and the water temperature averaged 23.2ºC.

Table 3-1. Daily mean ± standard error of mosquitoes trapped at Blues Creek Subdivision for seven days. Percent of *Cx. erraticus* in bold lettering.

Blues Creek Subdivision (June 4-10, 2001)			
Species Name	Adult Females	Mean ± SEM	%
An. crucians	680	97.1 ± 15.0	70.0
Cq. perturbans	161	23.0 ± 6.0	16.6
Cx. nigripalpus	5	0.7 ± 0.3	0.5
Cx. salinarius	63	9.0 ± 1.8	6.5
Cx. erraticus	**12**	**1.7 ± 0.5**	**1.2**
Ps. ciliata	7	1.0 ± 0.4	0.7
Ps. howardii	8	1.1 ± 0.6	0.8
Ma. dyari	3	0.4 ± 0.2	0.3
Ma. titillans	3	0.4 ± 0.3	0.3
Aedes spp.	30	4.3 ± 1.6	3.1

Table 3-2. Analysis of variance for the species and number of mosquitoes trapped with modified CDC light trap, carbon dioxide (250 ml/min) and lizard extract at Blues Creek Subdivision.

ANOVA Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	57001.66	9	6333.52	34.00	2.45E-20	2.04
Within Groups	11175.43	60	186.26			
Total	68177.09	69				

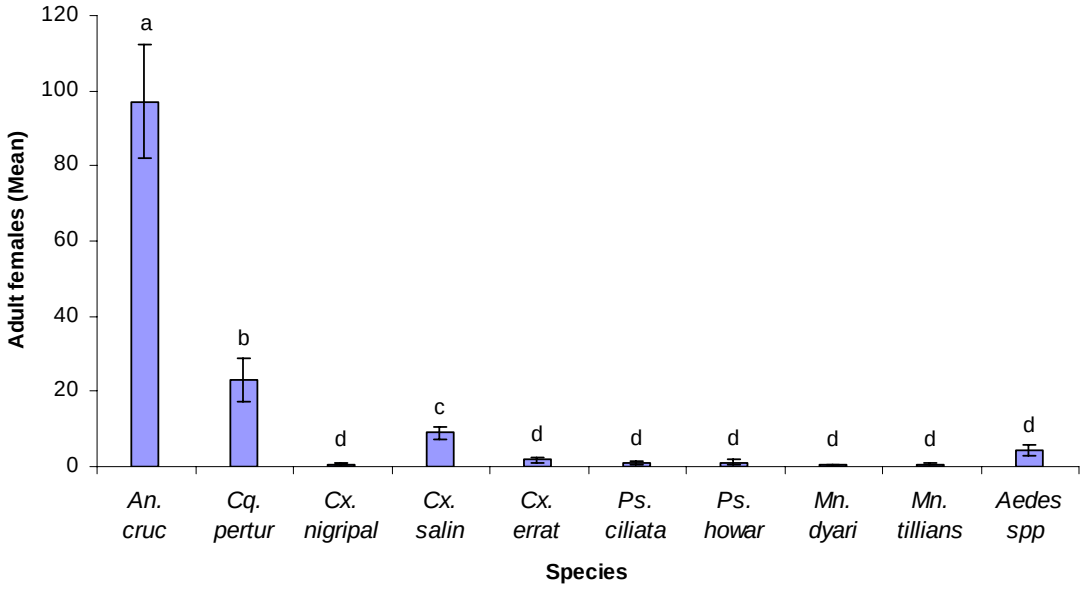


Figure 3-10. Daily mean number of adult mosquitoes trapped at Blues Creek Subdivision for seven days with modified CDC light trap, 250 ml/min carbon dioxide and lizard extract. Mean with same letter not significantly different.

James Park Property

A significant difference (p=<0.0001; df=12; F=4.14) was found between the number of mosquitoes trapped in a total of 13 species (Table 3-3). The number of *Cx. erraticus* (n=1510) contained 40.9% of the total mosquitoes (n=3691) trapped (Table 3-4). Figure 3-11 shows the mean number of mosquitoes trapped in each species. The ambient temperature for the seven trapping days averaged 27.4ºC and the water temperature averaged 26.8ºC. Trapping study was conducted from June 11, 2001 until June 17, 2001.

Table 3-3. Analysis of variance for the 13 species and number of mosquitoes trapped on the James Park property in modified CDC light trap, carbon dioxide and lizard extract.

ANOVA Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	299802.99	12	24983.58	4.14	5.407E-05	1.88
Within Groups	471057.43	78	6039.20			
Total	770860.42	90				

Table 3-4. Daily mean ± SEM for seven trapping days on the James Park property. Percent *Cx. erraticus* in bold.

Species	Mean ± SEM	Total	Percent
Cx. salinarius	18.86 ± 4.2	132	3.6
Ps. ciliata	5.14 ± 1.5	36	1.0
Ps. ferox	25.29 ± 4.4	177	4.8
Cx. quinquefasciatus	7.14 ± 1.6	50	1.4
Cx. erraticus	**215.71 ± 102.7**	**1510**	**40.9**
Cq. perturbans	7.14 ± 1.8	50	1.4
Ma. titillans	6.43 ± 1.9	45	1.2
Cx. pilosus	2.71 ± 0.7	19	0.5
Cx. restuans	33.29 ± 7.4	233	6.3
Cx. nigripalpus	95.14 ± 15.0	686	18.0
Ochlerotatus. spp	38.29 ± 6.7	268	7.3
An. crucians	69.86 ± 17.5	489	13.2
Aedes spp	2.29 ± 0.5	229	0.4

County Road 2082

Cx. erraticus (n=8) accounted for only 0.8% of the total mosquitoes (n=991) trapped on county road 2082 (Table 3-5). A significant difference ($p=<0.0001$; $df=6$; $F=15.99$) between the number of mosquitoes in each of the 10 species (Table 3-6), and no difference ($p=0.831$; $df=6$; $F=0.46$) was found in the number of mosquitoes trapped each day. The average ambient temperature for the seven day trapping was 27.1ºC and the water temperature averaged 23.9ºC. County road 2082 trapping study was performed on June 18, 2001 until June 24, 2001.

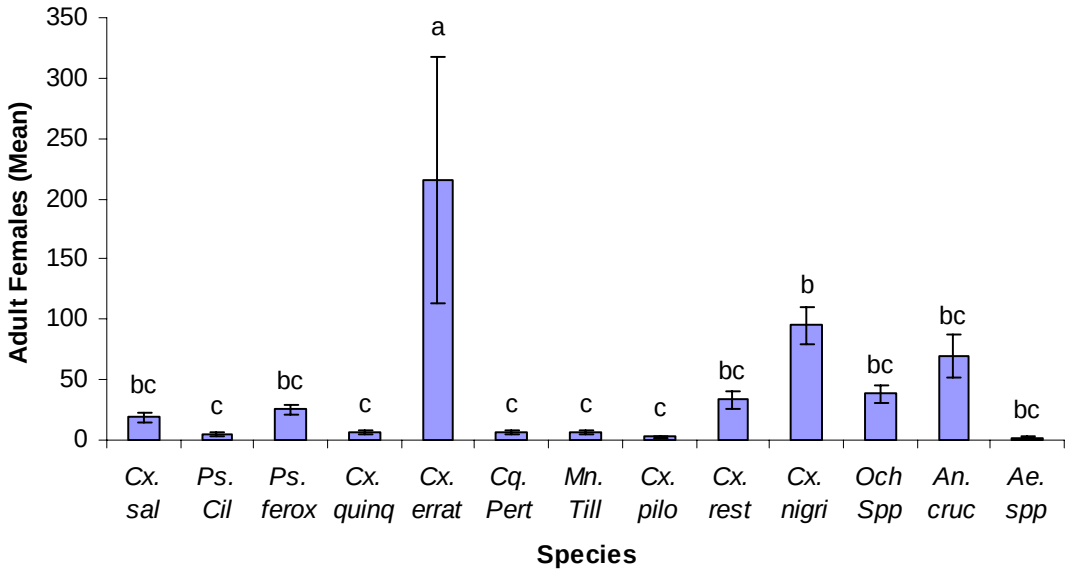


Figure 3-11. Daily mean number of adult mosquitoes trapped in seven days on the James Park property with modified CDC light trap with 250 ml/min carbon dioxide and lizard extract. Means with same letter are not significantly different.

Table 3-5. Daily mean ± SEM per seven trapping days along county road 2082. Percent *Cx. erraticus* in bold.

Species	Mean ± SEM	Total	Percent
Aedes spp.	17 ± 2.3	117	11.8
Cx. restuans	26 ± 3.8	181	18.3
Cx. erraticus	**1 ± 0.4**	**8**	**0.8**
Cx. quinquefasciatus	24 ± 3.2	171	17.3
Ps. ferox	5 ± 1.3	37	3.7
Ps. ciliata	12 ± 1.8	84	8.5
An. crucians	56 ± 10.5	393	39.7

Table 3-6. Analysis of variance for the seven species and number of mosquitoes trapped along county road 2082 using a modified CDC light trap, carbon dioxide and lizard extract.

ANOVA						
Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	14047.39	6	2341.23	15.99	1.82E-09	2.32
Within Groups	6149.14	42	146.41			
Total	20196.53	48				

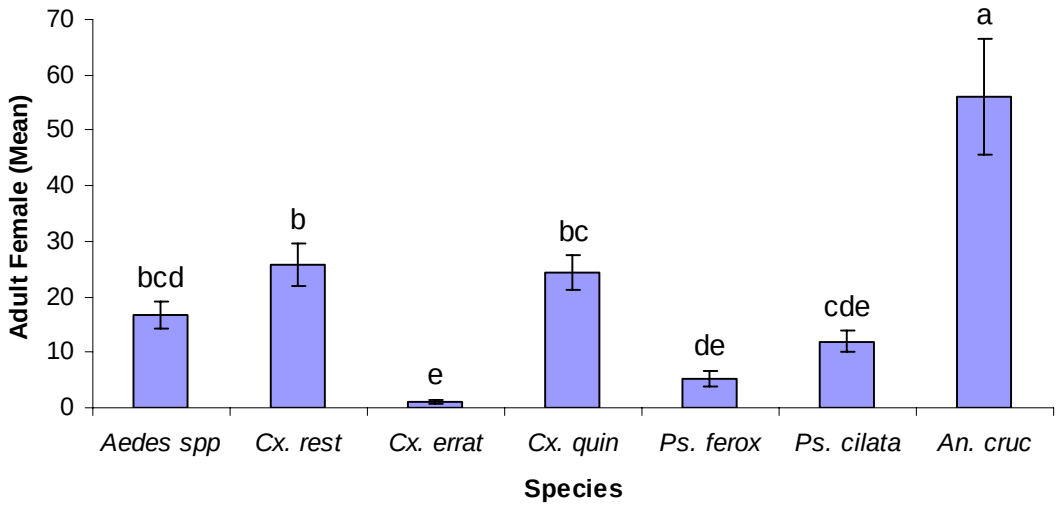


Figure 3-12. Daily mean number of adult mosquitoes trapped along county road 2082 for seven days using a modified CDC light trap, 250 ml/min carbon dioxide and lizard extract. Means with same letter are not significantly different.

Hatchet Creek

Significance (p=<0.0001; df=11; F=15.73) was found between the total numbers (n=2090) of mosquitoes trapped of the12 species (Table 3-7). Of the total number of mosquitoes trapped, *Cx. erraticus* (n=404) accounted for 19.3% (Table 3-8). Figure 3-4 shows the total mean number of mosquitoes trapped in each species. Average ambient and water temperature were 26.2ºC and 24.7ºC, respectively. The trapping study started on June 25, 2001 and continued until July 1, 2001.

Table 3-7. Analysis of variance for the 12 species of mosquitoes trapped along Hatchet Creek.

ANOVA Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	145197.67	11	13199.79	15.73	4.2E-15	1.92
Within Groups	60429.14	72	839.29			
Total	205626.81	83				

Table 3-8. Daily mean ± SEM on the 991 mosquitoes trapped along Hatchet Creek for seven days. Percent *Cx. erraticus* in bold.

Species	Mean ± SEM	Total	Percent
An. crucians	154.6 ± 36.6	1082	51.8
Cq. perturbans	16.7 ± 2.4	117	5.6
Ma. titillans	8.1 ± 2.2	57	2.7
Cx. quinquefasciatus	9.1 ± 2.2	64	3.1
Cx. erraticus	**57.7 ± 8.0**	**404**	**19.3**
Cx. nigripalpus	4.7 ± 0.9	33	1.6
Cx. salinarius	6.7 ± 1.3	47	2.2
Ps. ciliata	4.9 ± 0.9	34	1.6
Ps. ferox	8.4 ± 2.0	59	2.8
Ps. columbiae	10.1 ± 1.5	71	3.4
Aedes spp.	1.6 ± 1.3	11	0.5
Ochlerotatus spp.	15.9 ± 3.2	111	5.3

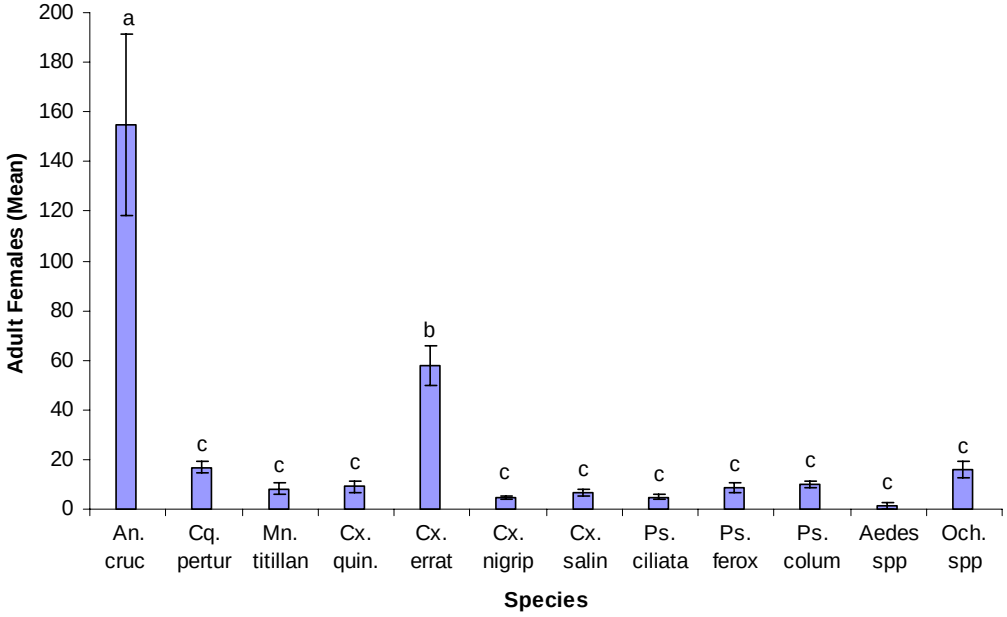


Figure 3-13. Daily mean number of mosquitoes trapped in seven days at Hatchet Creek. Means with the same letter are not significantly different.

University of Florida Equine Teaching Center

A significant difference (p=<0.0001; df=11; F=61.52) was found between the 12 species of mosquitoes trapped at University of Florida Equine Teaching Center (Table 3-9). Seven days of trapping yielded a total of 10,826 mosquitoes, of which 41.7% were *Cx. erraticus* (n=4516) (Table 3-10). The mean for *An. quadrimaculatus, Ur. sapphirina,*

Och. spp. and *Aedes spp.* were 2.29, 2.00, 1.29, and 1.00, respectively (Figure 3-14). The average ambient and water temperatures were 29.4ºC and 27.6ºC, respectively. This trapping study ran from July 2, 2001 until July 8, 2001.

Table 3-9. Analysis of variance for the 12 species and number of mosquitoes trapped at the University of Florida Equine Teaching Center using a modified CDC light trap, carbon dioxide and lizard extract.

ANOVA Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3388709.38	11	308064.49	61.52	4.2033E-32	1.92
Within Groups	360537.43	72	5007.46			
Total	3749246.81	83				

Table 3-10. Daily mean ± SEM of the species of mosquitoes trapped at the University of Florida Equine Teaching Center. Percent of total mosquitoes trapped with *Cx. erraticus* data in bold.

Species	Mean ± SEM	Total	Percent
An. crucians	164.1 ± 28.2	1149	10.6
An. quadrimaculatus	2.3 ± 0.5	16	0.1
Cq. perturbans	322.1 ± 49.4	2255	20.8
Ma. titillans	368.9 ± 36.8	2582	23.8
Cx. erraticus	**645.1 ± 63.1**	**4516**	**41.7**
Cx. nigripalpus	14.1 ± 3.1	99	0.9
Cx. salinarius	8.9 ± 2.0	62	0.6
Ur. sapphirina	2.0 ± 0.3	14	0.1
Ps. columbiae	6.0 ± 1.7	42	0.4
Ps. ferox	10.7 ± 1.4	75	0.7
Aedes spp	1.0 ± 0.4	7	0.1
Ochlerotatus spp	1.3 ± 0.4	9	0.1

Wade Smith Property, One Week Trapping

There is a significant difference ($p=<0.0001$; $df=9$; $F=105.63$) between the 10 species of mosquitoes trapped at the Wade Smith property (Table 3-11). A total of 11,682 mosquitoes were trapped, of which 63.3% were *Cx. erraticus* (n=7389) (Table 3-12). The mean of *Aedes* spp. and *Ps. ciliata* was so small that they do not show on the chart. Wade Smith trapping study was conducted from July 9, 2001 until July 15, 2001.

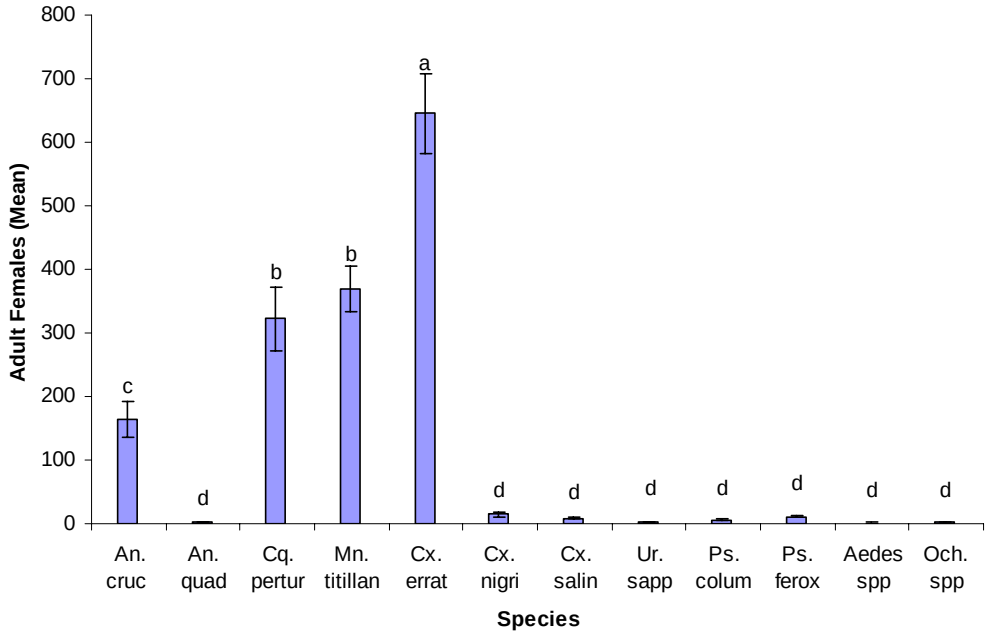


Figure 3-14. Daily mean number of mosquitoes trapped during seven day study at the Horse Teaching Unit. Means with same letters are not significantly different.

Table 3-11. Analysis of variance for the 10 species and number of mosquitoes trapped on the Wade Smith property for one week.

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	6684517.09	9	742724.1	105.63	2.1391E-33	2.04
Within Groups	421896.00	60	7031.6			
Total	7106413.09	69				

Table 3-12. Daily mean ± SEM of the species of mosquitoes trapped on the Wade Smith property for seven days. Percent of total mosquitoes trapped with *Cx. erraticus* data in bold.

Species	Mean ± SEM	Total	Percent
An. crucians	206.1 ± 16.2	1443	12.4
Cx. erraticus	**1055.6 ± 92.2**	**7389**	**63.3**
Cq. perturbans	207.9 ± 28.2	1455	12.5
Ochlerotatus spp	7.1 ± 1.5	50	0.4
Ps. columbiae	3.1 ± 0.5	22	0.2
Ps. ferox	2.1 ± 0.5	15	0.1
Ma. titillans	182.9 ± 21.9	1280	11.0
Aedes spp	0.9 ± 0.3	6	0.1
Cx. salinarius	2.3 ± 0.6	16	0.1
Ps. ciliata	0.9 ± 0.3	6	0.1

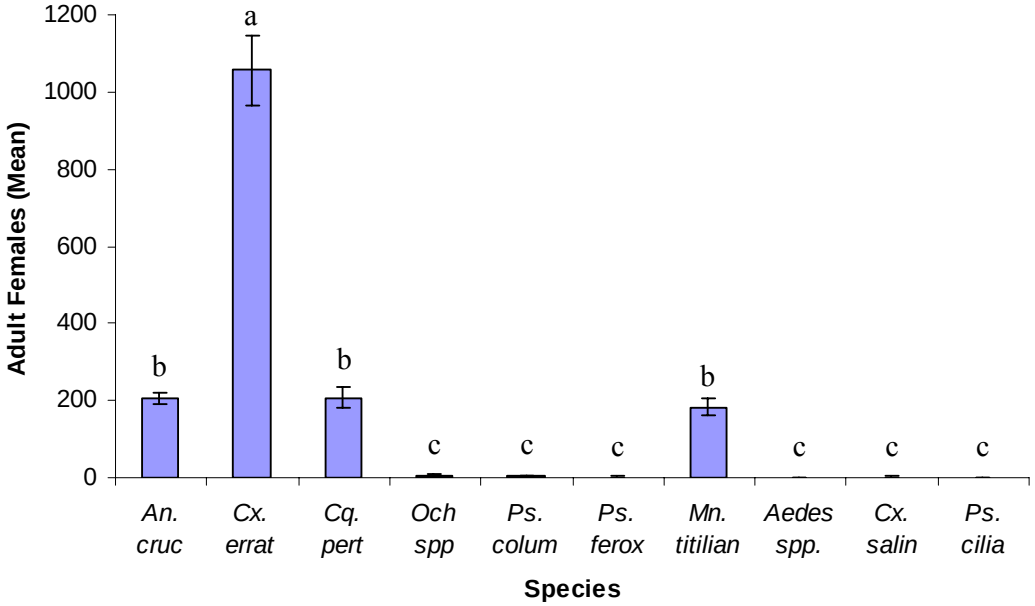


Figure 3-15. Daily mean number of mosquitoes trapped during seven day trapping study on the Wade Smith property. Means with the same letters are not significantly different.

Comparison of Six Trapping Trials

After evaluating six aquatic habitats, it was found that four of the aquatic habitats had submerged and floating vegetation, creating and providing a most suitable environment for *Cx. erraticus* (Figure 3-16).

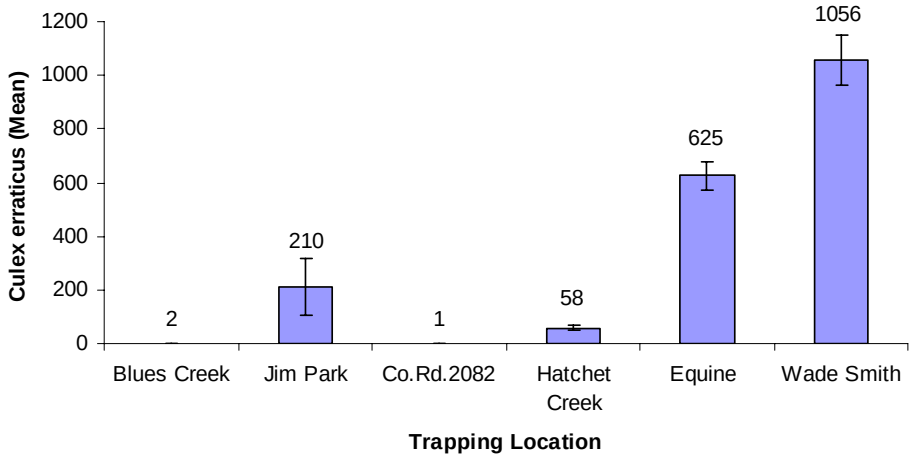


Figure 3-16. Habitat locations for *Cx. erraticus*. Means with same letters are not significant. Numbers above letters indicate the daily mean for *Cx. erraticus* trapped in those habitats.

The Wade Smith property produced 11,682 total mosquitoes with 10 species, of which 63.3% were *Cx. erraticus,* averaging 1055.6 per trapping day. The University of Florida Horse Teaching Unit produced a total of 10,826 mosquitoes in 12 species, of which 41.7% were *Cx. erraticus,* averaging 645 per trapping day. A total of 40.9% of 3,691 mosquitoes trapped on the James Park property were *Cx. erraticus* (n=1510). An average of 216 *Cx. erraticus* was trapped per day during the seven day trapping study. Rainfall started on the third day and lasted through day five of the trapping study, causing an increase in the depth of the pond. The grassy shallow margins were no longer visible and there was a decrease in the catch rate of *Cx. erraticus* and an increase in *An. crucians*. An average of 57.7 *Cx. erraticus* (n=404) were trapped daily at Hatchet Creek, making 19.3% of the 2,090 total mosquitoes trapped *Cx. erraticus*. The total number of mosquitoes trapped along county road 2082 was 991 with *Cx. erraticus* totaling eight, for a 0.8% catch rate and averaging one each trapping day. This trapping site was similar to Blues Creek where the total mosquitoes trapped were 972, with only 12 being *Cx. erraticus* averaging 1.7 per day. The latter two sites were void of aquatic vegetation. The mean number of mosquitoes trapped at each site is shown in Table 3-13.

Wade Smith Property, 30-Day Trapping Study

Three modified CDC light traps with CO_2 were randomly placed in one of the three selected sites around the five acre pond. Three treatments (CO_2 only, LE and CO_2, and hexane standard with CO_2) were randomly placed at one of the three sites and rotated the beginning of every fourth day for 30 days. Each treatment was rotated in each site a total of 10 times throughout the 30-day study. The traps were set each day at 6:00 pm and collected at 7:00 am the next morning.

Table 3-13. Means per day of mosquito species trapped in each habitat for seven days using modified CDC light trap, carbon dioxide and lizard extract.

Species	Bl Crk	J. Park	CR 2082	Hat Crk	HTU	W. Smith
Aedes spp.	4.29	2.29	16.71	1.57	1.00	0.86
An. crucians	97.14	69.86	56.14	154.57	164.14	206.14
An. quadrimaculatus					2.29	
Cx. erraticus	1.71	215.71	1.14	57.71	625.43	1055.57
Cx. nigripalpus	0.71	95.14		4.71	14.14	
Cx. salinarius	9.00	18.86		6.71	8.86	2.29
Cx. quinquefasciatus		7.14	24.43	9.14		
Cx. pilosus		2.71				
Cx. restuans		33.29	25.86			
Cq. perturbans	23.00	7.14		16.71	322.14	207.86
Ma. dyari	0.43					
Ma. titillans	0.43	6.43		8.14	368.86	182.86
Och. spp.		38.29		15.86	1.29	7.14
Ps. ciliata	1.00	5.14	12.00	4.86		0.86
Ps. columbiae				10.14	6.00	3.14
Ps. ferox		25.29	5.29	8.43	10.71	2.14
Ps. howardii	1.14					
Ur. sapphirina					2.00	

Key: Bl Crk= Blues Creek; J. Park=James Park; CR 2082=county road 2082; Hat Crk= Hatchet Creek; HTU=Horse Teaching Unit; W. Smith=Wade Smith

Sites one, two and three were based on 360 observations with a significant difference in the number of species trapped in site one ($p=<0.0001$; $df=11$; $F=238.89$), site 2 ($p=<0.0001$; $df=11$; $F=323.21$) and site 3 ($p=<0.0001$; $df=11$; $F=323.21$). A significant difference was also found in the treatment ($p=<0.0001$; $df=2$; $F=118.70$), ($p=<0.0001$; $df=2$; $F=181.55$), and ($p=<0.0001$; $df=2$; $F=199.78$), respectively. A significant difference was found in the interaction between the species of mosquitoes and treatment ($p=<0.0001$; $df=22$; $F=42.43$), ($p=<0.0001$; $df=22$; $F=73.44$) and ($p=<0.0001$; $df=22$; $F=34.75$) (Table 3-14, 3-15, 3-16). The 360 observations were based on three treatments; modified CDC light trap with CO_2, modified CDC light trap with CO_2 and hexane, and modified CDC light trap with CO_2 and lizard extract. Each treatment

trapped 12 species of mosquitoes found in the habitat during the 10 replications for a total of 360 observations.

Table 3-14. Analysis of variance for site 1 evaluating the number of species of mosquitoes and interaction with the three treatments for the 10 replications performed in 30 days.

ANOVA Source of variation	df	SS	Mean Square	F Value	Pr > F
Species	11	6576853.10	597895.74	238.89	<.0001
Treatment	2	594148.89	297074.45	118.70	<.0001
Species per Treatment	22	2336378.67	106199.03	42.43	<.0001

Table 3-15. Analysis of variance for site 2 evaluating the number of species of mosquitoes and interaction with the three treatments for the 10 replications performed in 30 days.

ANOVA Source of variation	DF	Type III SS	Mean Square	F Value	Pr > F
Species	11	5420201.55	492745.60	323.21	<.0001
Treatment	2	553552.78	276776.39	181.55	<.0001
Species per Treatment	22	2463245.48	111965.70	73.44	<.0001

Table 3-16. Analysis of variance for site 3 evaluating the number of species of mosquitoes and interaction with the three treatments for the 10 replications performed in 30 days.

ANOVA Source of variation	DF	Type III SS	Mean Square	F Value	Pr > F
Species	11	5526739.27	502430.84	199.78	<.0001
Treatment	2	512090.41	256045.21	101.81	<.0001
Species per Treatment	22	1922731.24	87396.88	34.75	<.0001

Four species of mosquitoes, *Cx. erraticus, Cq. perturbans, An. crucians* and *Ma. titillans*, were the most frequently trapped species, and each had the highest mean catch rates in all three sites. The remaining eight species; *Cx. nigripalpus, Och.* spp., *Ps. columbiae, Cx. quinquefasciatus, Cx. salinarius, Ps. ferox, Aedes* spp. and *Ur. sapphirina*, had low mean catch rates (Table 3-17).

Table 3-17. Wade Smith property 30-day trapping study means ± SEM of the species of mosquitoes for the three sites around the 5-acre pond.

Site 1		Site 2		Site 3	
Species	Mean ± SEM	Species	Mean ± SEM	Species	Mean ± SEM
Cx. errat	463.97 ± 59.20	*Cx. errat*	409.4 ± 58.5	*Cx. errat*	474.1 ± 55.0
Cq. pert	182.4 ± 20.06	*Cq. pert*	155.3 ± 17.8	*Cq. pert*	176.7 ± 17.6
An. cruc	175.67 ± 12.27	*An. cruc*	139.1 ± 9.2	*An. cruc*	154.4 ± 12.6
Mn. titil	138.47 ± 15.36	*Mn. titil*	132.4 ± 14.3	*Mn. titil*	141.9 ± 13.6
Ae. spp	11.1 ± 1.16	*Ae. spp*	10.9 ± 1.3	*Ae. spp*	11.7 ± 1.2
Cx. nigri	10.4 ± 1.11	*Cx. nigri*	10.2 ± 1.0	*Cx. nigri*	11.5± 1.5
Och. spp	10.03 ± 1.20	*Och. spp*	8.9 ± 1.0	*Och. spp*	9.5 ± 0.9
Ps. col	9.17 ± 0.92	*Ps. col*	8.4 ± 0.8	*Ps. col*	9.4 ± 0.8
Cx. quinq	7.33 ± 0.72	*Cx. quinq*	8.3 ± 1.2	*Cx. quinq*	7.2 ± 1.1
Cx. sal	6.9 ± 1.13	*Cx. sal*	5.3 ± 0.7	*Cx. sal*	6.3 ± 1.0
Ps. ferox	5.33 ± 0.51	*Ps. ferox*	4.5 ± 0.6	*Ps. ferox*	4.1 ± 0.6
Ur. sapp	3.73 ± 0.62	*Ur. sapp*	4.3 ± 0.6	*Ur. sapp*	3.3 ± 0.6

There are no significant differences found in the number of mosquitoes trapped in each species for all three sites.

Anopheles crucians was found in higher numbers in site 1 because it is the heaviest wooded area of the three sites. Site 1 also had moderate submersed and floating vegetation with shallow grassy margins and cat tails, which is indicative for *Culex erraticus*, *Cq. perturbans* and *Mn. titillans*. Figure 3-17 shows the number of mosquitoes in each species trapped during the randomized rotation of the three treatments in site 1 during the 30-day study. Figure 3-18 shows the percent of *Cx. erraticus* in comparison to the other 11 species of mosquitoes trapped in site 1 with randomized rotation of all three treatments. Figure 3-19 shows the number of mosquitoes in each species trapped during the randomized rotation of the three treatments in site 2 during the 30-day study. Figure 3-20 shows the percent of *Cx. erraticus* in comparison to the other 11 species of mosquitoes trapped in site 2 with randomized rotation of all three treatments.

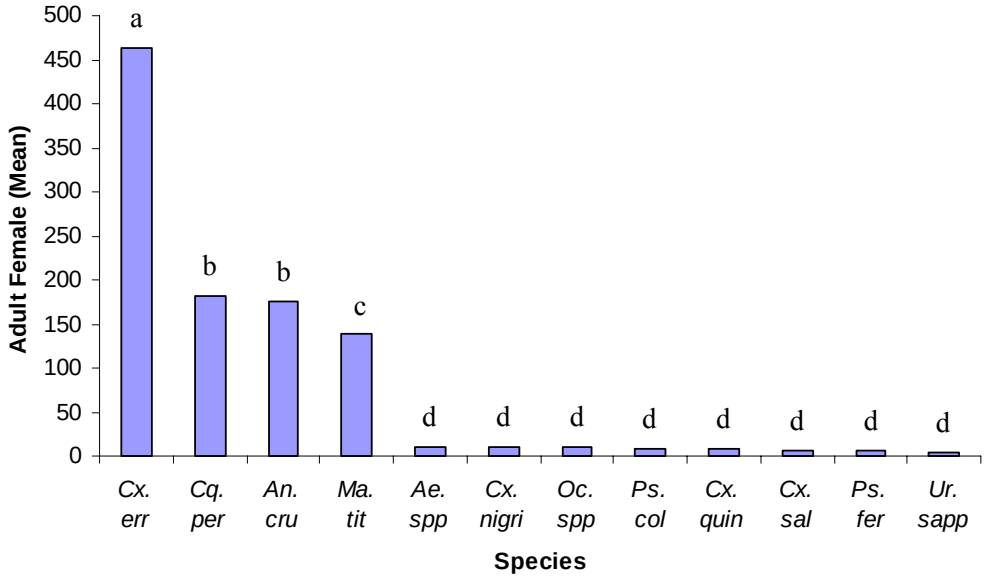


Figure 3-17. The mean of the 12 species of mosquitoes trapped in site 1 during the Wade Smith property 30-day trapping study are in numerical order. Means with same letter are not statistically significant.

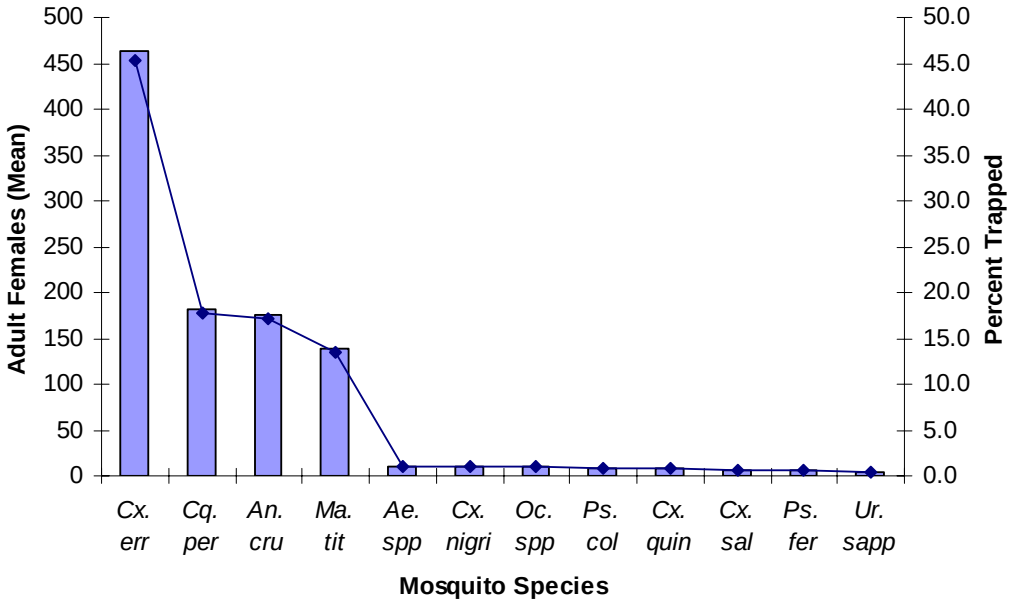


Figure 3-18. Mean number of adult female mosquitoes with percent of each species trapped in site 1 during randomized rotation of carbon dioxide, lizard extract and hexane.

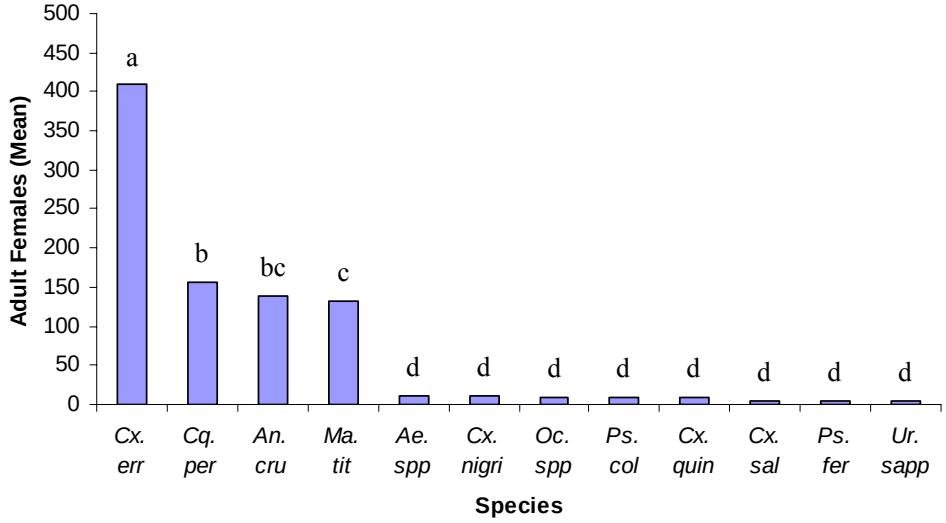


Figure 3-19. The mean of the 12 species of mosquitoes trapped in site 2 during the trapping study are in numerical order. Means with same letter are not statistically significant.

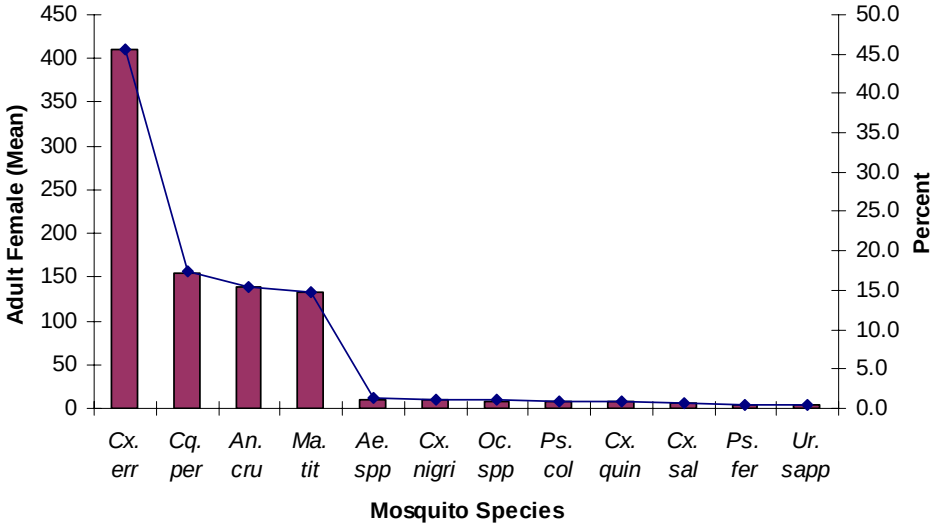


Figure 3-20. Mean number of adult female mosquitoes with percent of each species trapped in site 2 during randomized rotation of carbon dioxide, lizard extract and hexane.

Figure 3-21 shows the number of mosquitoes in each species trapped during the randomized rotation of the three treatments in site 3 during the 30-day study.

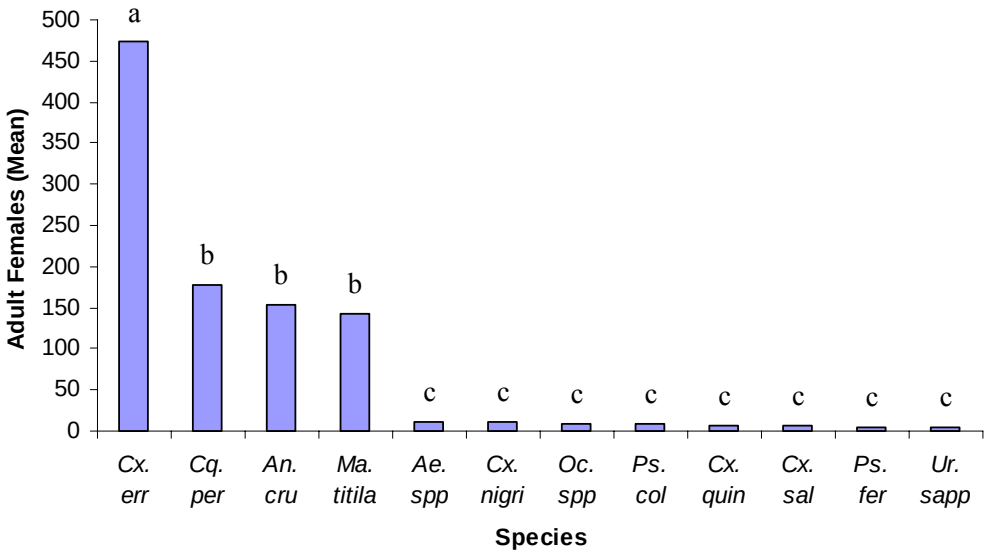


Figure 3-21. The mean of the 12 species of mosquitoes trapped in site 3 during the Wade Smith property 30-day trapping study is in numerical order. Means with same letter are not statistically significant.

Figure 3-22 shows the percent of *Cx. erraticus* in comparison to the other 11 species of mosquitoes trapped in site 3 with random rotation of all three treatments.

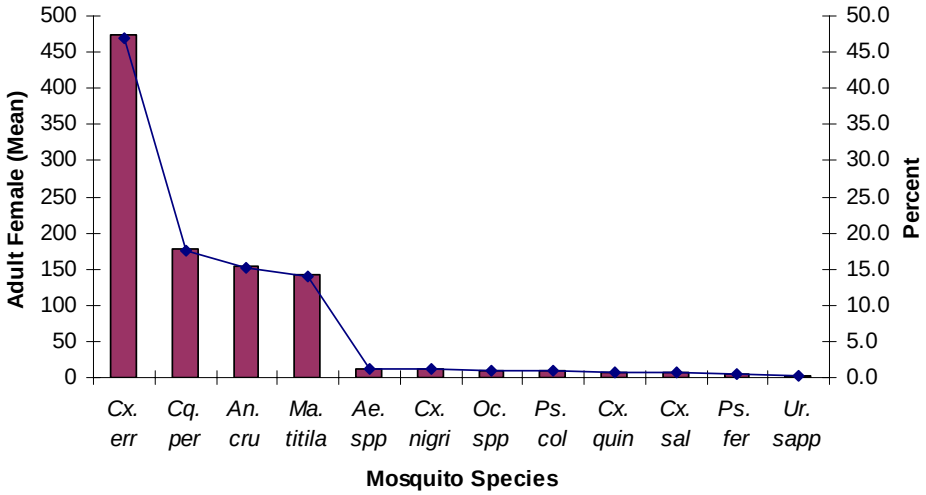


Figure 3-22. Mean number of adult female mosquitoes with percent of each species trapped in site 3 during randomized rotation of carbon dioxide, lizard extract and hexane.

The CO_2 treatment was not significant (p=0.1315; df=2; F=2.04) when compared to the three sites. Carbon dioxide was significant (p=<0.0001); df=11; F=205.24) when

compared to the 12 species of mosquitoes, and significance was found (p=0.04; df=22; F=1.59) when compared to the interaction of the three sites and the species trapped (Table 3-18).

Table 3-18. Analysis of variance for comparison of carbon dioxide treatment to the interaction with the number of mosquito species and for the 10 replications performed in 30 days.

ANOVA Source of variation	DF	Type III SS	Mean Square	F Value	Pr > F
Site	2	6768.375	3384.187	2.04	0.1315
Species	11	3742562.45	340233	205.24	<.0001
Site per Species	22	57875.169	2630.69	1.59	0.0473

Figure 3-23 shows the mean number of mosquito species attracted to the CO_2 treatment when compared to the interaction of the three sites.

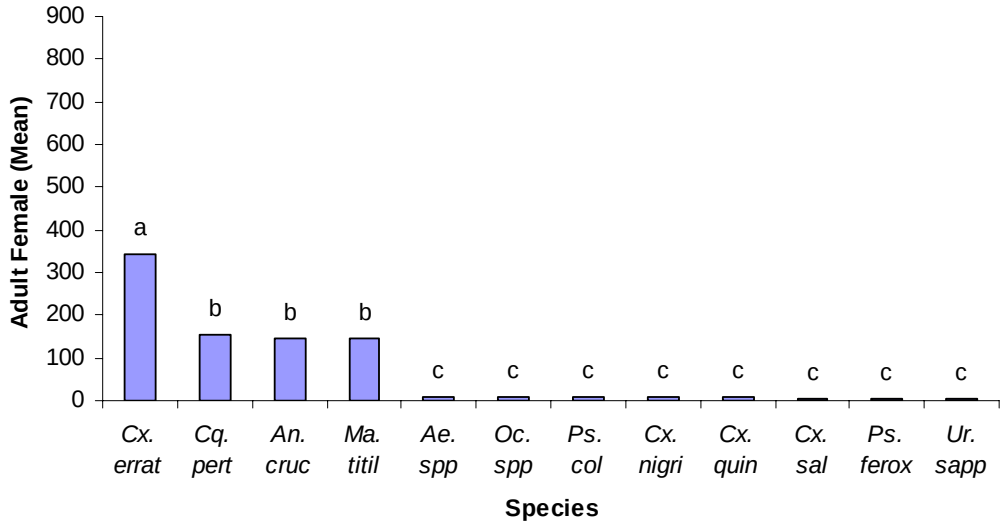


Figure 3-23. The mean of the mosquitoes attracted to treatment carbon dioxide when compared to the interaction of the three sites and species of adult mosquitoes. The means are in numerical order. Means with same letter are not statistically significant.

When hexane was used as a standard control, it was not significant (p=0.0858; df=2; F=2.48) when rotated in the three sites of the aquatic habitat during the 30-day study. Hexane was significant (p=<0.0001; df=11; F=122.81) when attracting the same

number of mosquito species as the other two treatments, although the mean numbers of *Cx. erraticus*, *An. crucians, Cq. perturbans*, and *Ma. titillans* decreased considerably compared to CO_2 and LE. The mean numbers of the remaining mosquito species were approximately the same as CO_2. Hexane is significant (p=0.045; df=22; F=1.60) when compared to the interaction of the three sites and the mosquito species in each site (Table 3-19).

Table 3-19. Analysis of variance comparing the hexane treatment to the interaction of the number of species of mosquitoes and the three sites.

ANOVA Source of variation	DF	Type III SS	Mean Square	F Value	Pr > F
Site	2	3587.5227	1793.761	2.48	0.0858
Species	11	979083.24	89007.57	122.81	<.0001
Site per Species	22	25464.4829	1157.477	1.6	0.045

Figure 3-24 shows the mean number of mosquito species attracted to the hexane treatment when compared to the interaction of the three sites.

Lizard extract (LE) was not significant (p=0.076; df=2; F=2.59) when rotated within the three sites for the 30-day study. The number of species attracted to the LE was significant (p=<0.0001; df=11; F=423.87), but when the interaction of the site and species were compared no significance (p=0.456; df=22; F=1.01) was found in the LE in all sites during the 30-day study (Table 3-20).

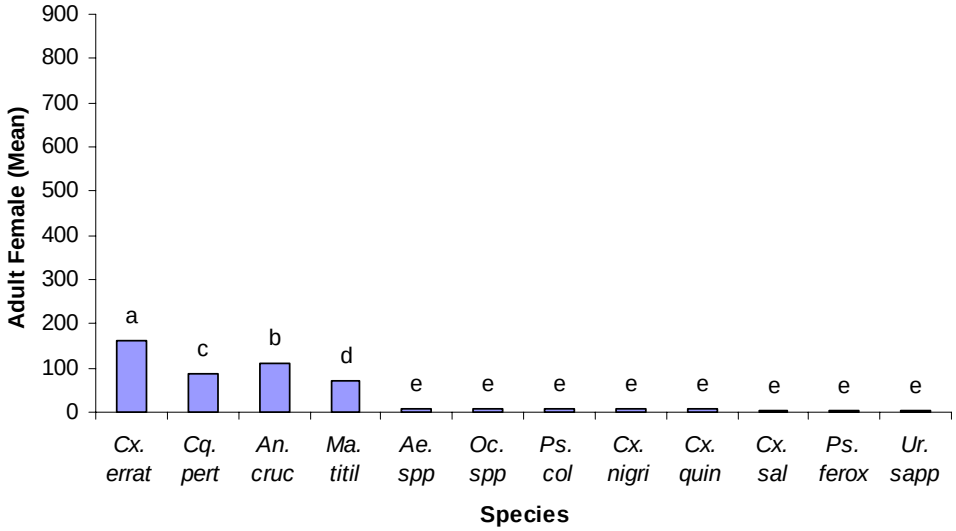


Figure 3-24. Hexane treatment compared to the interaction of the three sites and species of adult mosquitoes trapped. Means with same letters are not statistically significant.

Table 3-20. Analysis of variance of lizard extract when trapped in the three sites compared to the number of species of mosquitoes during the 30-day trapping study on the Wade Smith property.

ANOVA

Source of variation	DF	Type III SS	Mean Square	F Value	Pr > F
Site	2	21546.76	10773.38	2.59	0.0766
Species	11	19394871.23	1763170.11	423.87	<.0001
Site per Species	22	92066.91	4184.86	1.01	0.4561

Figure 3-25 shows the mean number of mosquitoes species attracted to the LE treatment when compared to the three sites throughout the study.

The LE treatment attracted a total of 48,188 mosquitoes within the 12 species resulting in a significant difference ($p=<0.0001$; df =11; F=420.79) between the CO_2 and the hexane standard. The CO_2 treatment trapped the same 12 species totaling 25,415 mosquitoes, and produced a significant difference to the hexane treatment ($p=<0.0001$; df=11; F=199.75). The hexane treatment trapped the lowest number of mosquitoes

(n=14,369) within the same 12 species (Figure 3-26). Table 3-21 shows the species and the mean ± SEM for the three treatments during the Wade Smith 30-day study.

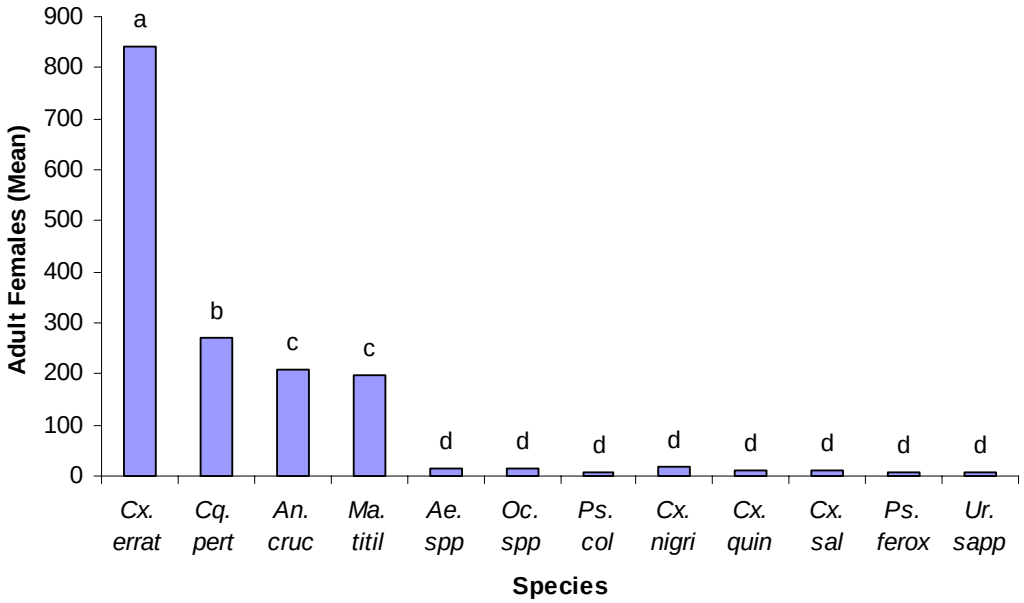


Figure 3-25. Lizard extract evaluated as an attractant when positioned by randomization in the three trapping sites during the 30-day study. The means with the same letters are not statistically different.

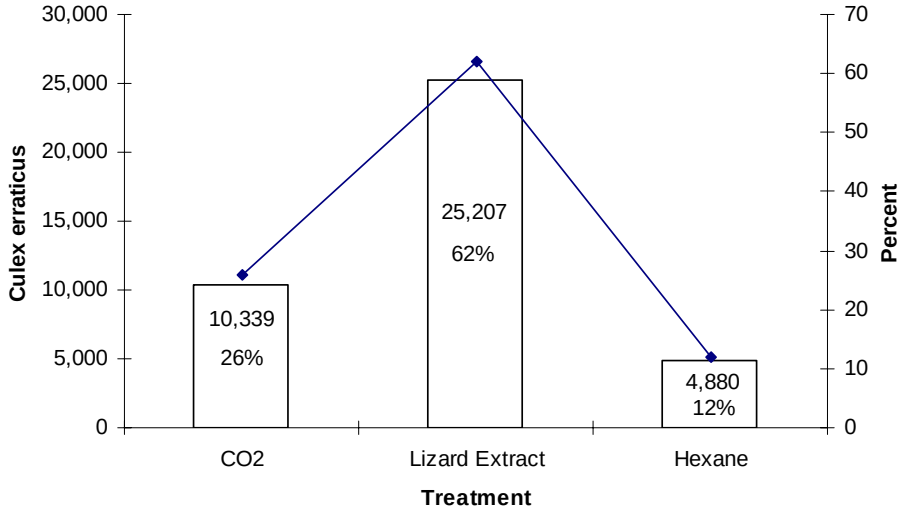


Figure 3-26. Treatments with the total number and percent of *Culex erraticus* trapped on the Wade Smith property during the 30-day study.

Table 3-21. The mean ± SEM for the mosquito species relative to the three treatments during the Wade Smith 30-day study

Species	LE	CO_2	Hexane	p-value	df	F
Aedes. spp.[1]	16.1 ± 1.3^{a}	10.16 ± 0.8^{b}	7.26 ± 0.7^{c}	<.0001	2	19.98
An. crucians[1]	209.9 ± 10.8^{a}	147.60 ± 7.7^{b}	108.30 ± 8.7^{c}	<.0001	2	30.92
Cq. perturbans[1]	270.77 ± 17.97^{a}	155.77 ± 9.52^{b}	85.87 ± 6.0^{c}	<.0001	2	58.16
Cx. erraticus[1]	840.23 ± 31.8^{a}	344.63 ± 20.7^{b}	159.07 ± 13.6^{c}	<.0001	2	228.39
Cx. nigripalpus[1]	16.83 ± 1.2^{a}	7.46 ± 0.6^{b}	10.10 ± 3.6^{b}	0.0123	2	4.63
Cx. quinquefasciatus[1]	11.96 ± 1.24^{a}	6.43 ± 0.6^{b}	6.63 ± 0.7^{b}	<.0001	2	11.84
Cx. salinarius[1]	10.46 ± 1.0^{a}	5.267 ± 0.6^{b}	3.36 ± 0.6^{b}	<.0001	2	19.92
Ma. titillans[1]	196.07 ± 14.74^{a}	145.73 ± 10.3^{b}	68.50 ± 3.01^{c}	<.0001	2	34.35
Och. spp.	13.36 ± 1.1^{a}	9.16 ± 0.8^{a}	11.40 ± 4.6^{a}	0.5698	2	0.57
Ps. columbiae	8.70 ± 0.9^{a}	7.66 ± 0.7^{a}	7.93 ± 0.8^{a}	0.6703	2	0.4
Ps. ferox	5.83 ± 0.6^{a}	4.23 ± 0.4^{a}	4.40 ± 0.7^{a}	0.1493	2	1.94
Ur. sapphirina	6.03 ± 0.7^{a}	3.03 ± 0.5^{a}	6.36 ± 3.9^{a}	0.5448	2	0.61
Trap mean[2] ± SEM	4818.8 ± 177.0^{a}	2541.5 ± 101.4^{b}	1436.9 ± 79.3^{c}			

[1]Significant position effect ($p<0.05$). [2]Trap mean = trap sum of all mosquito species divided by 10 replications

Discussion

Florida has 77 known species of mosquitoes that are found throughout the state, while some are isolated in certain areas because of the specific required environmental conditions (Darsie and Ward 2005, Florida Coordinating Council on Mosquito Control 1998, Reinert 2001). Many of these species have been under extensive study while others, such as *Cx. erraticus*, have had minimal attention. Locating a specific species of mosquito such as *Cx. erraticus* requires a sampling program which includes an experimental design, method of data collection and a statistical analysis program to validate the samples.

Various types of light traps are used to sample adult mosquito populations for laboratory studies (Artsob et al.1983, Chamberlain et al.1964, Malainual et al.1987, Olson et al.1979, Reeves 1968, Srihongse et al.1980). CDC light traps baited with CO_2 were found to increase the numbers of species of mosquitoes, as well as the number of mosquitoes in many species including *Cx. erraticus* in this study (Allan et al. 1981, Leake et al.1986, LeDuc et al.1975, McNelly 1995, Newhouse et al.1966, Reisen et al 1983a, Siverly and DeFoliart 1968). Light traps modified with different colored, flickering light-emitting diodes (LEDs) have also been used with much success in locating and increasing the sample sizes of various populations of mosquitoes including *Cx. erraticus* (Hoel 2005, Burkett et al.1998).

Headlee (1932) was one of the first to find that mosquitoes respond favorably to transmitted light. *Culex erraticus* was found to be attracted to various wavelengths of different colored lights, especially to blues and green, therefore the CDC light traps were modified with four blue and one green LED light source for maximum attraction in this

study (Ali et al.1989, Barr et al.1963, Breyev 1963, Herbert et al.1972, Muir et al.1992, Thurman and Thurman 1955).

Culex erraticus responded favorably to many chemical attractants including 1-octen-e-ol (octenol) (Kline et al.1991a, b, Takken and Kline 1989), lactic acid (Kline et al.1990a), acetone (Bernier et al. 2003), and phenols (Kline et al.1990b) have also been used as mosquito attractants to bait light traps. However, this study is the first to use a modified CDC light trap baited with CO_2 and lizard extract.

Using live *A. carolinensis* lizards in baited traps in the wild was prohibited by the Institutional Animal Care and Use Committee (IACUC) during this study. The removal of respired CO_2 and the visual cue of the lizards' movement in the wild are important to mosquito's ability to locate its host (Allan et al.1987, Brown 1951, 1953, 1956; Brown et al.1951, Gillett 1972, Khan et al.1966, Kline 1994a, Sippel and Brown 1953, Wood and Wright 1968). Therefore, washing of deceased colony reared lizards with filtered hexane was necessary to remove skin compounds that may be used by *Cx. erraticus* as attractants during host location. In order to evaluate the lizard extract as an attractant by *Cx. erraticus*, it was important to locate a high population of these mosquitoes so a 30-day randomized trapping regime could be performed.

Culex erraticus favors aquatic habitats that have submersed and floating vegetation (Klein 1985; Robertson et al.1993). In this study two habitats void of aquatic vegetation and four with vegetation were selected to sample for the presence of *Cx. erraticus.*

The two habitats void of aquatic vegetation were located in Blues Creek Subdivision behind the clubhouse and a forested area along county road 2082 (CR. 2082). Both habitats were very similar in environmental conditions including very little

aquatic vegetation, and muddy soil with strong stagnant odors. Blues Creek was thick with small shrubbery and a sporadic canopy of large trees. Along CR. 2082 a deep man made ravine for water run-off was lined on both sides by immense oak trees which prohibited most sunlight from reaching the ground. There were many artificial containers discarded from passing motorists over time, which created artificial habitats for some of the species of mosquitoes trapped including *An. crucians*. There were 10 species of mosquitoes trapped at Blues Creek and seven species trapped along CR.2082. Trapping seven consecutive days in each of these habitats yielded more *An. crucians* than other species of mosquitoes. It is not known at this point if the lizard extract had an influence on attracting the *Anopheles*, because it is known that these mosquitoes have a high affinity for CO_2 especially when delivered through the modified CDC light trap (Dekker and Takken 1998, Kline 1999).

Two species associated with submersed and floating vegetation were trapped at Blues Creek in minimal numbers, *Cq. perturbans* (n=161) and *Cx. salinarius* (n=63). *Coquillettidia perturbans* and *Cx. salinarius* are both attracted to CO_2 baited light traps (Buckley et al 1994, Howard et al.1988, Schreck et al. 1972). The trapping site in Blues Creek Subdivision is part of a larger aquatic system that contains different types of aquatic vegetation and explains why a *Cq. perturbans* and *Cx. salinarius* were trapped in an area that is virtually free of submersed vegetation. Also there is a field adjoining the trapping site along CR.2082 that has an open field with a low area containing water, and this too is another reason for the *Anopheles* and *Culex* species trapped at CR.2082 and Blues Creek. *Psorophora* spp. was trapped in low numbers in both habitats, which is favored by this species. *Culex erraticus* was trapped in very low numbers for both

habitats, therefore suggesting that either this species requires immediate vegetative aquatic habitats or the lizard extract worked as a repellent for this species of mosquito.

The James Park property produced 661 *Cx. erraticus* during the first two days of trapping using the lizard extract. This aquatic habitat had shallow grassy margins around the circumference of the pond with a moderate amount of small duckweed (*Lemna* spp.) and water hyacinths. On the third day of trapping, heavy rainfall began and continued throughout the fifth day. The wind and water increased during this time, and it was found that a decrease in the number of *Cx. erraticus* and *Cx. nigripalpus* took place through the end of the seven days trapping regime. At the end of the seven days, a total of 1,510 *Cx. erraticus* and 686 *Cx. nigripalpus* were trapped. Wind speeds of 0.25 to 0.49 m/s reduced the number of *Cx. nigripalpus* and *Cx. erraticus* by 75% (Bidlingmayer 1974). Robertson et al. (1993) found that when the water level rises significantly, the number of *Cx. erraticus* declined and an increase in *An. quadrimaculatus* took place. Considering that 661 *Cx. erraticus* were trapped in two days, this is an average of 330 per day, with a probable total yield of at least 2,313 for the seven days if the rain and wind had not occurred, however, this is merely speculation. The lizard extract, however, did function as an attractant for eight of the species of mosquitoes, including the target mosquito *Cx. erraticus.*

Hatchet Creek trapping produced a total of 2,090 mosquitoes of which 51.8% were *An. crucians*, the most frequently trapped species of mosquitoes with a total of 1,082 for the seven days. The total number of *Culex erraticus* was 404 and comprised 19.3% of the total trapped. Robertson et al. (1993) found a direct correlation of increasing numbers of species of *Anopheles* and a decrease in *Cx. erraticus* when the water surface is densely

matted with aquatic vegetation, especially *Spirodela oligorrhiza* (Kunte), which is the case with Hatchet Creek. The other remaining 10 species, *Cq. perturbans, Ma. titillans, Cx. quinquefasicatus, Cx. nigripalpus, Cx. salinarius, Ps. ciliata, Ps. ferox, Ps. columbiae,* and *Aedes* and *Ochlerotatus* species were also trapped in low numbers at this site, but were trapped in moderate numbers at the James Park property where the water surface was not crowded with aquatic vegetation. So it is unlikely that the lizard extract acted as a repellent in this particular site. Therefore, the decreased number of mosquitoes trapped in each species could have been attributed to the dense mat of vegetation on the water surface, which has been shown to have a direct correlation to the numbers of emerging mosquito larvae, especially *Cx. erraticus* (Bradley 1932, Furlow and Hays 1972, Smith 1910, Smith and Enns 1967).

A total of 10, 826 mosquitoes were trapped during the seven day study at the University of Florida Horse Teaching Unit. *Culex erraticus* comprised 41.7% (5,416) of the total catch with *Mn. titillans* and *Cq. perturbans* catch rates of 23.8% (2,582) and 20.8% (2,255) respectively. It is interesting to note that the *An. crucians* total catch was 10.6% (1,149), somewhat lower than that of *Cx. erraticus.* Species of *Psorophora, Uranotaenia, Ochlerotatus* and *Aedes* have been trapped using various lights and wattage and have been found to be attracted to these light sources (Ali et al.1989; Burkett et al.1998; Herbert et al.1972). The water level at the Horse Teaching Unit was approximately two feet below normal level and the circumference around the pond was lined with grass and duckweed with moderate amounts of water hyacinth. Furlow and Hays (1972) reported that *An. crucians* exhibited a strong preference for emergent vegetation growing out of the water, such as *Typha latifolia* L. (cattail). Furlow and Hays

also found that *Anopheles* was absent in duckweed only pools. It appears that environmental conditions are a major contributing factor in the number of mosquitoes and species trapped in this location because the lizard extract attracted considerably more *Cx. erraticus* than other mosquitoes.

The Wade Smith property outperformed all of the aquatic environments sampled in this study. During the seven day sampling, a total of 11,682 mosquitoes were trapped with 63.3% (7,389) being *Cx. erraticus*. *Anopheles crucians* and *Cq. perturbans* totaled 12.4% (1,443) and 12.5% (1,455) of trapped mosquitoes. This site was similar to that of the Horse Teaching Unit with exception that the 5-acre pond was totally surrounded with large trees, creating a cooler environment, and the shoreline was shallow with clear water and the presence of grassy margins mixed with moderate amounts of duckweed. Because of the success with capturing a high number of *Cx. erraticus* in seven days, it was determined that the 30-day randomized trapping study would be performed at this site.

As part of the experimental design for evaluating the lizard extract as an attractant, three different treatments were rotated through three different sites around the 5-acre pond. At the end of the 30-day trapping study, it was found that site position was not significant in the total number of mosquitoes in each species trapped. However, site did play a significant part in the number of species trapped and a significant difference was found within each treatment.

There was no significance found between sites for the number of mosquitoes trapped in each species. However, all three sites showed a significant difference in the number of *Cx. erraticus* compared to the other 11 species trapped. The four most

frequently trapped species in the three sites in numerical order are *Cx. erraticus, Cq. perturbans, An. Crucians*, and *Ma. titillans.*

The Wade Smith property habitat has the submersed, floating and shallow grassy margins that are favorable for *Cx. erraticus, Cq. perturbans, An. crucians*, and *Ma. titillans. Mansonia titillans* and *Cq. perturbans* have siphons that are modified for piercing submersed aquatic vegetation for oxygen consumption. *Anopheles crucians* is found in higher number in aquatic habitats where the plant cattails are present. These plants were present in many areas around the habitat.

Anopheles crucians was found in slightly higher numbers in site 1, possibly due to the area being conducive because of the forested area that borders this site, especially with cattails in the shallow grassy margins of the pond. Furlow and Hays (1972) reported that cattails are the emergent vegetation for which *An. crucians* have a strong preference.

The CO_2 treatment was not significant between the three sites. It was significant in the number of species of mosquitoes trapped in each site. A higher number of *Cx. erraticus* were trapped using CO_2 only within all three sites as compared to the other 11 species trapped. There was no significant difference between *Cq. perturbans, Anopheles crucians*, and *Ma. titillans* trapped in all three sites with CO_2 only. Hoel (2005) used modified CDC light traps with the same color LEDs and 500 ml/m of CO_2 compared to the 250 ml/m of CO_2 used in this study, and the numbers of mosquitoes in the same species found in Hoel's study at the Florida Horse Teaching Unit were slightly higher than those found in the species in this study.

The use of hexane as a control standard for this study is perhaps the first recording of hexane being evaluated as an attractant. When the total number of species and

numbers of mosquitoes were computed, there was no difference in the number of mosquitoes trapped in each site using the three treatments. The overall numbers of mosquitoes were considerably less in each species using hexane than that found in the other two treatments. *Culex erraticus* had a daily mean catch rate of 162.7 mosquitoes using hexane, which are approximately 50% less than that found in CO_2 and 250% less than that found in lizard extract. However, there was a slight non-significant increase in the number of *An. crucians* compared to the *Cq. perturbans* and the *Ma. titillans* in the hexane treatment, but not enough to designate that hexane is an attractant for *Anopheles* species.

The lizard extract outperformed CO_2 and hexane in the trapping experiments of *Cx. erraticus*. Lizard extract attracted approximately 150% more *Cx. erraticus* than CO_2 and 250% more than the hexane treatment. The lizard extract also showed a significant difference in the number of *Cq. perturbans* attracted in comparison to *An. crucians* and *Ma. titillans* when rotated within all three sites.

Now that the experimental design has been carried through for 30 days and all of the mosquitoes were collected, identified and counted, the statistical analysis of the data clearly showed that lizard extract attracted significantly more *Cx. erraticus* than the CDC light traps baited with CO_2 and hexane. It is fascinating to see that a small compound from the cuticular structures of a lizard can make a difference in a specific species of mosquito making a deliberate choice to be attracted to a compound when the original host is absent.

CHAPTER 4
TESTING THE ABILITY OF *CULEX ERRATICUS'S* TO LOCATE *ANOLIS CAROLINENSIS* WITH THE AID OF AN OLFACTOMETER

Introduction

The variety and numbers of visual, gustatory, physical and olfactory stimuli available to adult female mosquitoes are so numerous and complex that researchers have developed testing procedures to unlock the codes that affect mosquito attractancy and repellency to hosts and habitats (Allan et al.1987, Brown 1956, Butler and Okine 1995, Dethier 1953, 1957, 1963 1976; Gillies and Wilkes 1969, Hocking 1964, 1971; Jacobson 1990, Kline et al.1990, Laarman 1975, Wright 1958). Many plants and animals produce odors as a natural occurrence through daily metabolic activities, and insects interact with these odors and use them as an open line of communication to the target source.

Mosquito Attractants

Semiochemicals are chemicals that elicit a response between mosquitoes and hosts (Law and Regnier 1971). Brown et al. (1970) reported that kairomones are advantageous to the receiving organisms, while pheromones elicit a response within the species. Chemical cues that are important in the life of mosquitoes come from host, oviposition, sex and predator/parasite attractants. Also, microbial and microbial chemicals including hydrocarbons, fatty acids, amino acids and hormones play an important part in the day-to-day activities of insects, especially vectors of disease (Lewis et al. 1975a,b,).

Gouck (1972) and Service (1971) found evidence that some arthropods can distinguish between male and female hosts, ages and possibly individuals in certain

ethnic groups. Gouck and Service based their assumptions on the research by Roberts and O'Sullivan (1948) where *Anopheles* mosquitoes are more attracted to the aboriginals than to the Caucasians of Australia. *Aedes aegypti* was found to be more highly attracted to some individuals than others when Khan et al. (1965) used probing time (PT_{50}) to measure the level of attractiveness. Also, McKenzie (2003) reported that artificial membranes worn by individuals were tested in an olfactometer and results showed that some members of the test group were more attractive to *Aedes aegypti* than others.

Thomas (1951) found that both *Anopheles albimanus* and *Anopheles gambiae* are more highly attracted to adults than to children, and Freyvogel (1961) reported that *Ae. aegypti* produced the same results toward adults. Through the use of an olfactometer, it was reported that men are more attractive to *Ae. aegypti* than women (Rahm 1958). However, a temporal variation in attractiveness was found by Roessier (1963) when he tested women during their menses and found them to be more attractive to mosquitoes than were men. Mosquitoes are small, but sturdy, and bring with them a complicated melting pot of abilities to locate its host.

Host Seeking

When adult female mosquitoes are involved in host location, they rely on a complex system within the insect to decode the signals received from the host (Klowden 1996, Lehane 1991). Lemon and Getz (1999) reported that insects have an extremely complex hard-wired system that functions to perceive and sort out the many odors that are present in the plume at one time and in varying concentrations. The antennae and palpi have pore containing olfactory sensilla that first perceive the odors and send the signals through this pathway of complex neurons, which the insect then sorts out whether the cues are related to mating, oviposition, food or host location (Steinbrecht 1996).

Mustaparta (1984, 1996) evaluated the discrimination site for olfaction cues in the mosquito brain and found that the corpora pedunculata in the protocerebrum is perhaps the main location in the neural pathways where olfaction cues are sorted out.

The majority of mosquito-host interaction research has included mosquitoes in the genera *Aedes* and *Anopheles* because they were first recognized as serious vectors of diseases and they were easily reared in high numbers in laboratory conditions. However, the genus *Culex*, specifically the subgenus *Melanoconion*, needs more research in the area of host selection and feeding behaviors to determine if they are similar or different to mosquitoes in other genera.

Most mosquitoes are obligatory blood feeders looking for a host to fulfill the requirement of sequestering certain proteins and cholesterols for the development of eggs (Clements 1992). Some mosquitoes are autogenous and do not need a blood meal for their first batch of eggs but will require a blood meal for subsequent development of eggs, as do those mosquitoes that are anautogenous (Clements 1992).

As haematophagous insects became dependent on blood to develop eggs it became important for them to locate hosts. Vinson (1981, 1985) reported that long and short range host finding cues were necessary to make the finding and selection of hosts successful. With this information Vinson was convinced that certain species of blood sucking insects are specific in their choice of host preferences while others such as *Cx. erraticus* are said to be opportunistic (Crans 1964, Cupp et al. 2003).

Insects, especially those in the order *Diptera*, use their olfactory senses to move in and out of the odor zone in the plume to travel short and long distances detecting host cues (Nicolas and Sillians 1989). Carbon dioxide is that compound that has kept

researchers debating whether it acts as a host attractant, repellent or an activator for aggressive searching behavior (Gillies 1980).

It is suggested that the first step a mosquito uses to locate host is activated by CO_2, which sends the mosquito aggressively searching for the cues from the host. This is known as "flying in the host zone." Once the mosquito is in the host zone, other physiological controls take over until landing and probing takes place (Daykin et al. 1965).

Other views expressed on CO_2 indicate that when produced at minimal concentrations in combination with ambient temperature, it is an activator of mosquitoes instead of an orientation cue (Hocking 1971). Brown et al. (1951) reported that CO_2 is more of an attractant than the minimal amounts of odor coming from the hosts. But Khan and Maibach (1966) believe that CO_2, in combination with the body heat and moisture coming from the host, is extremely important for the mosquito when approaching the host closely. Carbon dioxide in combination with host odors, including sweat, heat, and lactic acid, help to activate and orientate the mosquito to the host (Warnes and Finlayson 1985).

Sutcliffe (1987) reported that once the mosquito sets out to locate a host, it is hungry and is in the appetitive searching mode, and the erratic flight behavior allows the moving in and out of the air plume, at the same time detecting host odors and/or other chemicals.

While traveling through this plume, mosquitoes can perceive all of the volatile components and compounds and distinguish between those that are attractive and repellent (Lemon and Getz 1999). Lemon and Getz also reported that mosquitoes can judge the distance of the host faster by the number of times a host has been visited and

based on the concentration of the host odors. Sutton (1953) found that the higher the concentration of host odors in the plume, the faster the mosquito hones in on the host. Lehane (1991) suggests that a mosquito's final decision to land and/or probe a host is made from the cues of the host, including water vapor, heat, degree of host specificity, gustatory preference and visual attraction.

Light intensity, color, shapes, movement, and patterns with edges that are pronounced are some of the most important visual cues used by mosquitoes (Brown 1951; Sippel and Brown 1953; Smart and Brown 1955; Wood and Wright 1968; Browne and Bennett 1981; Bidlingmayer 1994). During an experiment, Kennedy (1940) found that the *Ae. aegypti* mosquito based its flight orientation on the floor pattern in the laboratory. Also, it was found that *Aedes aegypti* uses visual contact with the ground to keep its course in an upwind orientation during the night so its flight direction is not interrupted while locating its host (Daykin et al.1965). The host is a visual cue and, along with its temperature and moist body content in combination with lactic acid, helps the mosquito to maneuver as it comes in closer contact with the host (Cork 1996).

The visual orientation by host-seeking females in locating their targets during flights must include olfactory or other cues because visual orientation to targets is appetitive flight, except for the attraction to movement (Bidlingmayer 1994). The human eye has greater power of separating light spectra as compared to a mosquito's eye. However, the aperture in a mosquito eye is wider than that in a human eye, therefore allowing the mosquito greater capacity of vision in the dark which it combines with odors to locate the host (Hocking 1964, Muirhead-Thomson 1940).

Bidlingmayer and Hem (1980) found that suction traps covered with unpainted plywood attracted more mosquitoes from greater distances than suction traps not covered with plywood. Gillies and Wilkes (1974) conducted research in West Africa and found that it may be a possibility that some *Culex* mosquitoes are more attractive to large ramp traps rather than smaller suction traps. Kline (1994a) suggests that mosquitoes also use size as a cue for locating their host. Understanding how humans and animals initiate the differentiation of odors is extremely complex and researchers are finding that olfactory perception in insects is just as complicated.

With the design and implementation of olfactometers, many of the questions concerning attractancy and repellency can be better understood, especially in the role of mosquitoes as competent vectors of diseases.

Olfactometer Systems

The olfactometer is an instrument that examines different areas of vector-host interaction including biting, probing, landing and host seeking. Olfactometers measure the interaction of insects with compounds or hosts in the laboratory as closely as possible to the manner in which this behavior occurs in the environment. Olfactometers are designed to test isolated responses of insects to odors and compounds produced either naturally or synthetically (Butler and Okine 1995, Mookherjee et al.1993).

Some of the earlier olfactometers were constructed in a simple y-tube design with air flow creating an air plume, giving the mosquito a choice to move toward or away from the target odor (Bolton et al.1981, Dethier 1963, Eiras and Jepson 1994, Feinsod and Spielman 1979, Floore et al.1985, Gouck 1972, Mackley et al.1981, Mookherjee et al.1993, Muhammed et al.1975, Thorpe and Jones 1937).

Dethier (1963) developed an olfactometer that used and measured the effect of temperature, light, airflow, relative humidity, and the host response to the provided stimulant for Diptera. Further development of the y-tube design increased the number of ports so that odors and compounds could be tested singly or in combination with each other.

Many mosquitoes use host odors, carbon dioxide, environmental odors and visual cues singly and in combination with other stimulants to locate their host with the possible expectation of receiving a blood meal (Adams 1999, Bar-Zeev et al.1977, Cork 1996, Takken 1991,1996;). Establishing test systems that evaluate attractants and repellants is important in order to isolate how a particular species of mosquito reacts to stimulant(s). Therefore development of olfaction systems similar to the one used in this study have become more sophisticated over time as the quest for understanding the sensory perception of insects continues.

A Multi-Choice Olfactometer

The development of the laboratory olfactometer used in this research was supported by a grant by International Flavors and Fragrances, Incorporated and designed by Dr. J.F. Butler. This pie-shaped, 10-port olfactometer monitors, electronically validates, and data logs the amount of activity at each of the 10 artificial hosts simultaneously (Butler and Katz 1987, Butler and Okine 1995, Marin et al.1991, Okine 1994, Wilson et al.1991). Air flow at a speed of approximately 0.6 ml/min is introduced into the system at each of the 10 ports and removed through the top of the olfactometer at the same rate by a negative pressure which mimics the plume found in the environment. The environmental atmospheric conditions are duplicated as closely as possible by supplementing the air

flow going into the olfactometer with moist CO_2 at a rate of 250 ml/min through a computer controlled valve timed at intervals of four minutes on and six minutes off.

Each of the 10 ports is equipped with a fiber optic light source that mimics dusk and dawn time periods and can be turned on or off depending on insect feeding behavior. Each port is also lit by 1R diodes to present an infrared target at approximately 150 Hz. Each port has an amplifier that differentially amplifies the insects contact up to 10,000 times, and this data is recorded to a computer file in ten minute increments as bite-seconds. The program Strawberry Tree transfers the data to a computer where it is processed using macro programs written by N.C. Hostettler of the University of Florida Department of Entomology and Nematology in Gainesville, Florida.

The olfactometer is situated in a temperature (26ºC and 32ºC) and light controlled room known as a Faraday Cage (Lindgren Enclosures, model No. 18-3/5-1, U.S. Patent No. 5,134,892) (Butler and Okine 1995, Okine 1994) (Figure 4-1).

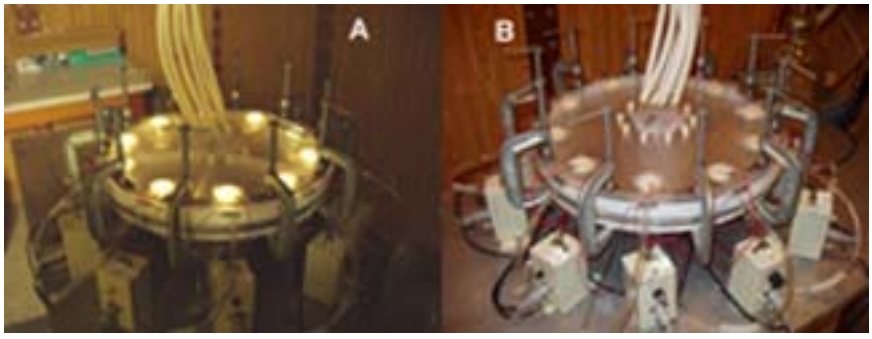


Figure 4-1. Photo A: olfactometer with fiber optic light source light on, depicting the dusk and dawn biting period. Photo B: olfactometer lights off.

In this study the olfactometer was used to evaluate the ability of *Cx. erraticus* to detect the presence of *A. carolinensis* by measuring the landing rate activity of the mosquito. The olfactometer was also used to measure and evaluate the attractancy of *Cx. erraticus* to the lizard extract (Chapter 3) for five purposes. The first purpose was to

determine if *Cx. erraticus* could detect the presence of the extract, and second to determine the amount of lizard extract (LE Chapter 3) that *Cx. erraticus* is attracted to in the laboratory. The third purpose was to evaluate this determined material and the dosage amount in the field. The fourth purpose was to evaluate the lizard extract in laboratory conditions through replication to compare responses to concentrations used in trapping field data. Finally, the olfactometer was to evaluate whether malaria infected *A. carolinensis* lizards were more attractive than noninfected lizards.

Materials and Methods

Culex erraticus Wild Colony

Culex erraticus trapped in the wild were brought into the laboratory and placed in screened mosquito cages (30.5 cm x 30.5 cm x 30.5 cm) with a 100 ml specimen vial of 10% sugar water with a wick made of filter paper. This was done in an attempt to establish a colony to have a source of mosquitoes for fertility. Each mosquito cage was kept in a temperature controlled rearing chamber at 27ºC and 80% RH in combination with a 16:8 light/dark (LD) photo regime.

Anolis carolinensis Colony

An *Anolis carolinensis* lizard colony was maintained in an animal control room inspected and approved by the Institutional Animal Care and Use Committee (IACUC) Project No.A644 and the Environmental Health and Safety (EH&S) Department at the University of Florida Project No.BA-2030. The lizards were housed in a screened cage (60.96 cm x 60.96 cm x 60.96 cm) with room temperature maintained between 30ºC and 32ºC and 80% relative humidity and 16:8 LD photo regime (Figure 4-2).

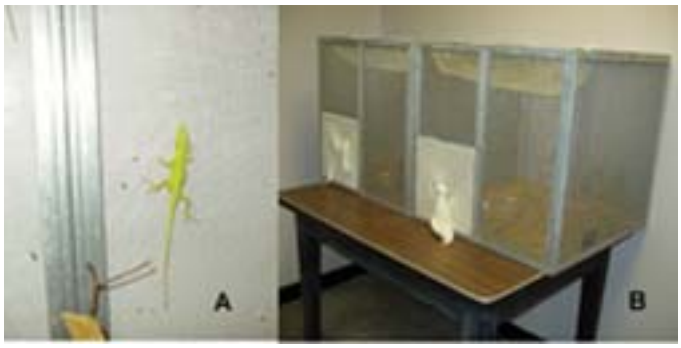


Figure 4-2. Photo A: *Anolis carolinensis* inside cage. Photo B: *Anolis carolinensis* cages.

The lizards were supplied with fresh water and *Musca domestica* Linnaeus (house flies) daily along with powdered milk as a source of calcium for the flies and indirectly supplied via the flies to the lizards as a food source. Fresh branches were placed in each cage twice a week. The cages were cleaned twice weekly and an automatic timer control regulated lights on a 16:8 LD photo regime. Daily lizard counts, temperature, relative humidity, feeding, water, overall room sanitation, attending veterinarians and emergency contact number were recorded on proper forms on the outside of the door to the animal control room, as required by IACUC. Each lizard was identified by toe clipping of two separate digits that are unique to each lizard. For example, RF1RR1 indicates that the first inside digit nearest the body on the right front leg is clipped and the first inside digit nearest the body on the right rear leg is clipped. This form of identification is the most common method used in zoological research involving lizards.

Lizard Extract

A crude lizard extract used in field and laboratory experiments was obtained from deceased *Anolis carolinensis* lizards that died in colony. Dead lizards were washed in 100 ml of hexane for 24 hours to obtain lizard extract (LE). The lizards were removed from the hexane and incinerated as per protocol and the 100 ml of lizard extract was concentrated under nitrogen to 10 ml. The concentrated lizard extract was used in the

olfactometer to determine attractant activity and establish the optimum dosage. LE was also evaluated by gas chromatography to determine presence of compounds in the crude extract.

Gas Chromatography of Lizard Extract

The 10 ml of prepared *A. carolinensis* lizard extract was concentrated to 5 ml under nitrogen. The 5 ml of *A. carolinensis* lizard extract was fractionated by putting the 5 ml of lizard extract through a column of silicic acid (300 mg silicic acid in Pasteur pipet) four times, each time using a clean pipet with new silicic acid and a separate sterilized 25 ml beaker used to contain the wash as it leaves the pipet. After washing the 5 ml through the silic acid four times, all of the eluates are combined in one sterilized 25 ml beaker. This fraction contains the hydrocarbons and any oxygen-containing molecules will stick to the columns. The 0.2 µl and 0.4 µl of the fractionated lizard extract was injected into the Gas Chromatography with Mass Spectrometery AT1 instrument with a nonpolar column. In order for the injected sample to process properly, the column was heated from 200ºC at 4ºC/min to a maximum of 300ºC. A gas flow rate of 25 meters in 1.91 minutes or 21.8 cm/second was achieved with a helium carrier. The results were recorded by a Hewlett Packard 3390A integrator (Appendix B and C).

Bovine Blood

Bovine blood used as a feeding source for mosquitoes was obtained from JenCo. (Fellowship, Florida). Four liters of refrigerated bovine blood treated with 12.0 grams of sodium citrate (anticoagulant) and antibiotics (Nystatin 125,000 units and Kantrex 125 mg) to retard spoilage were used in the olfactometer attractant artificial host agar (McKenzie 2000) and blood feeding pads for the mosquito colony.

Non-Attractant Agar

The non-attractant artificial host agar used in olfactometer trials was prepared by combining 133 ml of BSS® Plus intraocular irrigating solution (Alcon Labs, Ft. Worth, TX), known as part one, and 1.66 grams agar (U.S. Biochemical Corp., Cleveland, OH). Part one contained 0.395 mg sodium chloride, 0.433 mg dibasic sodium phosphate, 2.19 mg sodium bicarbonate, sodium hydroxide and/or hydrochloric acid, which adjusts the pH of the solution when added to water during eye surgery. Part one components were found not to be attractive to blood feeding insects (Galun and Margalit 1970; McKenzie 2000, 2003).

The non-attractant agar was prepared by combining 133 ml of part one BSS solution with 1.66 grams of agar in a 250 ml beaker. It was then heated in a microwave oven for two minutes, pausing to stir each time the solution boiled to the rim of the beaker. The prepared agar solution was poured into two 60 cc syringes that had a small hole cut out of the end with the plunger inserted to accommodate 60 cc. Both filled syringes were placed in a larger beaker and placed in the refrigerator for 24 hours.

Attractant Agar

Attractant agar for olfactometer studies was made by combining part one BSS® Plus intraocular irrigating solution (Alcon Labs, Ft. Worth, TX) with a sterile solution referred to as part two and 1.66 grams agar (U.S. Biochemical Corp, Cleveland, OH), making an artificial agar that has certain components known to be attractant for blood feeding insects (Galun 1967, Galun et al. 1985a; McKenzie 2003). Part two solution contains 3.85 mg calcium chloride dehydrate, 5 mg magnesium chloride hexahydrate, 23 mg dextrose, 4.6 mg glutathione disulfide, and also contains sodium hydroxide and/or

hydrochloric acid, which adjusts the pH of the solution when added to water in preparation for eye surgery. When both parts one and two are combined according to the corporate insert for the BSS® Plus Ocular Solution, the approximate pH is 7.4.

The attractant agar was prepared by combining 100 ml of parts one and two ocular solution with 1.66 grams of agar and heating in a microwave oven as previously directed. The cooked agar was allowed to cool for approximately five to seven minutes. While being stirred slowly, 33 ml of the bovine blood was poured into the solution being careful not to overheat the blood or allowing it to turn brown. The color of the medium should be red. The prepared agar solution was poured into two 60 cc syringes as previously directed and placed in a refrigerator for 24 hours.

Artificial Host

Artificial hosts used in the olfactometer were made out of 35 mm film canister lids filled with blood agar. A 35 mm plastic film canister lid was used as the holding chamber for the artificial agar medium. Cutting a 4 mm securing ring from the top of the 35 mm canister, the ring was added to snap the silicone membrane (Butler et al.1984, Burkett 1998) in place securing and enclosing the artificial agar medium in the film canister lid. A size 18 syringe needle was used to place a hole in the top of the lid to insert the electrical probe that connected to the amplifer. Each amplifier could be adjusted to record data at a 1,000, 5,000 or 10,000 amplication rate. This adjustment was necessary depending on the type of insect activity and type of insect being used in the system. These artificial hosts were placed membrane side down in the Plexiglas covering the olfactometer over a fiber-optic light which imitates dusk and dawn infrared sources on each host equally (McKenzie 2003) (Figure 4-3).

Figure 4-3. Artificial host in position in Plexiglas on top of the olfactometer with a red wire electrical probe inserted in the medium and connected to the amplifier. A blue wire is connected to the wire sensor and amplifier.

Statistical Analysis

The three trials totaling 15 replications of *Cx. erraticus* detecting the presence of *A. carolinensis* lizards were set up as a randomized paired *t*-test in the laboratory olfactometer. The Medusa 2.1.2 software designed by N. Hostettler in Gainesville, Florida, was used to analyze the total number of landings at each port identified as lizard or no lizard. The data was normalized using the square root (SQRT) of n+1 using a one-tailed *t*-test to evaluate if significant differences were found between the mosquitoes' ability to locate the olfactometer port in which the lizard was present in. Analysis of variance (ANOVA) was used to show the main and interaction effects of categorical independent variables on an interval dependent variable.

Objective 1 Procedure

The olfactometer was set up to determine if *Cx. erraticus* could determine the presence of *A. carolinensis* by measuring the landing rate activity of the mosquito. This

was done by comparing mosquito activity between sensors over airstreams with and without lizards present. Three trials in the olfactometer yielded five paired replications per trial utilizing five different *A. carolinensis* lizards, 160 field collected *Cx. erraticus* female mosquitoes (32/replication), cylinder CO_2, attractant artificial host medium, and olfactometer fiber-optic lights on during trial. The pie-shaped olfactometer was divided into five separate replications of paired *t*-test systems with two ports in each replication, one treated with lizard and one without, 32 *Cx. erraticus* mosquitoes, one *A. carolinensis* lizard in the air source (Figure 4-4), and two 100 dram vials (5.08 cm x 10.8 cm) with tygon tubing.

Each of the five pie-shaped chambers were sectioned off and have two port holes where tygon tubing covered with bridal veil netting was inserted and extended from 100 ml vials. A second piece of tygon tubing exited the 100 dram vials and extended to the bottom of the olfactometer, connecting to the incoming fresh air containing environmental CO_2. Each of the tygon tubes covered with bridal veil were inserted into the port of the olfactometer directly under the wire sensor, which lay across the bottom side of the 35 mm film canister lid covered with the artificial membrane enclosing the attractant artificial host medium. The film canister had a small hole made by a size 18 syringe needle where the single wire probe was inserted into the medium and plugged into an amplifier. One lizard was randomly placed in one of the 100 dram vials connected to each of the paired air sources of the two choice pie-shaped chambers. When the mosquitoes perceived the lizard's CO_2 and/or body odors, they landed on the sensor and probed the attractant agar medium as if to take a blood meal at the same time completing the electrical circuit registering bite-seconds on the computer outside of the

Faraday cage. The overhead lights inside the Faraday cage were turned off while the fiber-optic lights recessively mounted in the base of the olfactometer were positioned directly under each wire sensor artificial host to mimic dusk and dawn time periods (Figure 4-4).

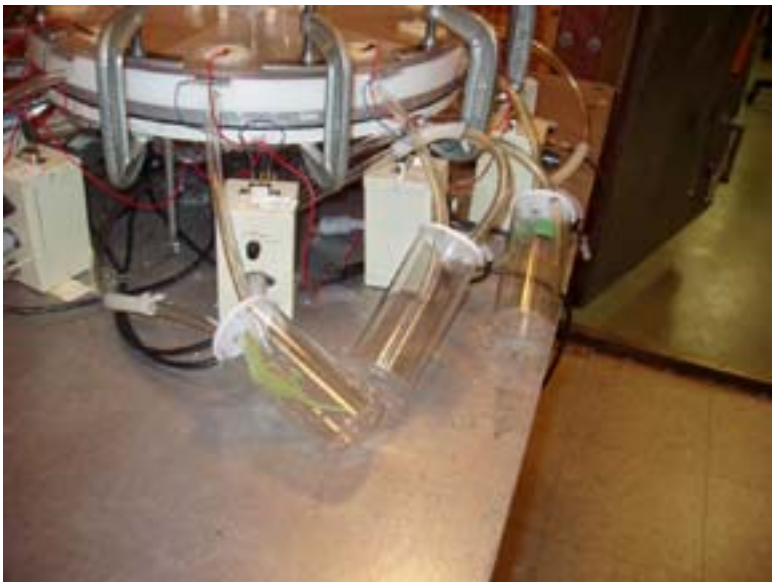


Figure 4-4. Olfactometer Trial 1 lizard vs. no-lizard paired *t*-test system.

Objective 2 Procedure

The olfactometer was set up to measure and evaluate the attractancy of *Cx. erraticus* to the lizard extract (LE) in the laboratory and the most attractive dosage would then be used in the field trapping study. To determine the attractiveness of lizard extract (LE), five microliter amounts (5, 10, 15, 20 and 25) were placed in the olfactometer (Figure 4-5). The five microliter amounts would be evaluated against a hexane control, both on artificial membrane host and in the air stream on perfume sticks in a randomized order. The microliter amount that performed best as an attractant would then be compared with hexane standard in paired *t*-tests and in an open choice test (Figure 4-1B).

The LE rate that was the most attractant would be compared to hexane standard placed on perfume sticks in tygon tubing and placed in randomized positions in the

olfactometer. The artificial hosts were deleted in this trial. The sensors were bent down at a 90º angle to cover the bridal veil covered end of the tygon tubing holding the perfume sticks with the LE and hexane standard in order to record the mosquito landing rate over the treated and untreated air source. The 10 holes cut in the Plexiglass centered over each of the 10 ports were closed off with blank 35 mm film canister lids covered with Saran Wrap. *Culex erraticus* mosquitoes (n=160) were released in the olfactometer with the fiber optic lights and 250 ml/min CO_2 flow rate on during each of the eight hour trials. One hundred sixty wild collected *Cx. erraticus* mosquitoes were used in each trial. Temperature and relative humidity was recorded inside the Faraday cage and inside the olfactometer air-stream being introduced into the system. All trials were run for eight hours with CO_2 and fiber-optic lights on. The data was transferred by the program Strawberry Tree to a computer where data was processed using macro programs written by N. C. Hostettler in Gainesville, Florida.

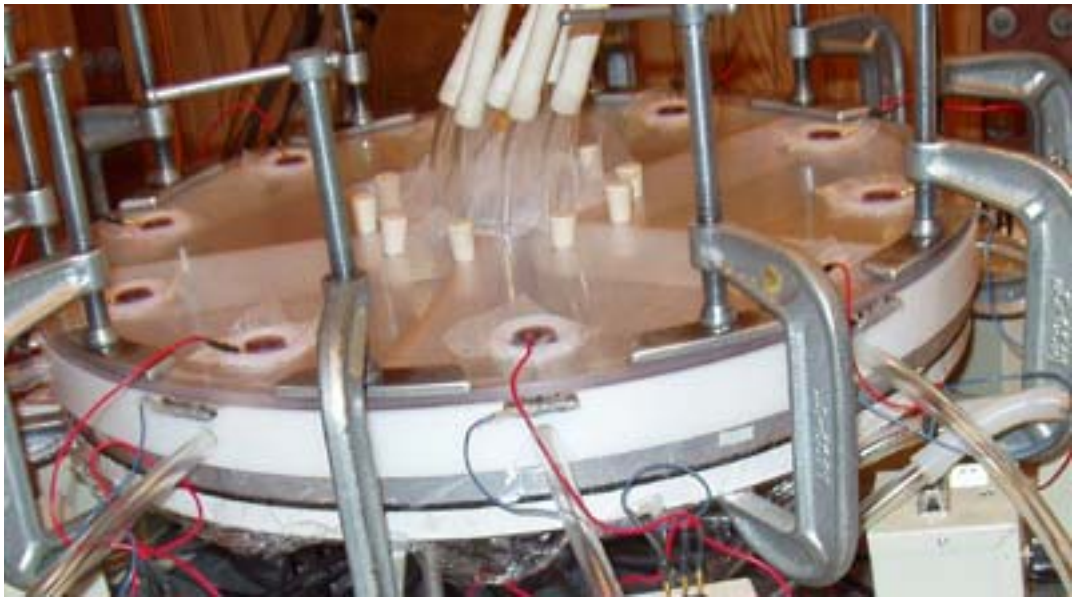


Figure 4-5. Olfactometer with 35 mm canister lids containing agar combined with BSS® Parts one and two.

Objective 3 Procedure

The olfactometer was set up to determine if *Cx. erraticus* has a preference for malaria infected *A. carolinensis* lizards over noninfected *A. carolinensis* lizards. The pie-shaped olfactometer was divided into five replications with two paired ports per replication (Figure 4-5). The starting port on the olfactometer is assigned by randomization and each port is randomly assigned a malaria infected lizard or a noninfected lizard (Figure 4-6).

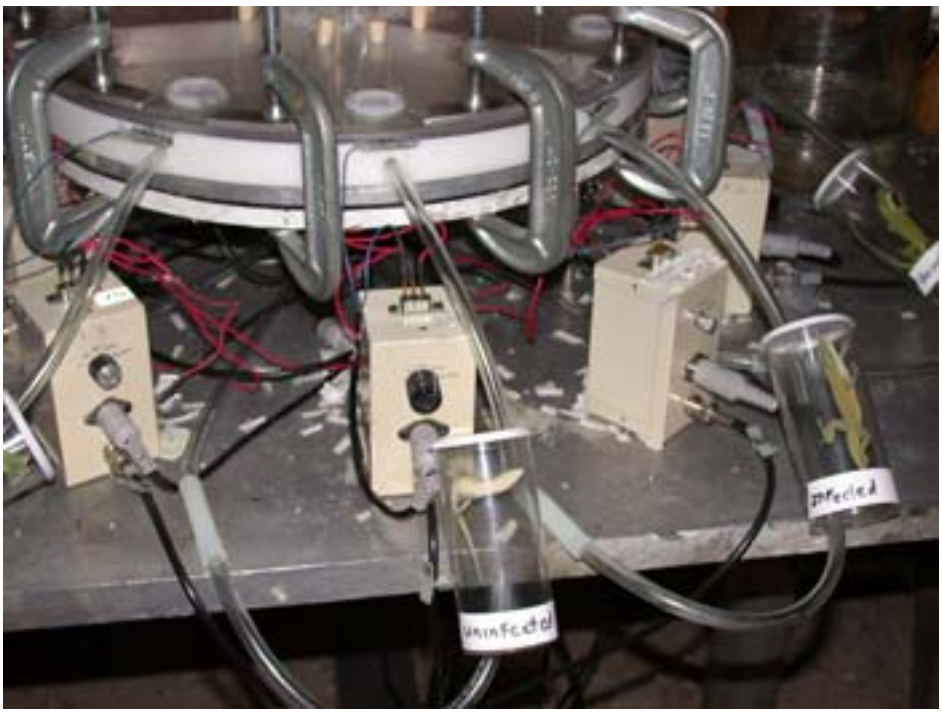


Figure 4-6. Malaria infected and noninfected lizards in paired *t*-test with no CO_2.

Thirty-two field collected *Culex erraticus* mosquitoes were placed in each replication with no artificial host or CO_2 used in this objective. The 35 mm film canister lids were closed with Saran Wrap and the incoming tygon tubing covered with bridal veil netting was inserted into each port around the olfactometer as done previously in objective 2. The wire sensor was bent at a 90º angle over the port hole containing the tygon tubing coming from the 100 ml vials containing the infected and noninfected

lizards. A two lead wire instead of a one-wire probe connected the sensor and amplifier because the recorded data was based on landing, not probing of the agar medium.

Temperature and relative humidity in the Faraday cage and air stream of the olfactometer were recorded. Lights in the Faraday cage were turned off and the fiber optic lights in the olfactometer remained on to mimic dusk and dawn, and the data was recorded as described previously.

Statistical Analysis

The pie-shaped olfactometer was divided into five replications with two paired ports per replication and 32 *Cx. erraticus* mosquitoes placed in replication to measure and evaluate the landing rate of preference to infected or noninfected lizards. The trials were set up as a randomized paired *t*-test in the laboratory olfactometer. The Medusa 2.1.2 software designed by N.C. Hostettler was used to analyze the total number of landings at each port identified as either infected lizard or noninfected lizard. The data was normalized using the SQRT of n+1 using a one-tailed *t*-test to evaluate if significant differences were found between the mosquitoes' preference for infected lizard over noninfected lizards. ANOVA was used to show the main and interaction effects of categorical independent variables on an interval dependent variable.

Results

Objective 1

The olfactometer was set up to determine if *Cx. erraticus* could determine the presence of *A. carolinensis* by measuring the landing rate activity of the mosquito. Five paired *t*-test replications were recorded for each of the three trials in objective 1, giving a total of 15 paired *t*-test replications that were initially run with the fiber optic-lights on. The SQRT of n+1 was used to normalize the data (Table 4-1) and an ANOVA

(Table 4-2) showed no significance between groups or within groups in this trial (α=0.05; p=0.75; F=2.62; df=5).

Table 4-1. Lizard vs. no lizard probing normalized data of a three trial 15 replication paired *t*-test in olfactometer. BSS® part two and three used in artificial host medium. Cylinder carbon dioxide introduced into the system at a flow rate of 250 ml/min with olfactometer fiber- optic lights left on during trial.

	Trial 1		Trial 2		Trial 3	
Replication	Lizard	No Lizard	Lizard	No Lizard	Lizard	No Lizard
1	74	76	66	60	74	28
2	35	124	97	171	26	44
3	12	19	80	37	40	48
4	95	45	53	23	42	22
5	22	34	31	29	86	24
Average	48	60	65	64	54	33

Table 4-2. Analysis of variance for the 3 trials performed with BSS® part one and part two in artificial host medium, 250 ml/min of carbon dioxide and olfactometer fiber-optic lights on.

ANOVA

Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	3638.05	5	727.61	0.53	0.75	2.62
Within Groups	32796.43	24	1366.52			
Total	36434.48	29				

In all three paired *t*-tests, the host finding mission of *Cx. erraticus* in selecting the presence of the lizard at a higher rate than that of the vacant port resulted in 55 and 52 average bite-seconds, respectively (Figure 4-7).

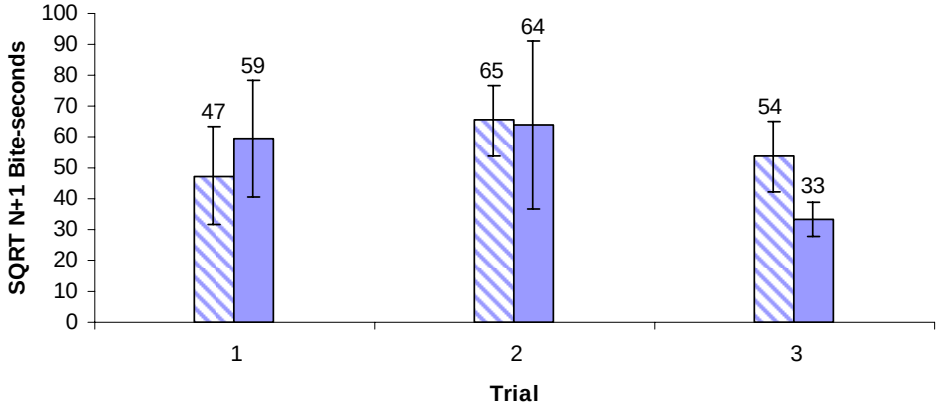


Figure 4-7. Lizard versus no lizard normalized data with average bite-seconds and standard error. Olfactometer fiber-optic lights on during the three trials (15 replications). The striped bars indicate lizard present and solid bars indicate no lizard present in study. Artificial host medium combined with cylinder carbon dioxide and fiber-optics lights on during this study.

Observations found in published data late in this study on nocturnal feeding habits of *Cx. erraticus* led to setting olfactometer trials run in darkness to determine if the fiber-optic light source in combination with the attractant artificial host and cylinder CO_2 was a major factor in the first objective trial not being significant.

The removal of the artificial host medium, cylinder CO_2, and turning the olfactometer fiber-optic lights out during the 15 paired *t*-test replications showed a statistical significance (α=0.05; p=0.002; F=4.20; df=5). The ability of *Cx. erraticus* to see the *A. carolinensis* lizard was prohibited because the lizards were suspended lower than the olfactometer. The SQRT of n+1 normalized the data and an ANOVA statistically analyzed the results (Tables 4-3, 4-4).

Table 4-3. Analysis of variance for the 15 paired *t*-replications performed in the olfactometer without attractant artificial host, cylinder carbon dioxide, and fiber optic olfactometer lights turned off.

ANOVA

Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	11366.07	5	11366.07	12.11	0.002	4.20
Within Groups	26270.19	28	938.22			
Total	37636.25					

Table 4-4. Lizard versus no lizard normalized data for Trials 1 through 3. No attractant artificial host, no carbon dioxide, and no fiber-optic lights used in these trials.

	Trial 1		Trial 2		Trial 3	
Replication	Lizard	No Lizard	Lizard	No Lizard	Lizard	No Lizard
1	61	12	103	61	154	29
2	67	15	127	44	2	1
3	66	18	25	22	16	16
4	64	20	80	34	36	21
5	43	22	65	40	75	45

The normalized data of the second set of three trials show deliberate landing on the sensors by *Cx. erraticus*, signifying its ability to locate the lizard by CO_2 and/or body odors (Figure 4-8). Removing the artificial host, cylinder CO_2, and turning the fiber optic lights off aided *Cx. erraticus* in successfully detecting the lizard's presence.

Objective 2

The olfactometer was set up to measure and evaluate the attractancy of *Cx. erraticus* to the lizard extract (LE) in the laboratory. The most attractive dosage would then be used in the field trapping study. Lizard extract (LE) in 5, 10, 15, 20 and 25 microliter amounts were compared to hexane in 9 paired *t*-tests in an open chamber olfactometer with the fiber optic lights on.

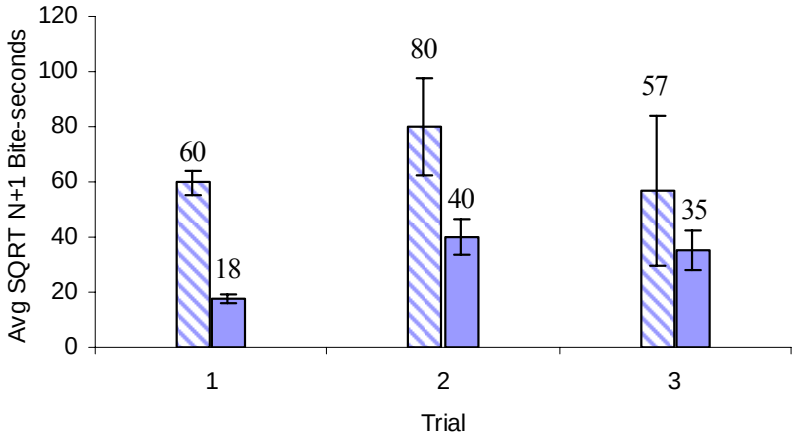


Figure 4-8. Lizard versus no lizard normalized data with average bite-seconds and standard error in olfactometer with LED lights off during the three trials (15 replications). Striped bars indicate lizard present and solid bars indicate no lizard present in study. No artificial attractant medium or carbon dioxide used.

ANOVA was used to analyze the data and resulted in statistical significance ($p=0.02$; $df=9$; $F=2.32$) with 10 microliters of LE having the most bite-second activity (Table 4-5).

Table 4-5. Analysis of variance for the evaluation of the 5, 10, 15, 20 and 25 µl of lizard extract with equal amounts of hexane in paired *t*-test.

ANOVA Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	9506.85	9	1056.32	2.32	0.02	2.00
Within Groups	36410.33	80	455.13			
Total	45917.18	89				

Results show that the mosquitoes consistently probed the artificial host agar for the control hexane in each of the replications approximately the same number of average bite-seconds regardless of the dosage (Table 4-6, Figure 4-9).

Table 4-6. Normalized data for nine trials with five replications per trial yielded a total of 45 replications. The bold numbers in the table represent the average number of bite-seconds for hexane (Hx) on attractant artificial host agar.

	Treatment 25 µl LE	Treatment 25 µl Hx	Treatment 20 µl LE	Treatment 20 µl Hx	Treatment 15 µl LE	Treatment 15 µl Hx	Treatment 10 µl LE	Treatment 10 µl Hx	Treatment 5 µl LE	Treatment 5 µl Hx
Avg. Bite-seconds	26	**32**	40	**30**	38	**30**	60	**29**	25	**24**

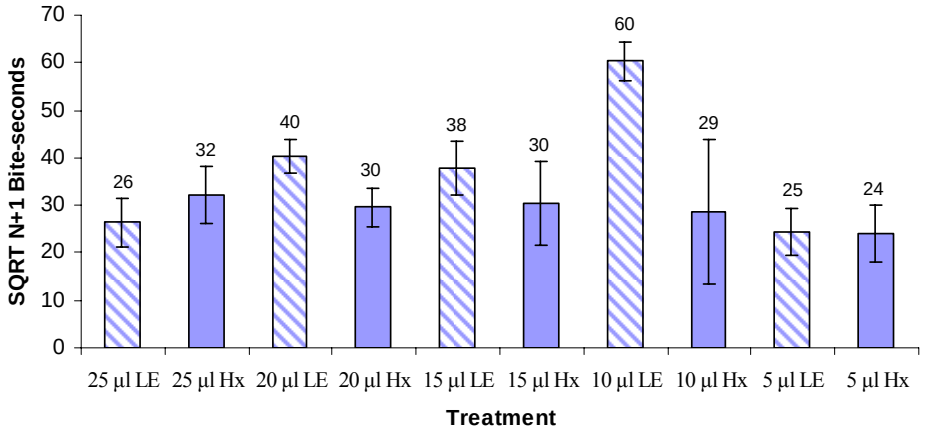


Figure 4-9. Striped bars represent lizard extract on artificial membrane and solid bars represent hexane control on artificial membrane. Artificial host agar in 35 mm film canister with BSS® part one and two was used to stimulate probing by mosquitoes to register bite-seconds. The 10 µl of lizard extract received significantly more average bite-seconds than all other treatments.

The evaluation of LE in five microliter amounts (5, 10, 15, 20, 25) on artificial host agar with BSS® part one and two in a randomized open chamber olfactometer resulted in the trials being statistically significant (α=0.05; p=<0.001; df =9; F=11.64) (Table 4-7).

Table 4-7. Analysis of variance for nine lizard extract open chamber artificial media comparisons with hexane control to determine difference between treatments.

ANOVA Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	28942.85	9	3215.87	11.64	1.79E-11	2.00
Within Groups	22107.33	80	276.34			
Total	51050.18	89				

The normalized data for the average bite-seconds of the 10 microliters of LE was also higher (n=71) than the other LE amounts. Also, the corresponding amounts of

hexane used as controls resulted in the normalized average bite-seconds being approximately the same regardless of dosage amount (Figure 4-10).

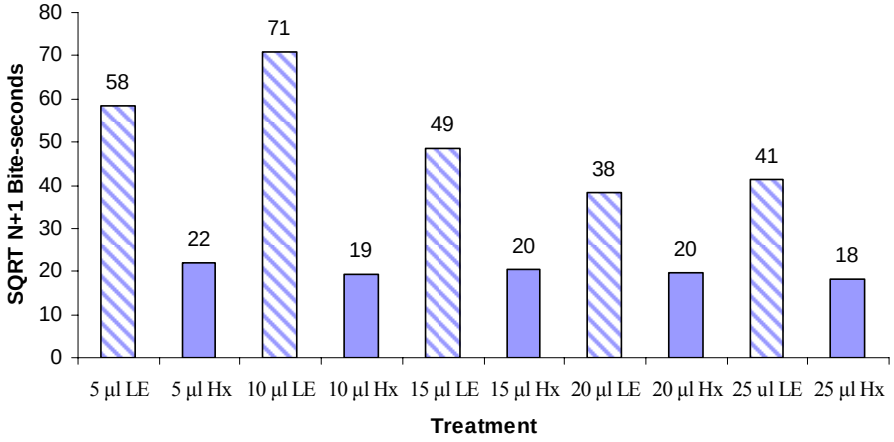


Figure 4-10. Normalized data graph for the nine trials of five microliter amounts of lizard extract compared to five microliter amounts of hexane as control. Both lizard extract and hexane are placed on perfume sticks artificial host media in an open chamber olfactometer. Striped bars represent lizard extract and the solid bars represent hexane.

With the olfactometer separated into six ports consisting of four LE microliter amounts (5, 10, 15 and 25), one 25 microliter of hexane standard, and one blood agar with BSS® part one and part two a statistical significance (α=0.05; p=0.01; df =5; F=3.56) is reported (Table 4-8). The LE was compared to the hexane standard and blood agar by directly applying all dosages to artificial membrane host (Figure 4-11).

Table 4-8. Analysis of variance of normalized data comparing four lizard extract amounts of hexane standard and one blood agar with BSS® part one and two in open olfactometer containing six ports.

ANOVA						
Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	6730.75	5	1346.15	3.56	0.01	2.34
Within Groups	27210.99	72	377.93			
Total	33941.75	77				

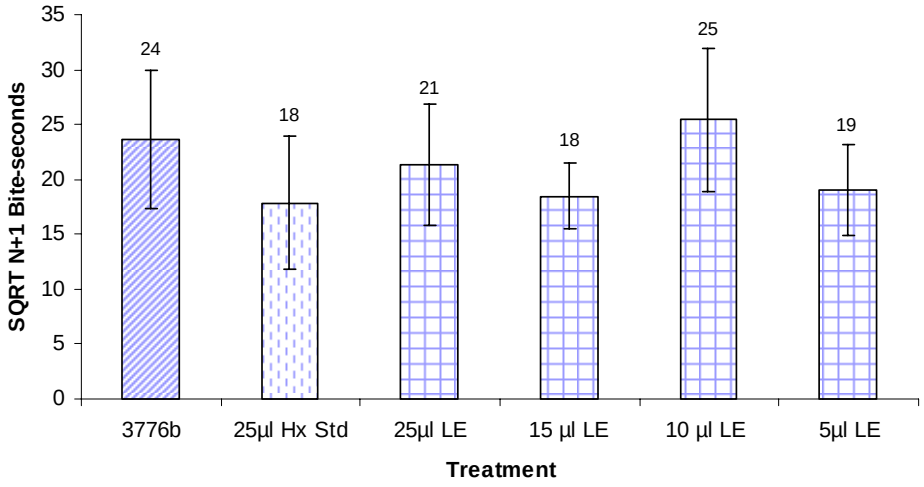


Figure 4-11. Comparing the normalized average data of the six ports with four different microliters of lizard extract to one 25 microliters of hexane standard and one blood agar with BSS® part one and two. The striped bar represent the blood agar 3776, the dotted line bar represent the hexane standard and the plaid bars represent the lizard extract.

All dosages were applied directly to artificial host only. The hexane standard received less bite-seconds than four of the treatments except the 15 µl LE. The 10 µl LE received more bite-seconds than all other treatments including the blood agar standard host (3776). However, the blood agar 3776 outperformed four of the LE treatments and the hexane standard.

The remaining four olfactometer ports compared three LE (10, 15 and 25 microliters) amounts to one 25 microliter hexane standard. The four remaining ports evaluated solely on odor from perfume sticks did not produce a significant difference (α=0.05; p=0.47; df =3; F=0.85) (Table 4-9).

Table 4-9. Analysis of variance of normalized data comparing three lizard extract amounts to one hexane standard in an open olfactometer containing four ports.

ANOVA						
Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	1365.55	3	455.18	0.85	0.47	2.80

Within Groups	25629.79	48	533.95
Total	26995.35	51	

No significance was found in or between these four treatments, but the 10 µl of LE received slightly more bite-seconds than the other three treatments (Figure 4-12).

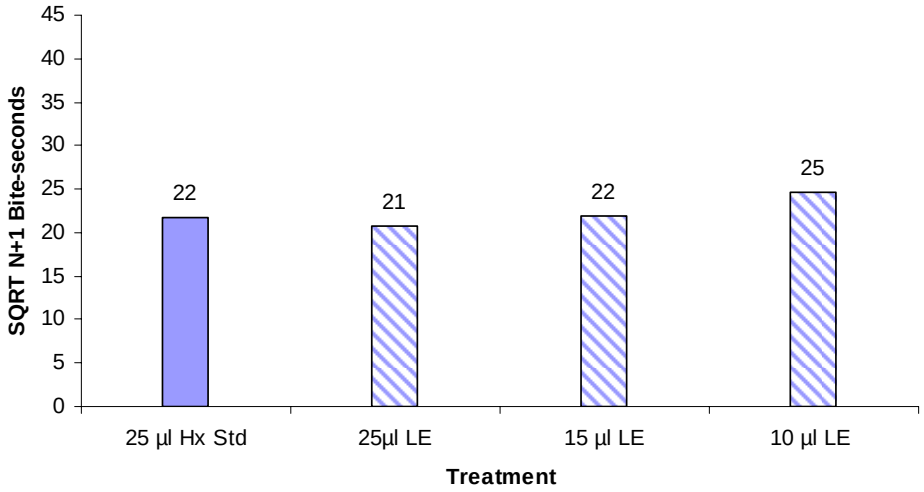


Figure 4-12. Normalized data graph comparing three lizard extract amounts to hexane standard. The striped bars represent the lizard extracts and the solid bar represents the hexane standard. All four treatments were evaluated in air stream only.

Combining both parts of the divided olfactometer trials into single trials resulted in a significant difference (α=0.05; p=<0.001; df=17; F=19.75) (Table 4-10). However, the results found through the ANOVA on the combined trial may show a large significance overall, but the environment inside each part of the combined trial was different. Therefore, separating the trials into their respective parts was probably best and a truer representation of the data was more accurately reported (Figure 4-13).

Table 4-10. Analysis of variance for combined parts 1 and 2 of divided olfactometer trial comparing 10 microliters lizard extract to 10 microliters hexane standard.

ANOVA Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	28059536	17	1650560.94	19.75	1.0067E-27	1.70
Within Groups	10528446	126	83559.09			

Total	38587982	143

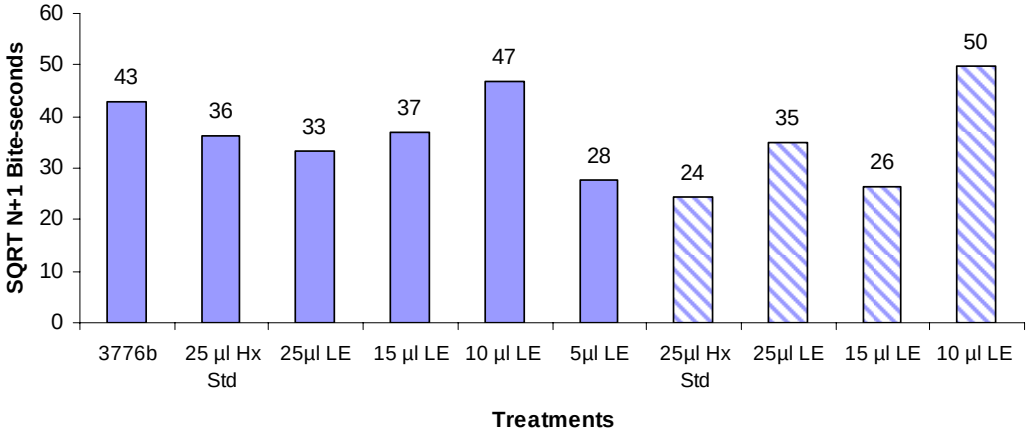


Figure 4-13. Normalized data graph for combined trial. Solid bars represent part 1 with six ports in olfactometer comparing four treatments of lizard extract to one treatment of hexane standard and one bovine blood agar with attractant. The striped bars represent part 2 with four ports comparing three treatments of lizard extract to one treatment of hexane standard on perfume stick.

Because the 10 µl LE received significantly more bite-seconds, two different tests were used to evaluate the 10 µl of LE as an attractant for *Cx. erraticus*.

The first test compared the 10 µl of LE to 10 µl of hexane standard with treated perfume sticks in the air stream and with bovine blood agar in five paired *t*-trials. As a result, the ANOVA and a standard two-sample *t*-test gave a significant difference (p=0.01; df=9; F=2.99) (p=<0.001; df=24) between the two treatments (Table 4-11, Table 4-12).

Table 4-11. Analysis of variance and t-test comparing 10 microliters lizard extract to 10 microliters hexane in five paired *t*-test (25 replications) on perfume sticks with mosquitoes probing bovine blood agar.

ANOVA						
Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	28903239	9	3211471	2.99	0.01	2.12
Within Groups	42954871	40	1073872			
Total	71858110	49				

t-test: Paired Two Sample for Means		
	LE	Hexane
Mean	1250	2364
Variance	1108635	1239094
Observations	25	25
df	24	
P(T<=t) one-tail	1.44E-09	

Table 4-12. Standard two-sample *t*-test on normalized data for test 1 evaluation of lizard extract compared to hexane standard. P-value represents (P (T<=t) one-tail) and data significant at α=0.05.

	Rep. 1		Rep. 2		Rep. 3		Rep. 4		Rep. 5	
	LE	Hex	LE	Hex	LE	Hex	LE	Hex	LE	Hex
Mean	44.21	29.24	51.65	31.81	36.90	25.45	54.80	41.21	49.46	35.05
Variance	170.16	166.32	8.37	165.45	41.35	133.36	194.28	389.51	76.70	163.86
Observations	5		5		5		5		5	
df	4		4		4		4		4	
p-value	0.002		0.01		0.01		0.01		0.02	

The 10 µl of LE received higher bite-seconds than the 10 µl of hexane standard in each of the five eight-hour trials. Significance between the LE was found only in replication three with no significance found between the hexane standards (Figure 4-14). The nine replications in the open chamber were randomized with *Cx. erraticus* having only two choices, the 10 µl LE or the 10 µl hexane. The two treatments were found to have a significant difference with the ANOVA (p=<0.001; df=17; F=18.03) and with a standard two-sample *t*-test (p=0.005; df=8) (Tables 4-13, 4-14).

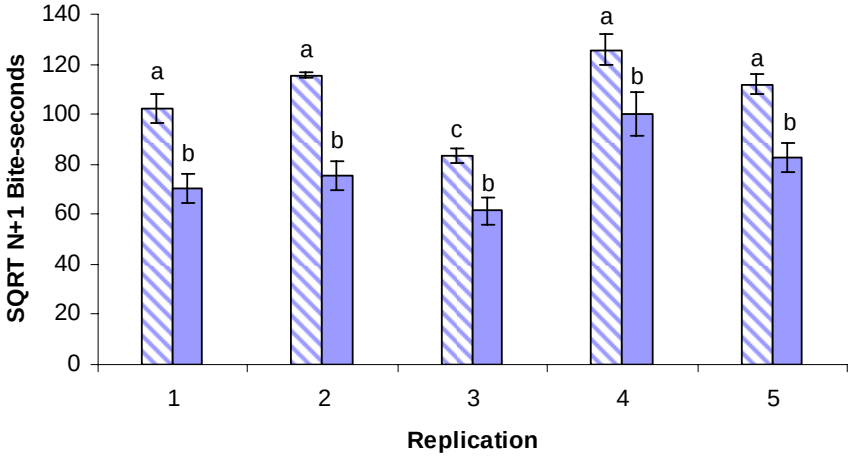


Figure 4-14. Normalized data graph for five paired *t*-trials comparing 10 microliters lizard extract with 10 microliters hexane standard on perfume sticks with bovine blood agar. Striped bars indicate the lizard extract and the solid bars represent the hexane standard. Standard error with small letters above bars show significance between treatments.

Table 4-13. Analysis of variance for test 2 in an open olfactometer comparing the 10 microliters of lizard extract to 10 microliters of hexane with treatments on perfume sticks only.

ANOVA						
Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	26222566	17	1542503.88	18.03	5.12E-26	1.70
Within Groups	10776623	126	85528.75			
Total	36999189	143				

Table 4-14. Normalized data in a standard two sample *t*-test for test 2 comparing lizard extract to hexane.

t-Test: Paired Two Sample for Means		
	LE	Hexane
Mean	60.90	29.13
Variance	905.58	160.18
Observations	9	9
df	8	
P(T<=t) one-tail	0.005	

The nine replications of comparing the 10 µl LE to 10 µl hexane were treated as paired *t*-test and the results indicated that *Cx. erraticus* found the LE more attractive than the hexane (Figure 4-15). Both 0.2 µl and 0.4 µl were injected into the gas

chromatograph AT1 instrument and both samples produced extremely small peaks, making the calculation of the retention indices difficult (Appendix B and C).

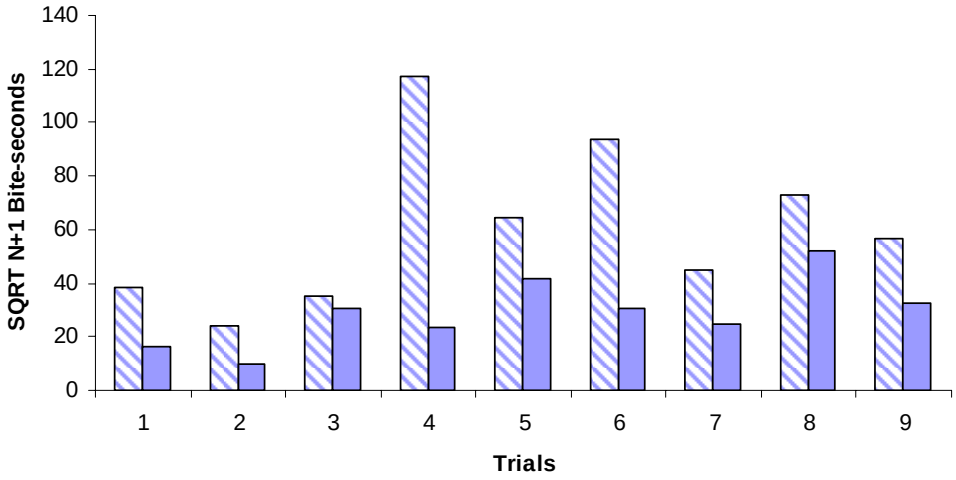


Figure 4-15. Normalized data graph for test 2 comparing the lizard extract to the hexane. The striped bars represent the lizard extract and the solid bars represent the hexane.

Objective 3

The olfactometer was set up to determine if *Cx. erraticus* has a preference for malaria infected *A. carolinensis* lizards over noninfected *A. carolinensis* lizards. Comparing infected lizards to noninfected lizards in three trials divided into five replications each (n=15 replications) resulted in no significant difference (p=0.98; df=9; F=0.24) when ANOVA was used to analyze the data (Table 4-15). Also, no significance was found in the standard two-sample *t*-test (p=0.17; df=14).

Table 4-15. Analysis of variance comparing infected lizards to noninfected lizards to determine the differences between treatments.

ANOVA Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	2801.77	9	311.31	0.24	0.98	2.39
Within Groups	25790.80	20	1289.54			
Total	28592.57	29				

Table 4-16. Standard two sample *t*-test comparing infected lizards to noninfected lizards.

	Noninfected Lizard	Infected Lizard
Mean	52.39	48.67
Variance	1263.00	1127.80
Observations	15	15
df	14	
P(T<=t) one-tail	0.17	

The landing activity of *Cx. erraticus* in selecting infected lizards to noninfected lizards was performed in the olfactometer without CO_2 and with the fiber optic-lights on during each trial (Figure 4-16). The normalized data graph with the average bite-seconds and standard errors represent the three trials totaling 15 replications.

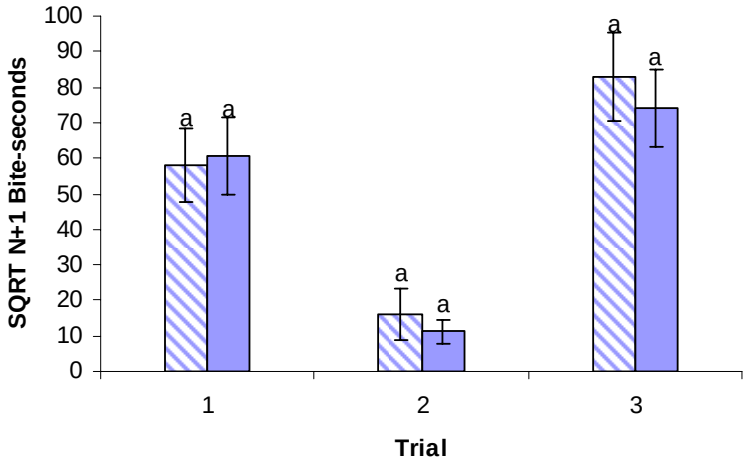


Figure 4-16. Normalized data graph for comparing *Culex erraticus* mosquito ability to select malaria infected to noninfected *Anolis carolinensis* lizard. Striped bars indicate malaria infected lizards and solid bars represent noninfected lizards.

Discussion

The decision to introduce CO_2 into the olfactometer for objective 1 was based on the fact that higher numbers of *Cx. erraticus* are attracted to CO_2 baited CDC light traps (Carestia and Horner 1968, Cupp et al. 2003, Kent et al. 2001, Kline 2002, Kline and Mann 1998, Roberts 1972). Also Van Thiel (1974) tested CO_2 in combination with air in an air current olfactometer and showed a 76% increase in mosquito attraction.

Carbon dioxide normally stimulates or enhances the biting activity of most mosquitoes (Service 1993). Mosquitoes use CO_2 as an activator that raises the awareness that a host is close and induces the mosquito to land and probe the host for feeding purposes (Khan et al. 1966).

With very little understanding of the host feeding behavior of *Cx. erraticus*, it was anticipated that the introduction of CO_2 in combination with the bovine blood treated with BSS® part one and two would heighten the stimulation for the mosquito to be attracted to the olfactometer port occupying the CO_2 from the lizard because of the other components found in vertebrate respiration (Mboera and Takken 1997; Kline et al. 1991a, 1994b). However, with *Cx. erraticus* not providing significance in locating the olfactometer ports occupied by the *A. carolinensis* lizard, it was apparent that the attractant power of the artificial medium, the CO_2, and the olfactometer fiber optic lights being left on possibly hindered the mosquito's ability to locate the port containing the lizard in order to probe the artificial host and register a higher number of bite-seconds.

Love et al. (1963) trapped higher numbers of *Cx. erraticus* between the hours of 6:00 pm and 9:00 pm in two different studies. This may suggest that the feeding behavior of *Cx. erraticus* gains momentum as a crepuscular feeder as the sun goes down, therefore the olfactometer fiber optic lights being on throughout the feeding trial may also contribute to the interruption of the appetitive searching mode of the mosquitoes. Sutcliffe (1987) showed that some haematophagous insects use various cues, including appetitive searching, activation, and visual cues as attractants to locate their host.

According to the study by Love et al. (1963), *Cx. erraticus* begins feeding at dusk when natural light is low but still available. While in the appetitive searching mode, *Cx.*

erraticus may use visual cues from *A. carolinensis* to locate the host and secondarily use the lizard's respired CO_2 and body odors to make the commitment for host contact (Allan et al. 1987). The design of the olfactometer does not allow the mosquitoes to visually see the lizards to make a commitment based on visual cues, therefore host location in this study evaluates detection by host CO_2 and host body odors.

Environmental concentrations of CO_2 can be a factor for attracting different species of mosquitoes (Reeves 1951). Carbon dioxide is a natural component in the atmosphere and is found naturally in the breath of humans and animals, and is found in combination with emanating odors from the skin of vertebrate hosts (Black 1968, Costantini et al. 1996, Costantini et al. 1998; Gillies 1980, Lehane 1991, Wessenling 1962). Kline and Mann (1998) showed that five species of *Culex* mosquito; *salinarius, nigripalpus, pilosus, restuans* and *erraticus*, responded to CO_2 and light. Of the five *Culex* species, *pilosus* and *erraticus* did not respond to light-only traps. The amount of CO_2 that *A. carolinensis* emanates during respiration may not be known, but it is known that the average weight of *Sceloporus occidentalis* Baird and Girard, the western fence lizard, is 15.76 grams and it respires approximately 1 ml CO_2 per hour. If *Sc. occidentalis* ingests a meal weighing 4% of its body mass, its metabolism increases briefly to about 3.5 ml of CO_2 per hour (W.A. Hopkin, personal communication).

The average weight of an *A. carolinensis* lizard is approximately 5 grams, and if weight is an indicator as to the amount of CO_2 respired normally and during increased metabolism of food, then *A. carolinensis* would respire one-third that of the *Sc. occidentalis*. Kline (1994a) reported that some species of mosquitoes may select its host according to the size of the host and the amount of CO_2 respired by the host. Not

knowing the sensitivity of *Cx. erraticus* to CO_2 or how the mosquito sorts out the environmental CO_2 from the small amount the lizard respires in its breath and from its skin, excluding the cylinder CO_2 from the system is necessary to allow only the CO_2 from the lizard and from the introduced air stream into the system.

Therefore, three new trials were run to eliminate the possible confusion and to establish the conditions for *Cx. erraticus* to locate *A. carolinensis.* The new trials were composed of 15 replications run in the same laboratory olfactometer excluding the cylinder CO_2, the artificial host medium, and the fiber-optic olfactometer lighting.

Culex erraticus convincingly located the lizards by deliberate approach and landing on the sensors as if to land on the host. With the 0.6 ml/min of atmospheric oxygen being introduced into the system, the environmental CO_2 in combination with the respired CO_2 and body odors from the lizard was perceived and acted upon by the mosquito. As for the first objective, it established that with deliberate intentions *Cx. erraticus* located *A. carolinensis* in laboratory conditions.

An important point in the vector-host relationship is that *Cx. erraticus* could not use visual cues to locate the *A. carolinensis* lizard in the olfactometer. The ability of *Cx. erraticus* to locate *A. carolinensis* by CO_2 and/or body odors is important in establishing that this recognition takes places in the wild. The approved protocol in this study would not allow the use of live lizards in field traps to sample mosquito populations, therefore devising an attractant that mimics *A. carolinensis* was important to the success of this study.

Hexane is a widely used solvent used to make many types of crude and complex extracts, including trailing pheromone components for the fire ant *Solenopsis invicta*

Buren, sex pheromone for *Periplaneta americana* L., toxic extracts for *Ae. aegypti* larvae, hydrocarbon profiles of adult *An. maculipennis* Meigen, mating pheromone and hydrocarbons of the horn fly, and insect attractant activity (Alvarez et al. 1987, Anyaele and Amusan 2003, Bolton et al. 1980, Mackley et al. 1981, Shaw et al. 1976, Seelinger and Schuderer 1985). Hexane was used in this study to make the crude lizard extract from deceased *A. carolinensis* lizards that died while in colony.

Washing the deceased lizards in 100 ml of hexane removed cuticular carbons and other host compounds that would be used in evaluating the amount that may be an attractant for *Cx. erraticus* in locating the lizard, increasing the catch rate in trapping study (chapter 3) and was used in the gas chromatography evaluation.

Concentrating the 100 ml of hexane lizard wash under nitrogen to 10 ml resulted in a thick, cloudy extract with a strong pungent odor, as found in several prior studies of vertebrate skin compounds and components (Burken et al. 1985, Roberts and Lillywhite 1980, 1983).

In this study, 5, 10, 15, 20 and 25 microliters of lizard extract was applied directly to the artificial attractant made of bovine blood and the BSS® part one and two, and was evaluated against hexane as a control standard to see which amount, if any, *Cx. erraticus* would find most attractive.

The bovine blood in combination with the cylinder CO_2 with the fiber-optic lights on was used to create stimulation in the mosquitoes to locate the LE mixed with the bovine blood, which had already been determined to be attractive. Significance was found with the 10 microliters of LE receiving the highest number of average bite-seconds. The number of bite-seconds the hexane received was approximately the same

regardless of the amount deposited directly onto the artificial host. Also, the number of bite-seconds the remaining LE amounts received was approximately the same as the hexane. The suppression of these two observations may have been caused by the forced feeding behavior of the mosquitoes to the blood agar, CO_2 and the lighted fiber-optic lights, which was the same as that found in the first objective of the lizard no-lizard trials.

Also, the lizard constituents and compounds found in the extract in combination with the artificial blood agar host may have caused confusion to the mosquitoes about which agar host to feed on since bovine blood has been found in *Cx. erraticus* blood meals previously (Williams and Meisch 1981).

The four amounts of LE were compared a second time on perfume sticks instead of being applied directly to the artificial bovine blood host and was evaluated in an open chamber instead of a paired *t*-test. Significance was found in favor of the 10 microliters LE, which received even higher average bite-seconds (n=71) than the first set of trials. When the LE amounts were applied directly to the perfume sticks and placed in the air stream, the mosquitoes possibly moved in and out of the plume zone and located the port more readily, and then probed the air stream directly above the tygon tubing containing the perfume stick with the LE applied to it. It is interesting that the number of bite-seconds increased for all LE amounts, while the number of bite-seconds decreased with the hexane standards. The comparison of LE to the hexane standard showed that 10 microliters of the thick, cloudy, pungent body compounds and odor of *A. carolinensis* is the optimum amount that *Cx. erraticus* perceives as a host attractant.

The cloudy color of the extract is possibly attributed to the vertebrate skin lipids that are known to retard the loss of water from the epidermis of the lizard. The skin

lipids are also known to elicit behavioral responses in intra- and interspecific interactions (Albone 1984). Epidermal lipids are known to be a barrier to microorganisms that can cause harmful problems and disruptions on the outside of the lizards' body (Nicolaides 1974). Most lizards shed their skin due to growth, and it was observed in the laboratory colony of *A. carolinensis* lizards that shedding of the skin was a common occurrence. This may be a defense mechanism the lizard uses to remove microorganisms that could be harmful. Various other researches have been performed on the cuticular compounds of lizard, which can vary from species to species (Birkby et al. 1982, Nicolaides et al. 1968, Nieminen et al. 1967, Lindholm et al. 1981). Roberts (1980) examined the epidermal lipids of lizards in the suborder Sauria and found nonpolar lipids on the *Iguana*, including cholesterol, diacyglycerols, alcohols, free fatty acids, aldehydes, triglycerides, wax esters and sterol esters.

All lizard skin contains an abundant and common type of sterols (Pesonen 1954). However, many of the researched constituents found on and in lizard skin are processed with Thin Layer Chromatography (TLC) which in itself cannot determine the presence of cholesterol because sterols, including 17-ketosteroids, lanosterol and agnosterol, are not identified separately from cholesterol by the TLC method (Nicolaides 1965). Therefore, it is not known if the components of the *A. carolinensis* lizard extract used in this study actually does or does not have cholesterol in it. It is known that mosquitoes, when searching for a blood meal, sequester sterols and cholesterol from the host in order to develop eggs. It is also suggested that mosquitoes do not sense the blood inside the host. It is possible that if cholesterol is present in combination with lipids and sterols on the outside of the vertebrate's skin, this may very well be a significant part of the code that

tells mosquitoes that if they land on the host, probing will reward them with their spoils (McKenzie 2003).

Gas chromatography was performed on the crude lizard extract along with hexane as a standard, and some very small peaks did appear different in the lizard extract. The peaks were so small in both the 0.2 µl and the 0.4 µl samples that the extract could possibly have been contaminated, or it may need to be concentrated perhaps to 2 ml or less. At this time it is unknown what these compounds are because gas chromatography does not identify those compounds that deviate from the straight chain carbons.

A major question has arisen out of this study. Is *Cx. erraticus* attracted to *A. carolinensis* malaria infected lizards compared to a noninfected lizards? This question was addressed in the last objective of this study where five infected and five non-infected *A. carolinensis* lizards were compared in 25 replications of paired *t*-test to see which group of lizards *Cx. erraticus* was attracted to most.

Thirty-two *Cx. erraticus* mosquitoes were released in each of the pie shaped replications where the respired CO_2 and body odors of malaria infected and noninfected *A. carolinensis* were allowed to enter each chamber. The cylinder CO_2 was removed during this comparison and the 35 mm film canisters were vacant and covered with Saran Wrap. With the sensor bent down covering the port holes where the lizard CO_2 and body odors entered each chamber, there was no significant difference in the landing rates during each of the 15 paired *t*-test replications. These replications were run with the fiber-optic lights on and it may well be that running this trial with the fiber-optic lights off may make a difference in the selection of *Cx. erraticus*.

The mosquitoes did respond to both infected and noninfected lizards, with the infected lizards receiving slightly more average bite-seconds than the noninfected lizards. At the time of this study, there were no indications that the malaria organism present in the lizard would provide an attractive component that would signal the mosquito differently. However, recent information in a newspaper article has suggested that the human *Plasmodium* organism may be able to manipulate its host into smelling more attractive to mosquitoes (International Herald Tribune, 2005).

Culex erraticus can use the respired CO_2 and body odors as locators to find *A. carolinensis* in the wild. In this particular study, it has not been established whether *Cx. erraticus* uses visual cues to locate *A. carolinensis*, because during the lizard/no-lizard test, the lizards were below the olfactometer and the mosquitoes' visual range. Crude extract made from the epidermis of *A. carolinensis* contains properties that are attractive to *Cx. erraticus*. *Culex erraticus* did not display a preference for infective to non-infective lizards.

The results from the lizard extract in the 30-day field trapping study supports the laboratory data that *Cx. erraticus* is able to detect the *A. carolinensis* lizard by respired CO_2 and/or in combination with the host odors emitted from the lizard.

CHAPTER 5
DETECTION OF THE SAURIAN MALARIA *Plasmodium floridense* IN THE ORGANS OF THE *Anolis carolinensis* LIZARD

Introduction

Malaria is a parasitic protozoan with species of *Plasmodium* infecting mammals, birds and reptiles. In 1909, Wenyon discovered *Plasmodium mabuiae* Wenyon and *Plasmodium agamae* Wenyon in the *Mabuya quinquetaneiata* Lichenstein the common skink and the *Agama agama* L. the rainbow lizard from Africa. Since Wenyon's discovery in 1909, approximately 90 saurian (reptile) malarias have been identified (Telford personal communication).

Early attempts to incriminate mosquitoes as vectors of saurian malaria were mostly unsuccessful with small numbers of mosquitoes developing oocysts on their midguts, and none of the mosquito's developed sporozoites (Ayala 1977, Huff 1941b).

Wood and Wood (1936) found that 10% of the western fence lizard *Sceloporus occidentalis* Baird and Girard in California were infected with what was later identified to be *P. mexicanum* Thompson and Huff (Garnham 1966). Huff (1941b) reported the first attempt to transmit *P. floridense* from infected eastern fence lizards *Sceloporus undulatus* Latreille by bite of *Culex pipiens* L. and *Aedes aegypti* (L.) mosquitoes. *Aedes aegypti* had one oocyst on its midgut and *Cx. pipiens* was found to be negative and both mosquitoes failed to produce sporozoites. It was not until 1944 that Thompson and Huff described *P. floridense*, which was found in *Anolis carolinensis* Voigt and in *S. undulatus* later in Georgia.

Jordan (1964) collected mosquitoes in southern Georgia in hopes of demonstrating sporogony of *P. floridense*, because the lizard malaria naturally occurs in *A. carolinensis* and *Sc. undulatus* (Goodwin 1951, Jordan 1964, Jordan and Friend 1971). Jordan (1964) was unsuccessful in her attempts to use lizard baited traps to attract mosquitoes. She found that local mosquitoes, *Aedes triseriatus* Say, *Aedes atlanticus* Dyar and Knab, *Coquillettidia perturbans* Walker, *Psorophora confinis* Lynch Arribalxaga and *Psorophora ferox* Von Humboldt were all negative for the *Plasmodium* organism. However several species of mosquitoes including, *Ae. aegypti*, *Culex territans* Walker, and *Culex quinquefasciatus* Say had one or more oocysts on the wall of the midgut. The *Culex* species containing the 70 oocysts was not identified to species, but it could have been a *Melanoconion* mosquito because they are prevalent in the research area. It wasn't until Ayala (1970b) and Ayala and Lee (1970) reported the first successful complete sporogonic development of saurian malaria.

Ayala and Lee (1970) successfully demonstrated under laboratory conditions that *Lutzomyia vexator* Coquillett and *Lutzomyia stewarti* Mangaberia and Galindo sand flies were able to complete sporogony of the *Plasmodium mexicanum* Thompson and Huff. The sporozoites were removed from the sand flies and found to be infective when injected by syringe into the *Sceloporus occidentalis* Baird and Girard western fence lizard. Until Klein (1985), the vectors of saurian *Plasmodium* were unknown, and the development of the malaria parasite in lizards was only successful by experimental inoculation (Thompson 1944, Thompson and Huff 1944a, b, Ayala and Lee 1970).

Klein et al. (1987) reported positive transmission of *P. floridense* to *A. carolinensis* by bite from *Cx. erraticus* mosquitoes and by intraperitoneal inoculation of sporozoites from the salivary glands of an infective *Cx. erraticus* mosquito.

The three-year laboratory study of Klein (1985) included 154 *A. carolinensis* lizards from various areas in Alachua County, Florida, with 41 (26.6%) of the *A. carolinensis* lizards from the wild infected with *P. floridense*. In the study, Klein placed 21 noninfected *A. carolinensis* lizards, restrained on tongue depressors, in a 4.5 cm x 15 cm plastic cylinder with an unknown number of presumed infected *Cx. erraticus* mosquitoes for a period of 24 hours. Klein did not specify if the *Cx. erraticus* mosquitoes fed on the lizards during the night and/or during the day. Klein reported that three *A. carolinensis* lizards were infected, with evidence of a prepatent period of 24 to 25 days after transmission by *Cx. erraticus.* Klein also reported that the parasitemia of each infected lizard was 1,780 and 4,280 per 10,000 red blood cells, with parasitemia peaking at 55 and 74 days respectively after feeding.

Although the numbers of transmission for *P. floridense* to *A. carolinensis* by *Cx. erraticus* were low during Klein's three-year study, he did show that utilizing lizard baited traps in the field attracted *Cx. erraticus* and *Cx. territans*, and that these two species did feed in the field and in the laboratory on *A. carolinensis.* He also completed the life cycle of *P. floridense* in both *Cx. erraticus* and *A. carolinensis* and he emphasized the need for determining whether there are subspecies of *Cx. erraticus* that could cause one population to be susceptible and the other population to be resistant to infection by *P. floridense*.

Blood smears have shown the exoerythrocytic (EE) stages in different saurian malaria species (Bray 1957, 1959, Garnham 1950, Garnham and Duke 1953, Lainson and Shaw 1969, Scorza 1971b, Telford 1970a). Thompson and Huff (1944a) reported the first detailed description of the EE stages of *P. mexicanum* in blood smears of lizard tissues. Thompson and Huff (1944a) and Mattingly (1965), suggested that the invasion of the types of non-erythrocytic cells of saurian and avian *Plasmodium* may signify that these two malaria parasites may have evolved from primitive ancestors, especially *P. mexicanum* because its EE stages are not restricted to one type of tissue (Huff 1945). Because of the different types of tissues invaded, the reptilian and bird malaria may come from one ancestral *Plasmodium* and that of mammalian may come from another (Bray 1957, 1963, Mattingly 1965).

Exoerythrocytic schizogony is affected by the age of the host, to which strain, if any, the parasite belongs, how large an infection is received, and how long a period the infection lasts (Garnham 1966). Only after sporozoite infection can phanerozoites be found in various organs and tissues located within the host (Bray 1957, Demina 1959, Huff 1952). In *P. gallinaceum*, when stained with Giemsa, phanerozoites have a bright blue cytoplasm and red nuclei. Manresa (1953) showed the occurrence and persistence of phanerozoites of *P. lophurae* in the blood of turkeys when they had been inoculated.

As stated by Garnham (1966), "the ability of the parasite to utilize wandering as well as fixed cells suggests that the distinction between *P. gallinaceum* and *P. elongatum* types of development is not absolute and it is possible that exoerythrocyte schizogony of both types is also seen in natural infections of saurian malarias."

Telford (1998) reported that phanerozoites of *Plasmodium sasai* Telford and Ball parasitized most tissues of the Japanese grass lizard *Takydromus tachydromoides* when the infection was by inoculation of blood. Telford reported that the phanerozoites were still evident in each lizard when they died ranging from two to 296 days post inoculation. In an earlier study, Telford (1996) found through histological examination of 422 hearts of *T. tachydromoides* that phanerozoites were present in newly infected juvenile lizards. He also discovered that the parasite was found in the lizards every month during this two-year study. Although phanerozoites of *P. floridense* have not been shown to take place in *A. carolinensis* lizards, it is presumed that if the phanerozoites of other plasmodium parasites is present in some lizards, most other lizards would also harbor parasites such as those found by Telford in the tissues *T. tachydromoides.*

The objective of this study is to show the phanerozoites of *P. floridense* in histological tissue samples in various organs of euthanized *A. carolinensis* lizards.

Materials and Methods

Anolis carolinensis Florida Colony

Anolis carolinensis were hand collected from the Cross Creek, Hatchet Creek area in Alachua County, Florida, and from the Ocala National Forest, Wade Smith property, and the James Park property in Marion County, Florida. Each lizard was toe clipped for identification and for collecting a small sample of blood, which was smeared on a microscope slide, air-dried, fixed in absolute methyl alcohol, stained with Giemsa, and examined under oil immersion using a compound microscope at 100x objective for examination of blood parasites. In addition, *A. carolinensis* were hand collected in Texas, where *P. floridense* has not been observed and purchased through Carolina Biological of North Carolina. Blood smears were made by toe clippings, and slides were

prepared as previous slides for examination of blood parasites. Both colonies of *A. carolinensis* lizards had subsequent blood smears to track parasite development. Infected and noninfected lizards were kept in separate cages in the same animal control room approved by the Institutional Animal Care use Committee (IACUC) Project No.A644 and Environmental Health and Safety Project No.BA-2030. The approved animal control room is maintained between 30°C and 32°C and 75% RH in combination with a 16:8 LD photo regime.

Anolis carolinensis Texas Colony

Anolis carolinensis lizards were purchased from Carolina Biological which were hand caught in Texas. It was necessary to acquire lizards from an area where *Plasmodium floridense* does not occur. The Texas lizards acclimated to the laboratory environment for two weeks before being toe clipped for identification and blood smears to check for infection of *P. floridense*. The Texas lizards were kept in separate cages in the same animal control room approved by the Institutional Animal Care use Committee (IACUC) Project No.A644 and Environmental Health and Safety Project No.BA-2030. The approved animal control room is maintained between 30°C and 32°C and 75% RH in combination with a 16:8 LD photo regime.

Culex erraticus Colony

Because *Cx. erraticus* failed to rear in laboratory colony, wild adults were trapped using a modified CDC light trap, brought into the laboratory, counted and placed in a Percival growth chamber at 27ºC and 80% RH with a 16:8 LD photoperiod. The wild mosquitoes were used in the transmission study, examination of the salivary glands for sporozoites, and midgut of mosquito for oocysts.

Rocky Creek Farm *Culex erraticus*

Mosquitoes were trapped at Rocky Creek Farm using a modified CDC light trap with CO_2 and lizard extract. They had the identifying characteristics of *Cx. erraticus* but the morphological color and shape were different. These mosquitoes were placed in a cage with infected *A. carolinensis* lizards. Mosquitoes were removed after one hour, as per IACUC protocol, and placed in a cage with 10% sugar water and an ovipositioning container. On day 12 after feeding on infected lizards, these mosquitoes were placed in another cage with noninfected *A. carolinensis* lizards for one hour. They were then transferred to the Interdisciplinary Center for Biotechnology Research (ICBR), BEECS (Biotechnologies for the Ecological, Evolutionary, and Conservation Sciences) Genetic Analysis Laboratory at University of Florida for DNA analysis of *P. floridense* presence.

Blood Smear Staining Procedure

Anolis carolinensis were periodically toe clipped for a small sample of blood to examine for various stages of *P. floridense* infection. The blood samples were placed on a clean microscope slide and smeared with the end of another slide in an even motion. Blood smears were air dried for several minutes then laid in a flat position on a staining tray and fixed with absolute methyl alcohol for one minute. The slides were placed in an upright position to allow the methyl alcohol to drain off the slides and for slides to air dry. Giemsa staining solution was made combining one ml of Giemsa stain with nine ml of buffered Fisher Scientific (SB108-500) solution having a pH of 7.0. Each slide was covered with the Giemsa stain solution and let stand for 50 minutes to one hour. Each slide was gently rinsed with regular tap water to remove excess Giemsa stain and placed back in upright position and allowed to air dry for approximately 10 minutes. Slides

were examined for various stages of *P. floridense* with a compound microscope at 100x oil immersion.

Staining of *Culex erraticus* Salivary Glands and Midgut

The salivary glands and midgut of wild *Cx. erraticus* were examined for sporozoites and oocysts, respectively. The mosquitoes' salivary glands and midgut were dissected in insect ringer's solution and placed on a microscope slide with several drops of Giemsa solution and a cover slip, then examined under a compound microscope.

Transmission of Plasmodium floridense

Parasitemia was checked in blood samples 24 hours prior to each transmission study by toe clipping the infected *A. carolinensis* lizards. The infected lizards were restrained on tongue depressors with surgical tape across the pelvic girdle and the shoulders to secure the legs of the lizard. Infected lizards were placed in a screen cage measuring 30.5 cm x 30.5 cm x 30.5 cm with 30 *Cx. erraticus* mosquitoes for one hour, as per the protocol by IACUC. After feeding, the engorged *Cx. erraticus* mosquitoes were placed in a separate oviposition cage with 10% sugar water solution and an oviposition container with water in a temperature and humidity controlled growth chamber at 25ºC and 80% RH with 16:8 LD photoperiod. On day 12, the blood fed mosquitoes were placed in the same size cage with noninfected *A. carolinensis* lizards restrained on tongue depressors for one hour, as per the IACUC protocol, in order to infect noninfected lizards with sporozoites. After blood feeding on noninfected lizards, the salivary glands and midguts were examined in each of the blood fed mosquitoes.

The *Cx. erraticus* mosquitoes trapped on the Rocky Creek Farm property are suspected of being a subspecies or different strain of *Cx. erraticus.* Therefore, 30 *Cx. erraticus* mosquitoes trapped at Rocky Creek Farm and 30 *Cx. erraticus* mosquitoes

trapped on the Wade Smith property were placed in two separate cages with two infected *A. carolinensis* lizards restrained on tongue depressors for one hour-feeding period.

Twelve days after feeding on infected *A. carolinensis* lizards in separate cages, 30 specimens of *Cx. erraticus* mosquitoes trapped at Rocky Creek Farm and 30 *Cx. erraticus* mosquitoes trapped on the Wade Smith property were placed in two separate cages with two noninfected lizards restrained on tongue depressors for a one-hour feeding period. After feeding on the noninfected lizards, the two samples of mosquitoes were delivered to the Interdisciplinary Center for Biotechnology Research BEECS Genetic Analysis Laboratory at University of Florida for DNA analysis for *P. floridense* (Appendix D) and for DNA comparison between both mosquitoes (Appendix E).

On day 15, after the wild *Cx. erraticus* fed on noninfected lizards, two blood samples were taken from the noninfected *A. carolinensis* lizards for parasitemia count and delivery to the Interdisciplinary Center for Biotechnology Research BEECS Genetic Analysis Laboratory at University of Florida for DNA analysis for *P. floridense*. The donor infected lizard and the noninfected lizards that became infected by mosquito bites were euthanized by a Florida licensed veterinarian for histological staining procedures.

Euthanasia of Anolis carolinensis

As per the protocol established by the Institutional Animal Care Use Committee (IACUC), a licensed veterinarian in the State of Florida must be listed on the outside of the animal control room where the *A. carolinensis* colony is kept. The licensed veterinarian is the only person allowed to euthanize the lizards in a humane manner by injection of Keteral®. The euthanized *A. carolinensis* lizards were dissected, tissue samples removed and placed in 10% formalin for 24 hours prior to microtoming. The

lizard remains were placed in a biohazard control bag and incinerated as per IACUC protocol.

Histological Staining of *Anolis carolinensis* Tissue

Following instructions by S.R. Telford (unpublished), dissected lizard tissues from one known infected lizard and two lizards fed on by infected *Cx. erraticus* mosquitoes were stained using xylene, ethanol absolute, ethanol 95%, ethanol 70%, distilled water, Giemsa Stain (liquid), Permount, nine Coplin jars, microscope slides and cover slips. Stock buffer solution with a pH of 4.6 was prepared by combining 28.4 grams of sodium phosphate dibasic with 19.2 grams of citric acid anhydrous and one liter of distilled water. The stock buffer solution was set to the side to be used in the working stock solution. Four ml of the stock buffer solution with a 4.6 pH was added to 16 ml of acetone and 80 ml of distilled water. A total of 27.5 ml of the working stock solution was added to 2.5 ml of Giemsa Stain and placed in the staining solution in a Coplin jar. The first rinse of absolute xylene was for two to five minutes to remove paraffin. The slides were rinsed a second time in absolute xylene for one to two minutes to remove remaining paraffin. The third rinse in absolute ethanol for two to three minutes removed the xylene from the tissues. The fourth rinse in absolute ethanol removed the remaining xylene. The fifth rinse was performed in 95% ethanol for two to three minutes to remove the absolute ethanol. The sixth rinse was in 95% ethanol for two to three minutes, and the seventh rinse was in 70% ethanol for approximately 30 seconds. The eighth rinse was in distilled water for 20 to 30 seconds to remove the remaining ethanol. The tissue slides were place in the Giemsa stain solution made with working stock for one hour. Destaining the slides of the Giemsa stain was done by reversing the order of rinses, but steps seven and eight were skipped, starting with step 6. It was noted not to leave the

slides in each solution, but, dip them in and out of each rinse solution three to seven times in each Coplin jar to remove the Giemsa stain. Differentiation occurs at 95% ethanol, the slides should not be held in these jars long during the destaining process. Next, permount was applied to a wet slide with cover slip. Slides were dried in a slide oven for approximately four days before using oil immersion to examine slides.

DNA Protocol

Six blood samples on Whatman paper were taken from *Anolis carolinensis* lizards infected with *P. floridense*. Two samples each of *Cx. erraticus* mosquitoes from different locales, one sample trapped on the Rocky Creek Farms Property and the second sample trapped on the Wade Smith property were analyzed because both samples of mosquitoes were thought to be different species. All DNA extractions were accomplished using a Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) following a modified protocol. By cutting the blood-soaked area of the Whatman paper from the rest of the paper and placing it into a 1.5 ml microcentrifuge tube, the blood samples were processed. One hundred eighty microliters of buffer ATL and 20 µl proteinase K were added and incubated overnight at 55ºC. For the mosquitoes, the entire animal was placed into a 1.5 ml microcentrifuge and, after the addition of 180 µl of buffer ATL; they were ground with a micropestle before adding 20 µl of proteinase K and incubated overnight at 55ºC. A negative control consisting of only the reagents and no DNA was carried through the DNA isolation to assure that there was no contamination during the DNA isolation. The samples were vortexed several times during the incubation period. Two hundred microliters of buffered ATL was added to each of the tubes; then they were vortexed and incubated at 70ºC for 10 minutes. Finally, 200 µl of 100% ethanol was

added to each tube and vortexed, and the solutions added to DNeasy Mini spin columns. The columns have a resin bed that captures the DNA while allowing the rest of the solution to pass through, and debris was held on the surface of the resin bed. The DNA was then washed with 500 µl of buffer AW_1, the flow-through discarded, and then washed with 500 µl of AW_2. The flow-through was again discarded and the column was placed in a clean, labeled 1.5 ml microcentrifuge tube. One hundred microliters of buffer AE was then added to each of the columns, allowed to incubate at room temperature for one minute, and then centrifuged to elute the DNA off the resin bed and into the 1.5 ml tube. The eluate was added back to the column, allowed to incubate for one minute and then centrifuged again to elute the most DNA possible off the column.

Polymerase chain reactions (PCR) were performed in 25 µl volumes containing 1X PCR buffer, 800 µM dNTPs (total concentration), 3.0 mM $MgCl_2$, 0.4 µM each primer, and 0.5 U of Taq polymerase. PCR parameters were five min at 95ºC followed by 39 cycles of 95ºC for one min, 52ºC for one min, 72ºC for one min, and a final 20 min extension at 72ºC. Standard precautions, including a negative control (template-free PCR reaction), were used to test for contamination and to ensure fidelity of the PCR reactions. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and sent to the ICBR DNA Sequencing core at the University of Florida for sequencing.

Five different sets of primers ITSA/ITSB (modified from White et al. 1990), 12SA/12SB and 16SA/16SB (Kessing et al. 1989), ITS_4/ITS_5 (White et al. 1990), CO8/CO13 were used on the mosquito samples only to assist in identifying the species of each mosquito. Of the five sets of primers, three (ITSA/B, 16SA/B, and $ITS_4/_5$) gave

amplicons that were sequenced, and two sets (16SA/B: 16SA: 5': CGC CTG TTT ATC AAA AAC AT-3' and 16SB: 5': CTC CGG TTT GAA CTC AGA TC-3'; and ITS4/ITS5: ITS4: 5' –TCC TCC GCT TAT TGA TAT GC-3' and ITS5: 5' –GGA ACT AAA AGT CGT AAC AAG G-3') had clear results. Sequencer 4.2 was used to view the chromatogram and to compare the sequence to GenBank sequences. To confirm the results of species identification of the mosquitoes, two more samples from the original two localities were submitted to the lab for analysis.

Results

A total of 113 lizards were hand collected from Alachua County and Marion County, Florida, between 2001 and 2005 (Table 5-1). The mean *Plasmodium* infection rate for all 113 anoles was 23.9%. The largest number of infected lizards was collected on the James Park and Wade Smith properties in Marion County, Florida. The James Park property borders the Ocala National Forest, and the Wade Smith property is approximately 100 yards from the Oklawaha River in Marion County, Florida.

Table 5-1. Number of *Anolis carolinensis* lizards collected from locations in Florida during 2001-2003. Number and percent infected with *Plasmodium floridense* from each location.

Location	Species	Number Collected	Number Infected (%)
Cross Creek	*A. carolinensis*	10	2 (20.0%)
Hatchett Creek	*A. carolinensis*	7	1 (14.3%)
Wade Smith property	*A. carolinensis*	47	9 (19.1%)
James Park property	*A. carolinensis*	33	11 (33.3%)
Ocala National Forest	*A. carolinensis*	16	4 (25.0%)
Total:	*A. carolinensis*	113	27 (23.9%)

Table 5-2 shows the random sampling of 260 *Cx. erraticus* mosquitoes from June 2003 through September 2003, and May 2004 through August 2004 for examination of the salivary glands and the midguts for sporozoites and oocysts, respectively. One *Cx.*

erraticus mosquito trapped on the James Park property appeared to have melonized oocysts on the midgut wall, but this was not conclusive because of weak Giemsa staining.

Table 5-2. Summary of the number of *Culex erraticus* trapped at various locations in Florida with number sampled.

Location	Species	Total Collected	Number Sampled	Number Infected (%)
Blues Creek	*Cx. erraticus*	12	3	0
Jim Park property	*Cx. erraticus*	1510	85	1** (1.2%)
Co. Rd. 2082	*Cx. erraticus*	8	2	0
Hatchett Creek	*Cx. erraticus*	404	35	0
Equine Center	*Cx. erraticus*	4516	45	0
Wade Smith property	*Cx. erraticus*	7389	90	0
Total		13839	260	1** (1.2%)

** Oocysts may be present but Giemsa staining of slide is poor.

Plasmodium floridense **Transmission Studies**

Twenty-six infected *A. carolinensis* lizards were used in seven transmission studies from 2002 through 2005 (Table 5-3). In four of the studies, five infected *A. carolinensis* lizards were restrained on tongue depressors, and in the remaining three transmission studies, two infected *A. carolinensis* lizards were restrained on tongue depressors (Figures 5-1, 5-2).

The 30 *Cx. erraticus* mosquitoes used in transmission study number 6 was trapped at Rocky Creek Farm after the hurricane flooded a crazing pasture. These mosquitoes keyed out to *Cx. erraticus* but were of different color and size (Figure 5-3). The Rocky Creek Farm mosquitoes and the Wade Smith property mosquitoes were sent over to the BEECS lab for DNA species comparison. Figure 5-7 shows *Cx. erraticus* feeding on Florida infected *An. carolinensis* lizard LF5RR123 during transmission study number seven.

Figure 5-1. Infected *Anolis carolinensis* lizard restrained on tongue depressor prior to being placed in feeding cage with *Culex erraticus* mosquitoes (photo by J.F. Butler)

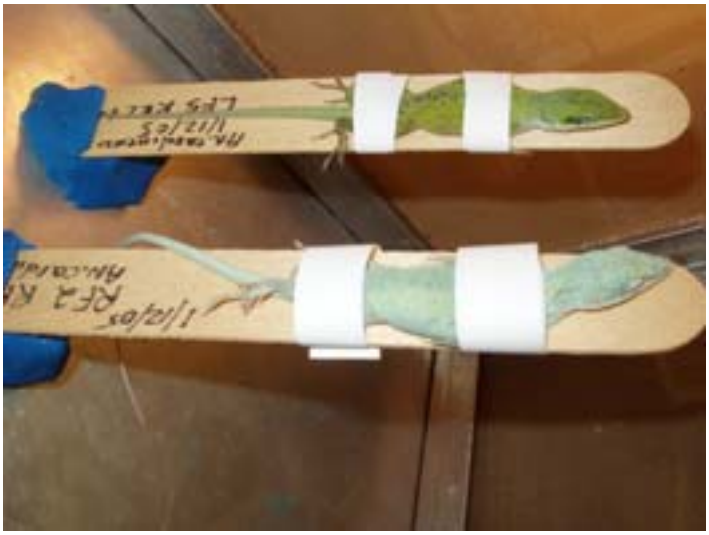


Figure 5-2. Infected *Anolis carolinensis* lizards restrained on tongue depressors in transmission cage (photo by J.F. Butler)

DNA Analysis of *Culex erraticus* Ribosomal DNA

Polymerase Chain Reaction (PCR) products amplified from mosquito DNA with primers 16SA/B was approximately 760 base pair (bp). The DNA sequences from PCR products from mosquito samples, RCF, RCF2, WSP, and WSP2 were aligned in Sequencer software. Of the 760 bp sequenced there were two nucleotide differences among the four sequences. Samples WSP and RCF were identical, and WSP2 and RCF2

differed by two base pairs. Since there were only two nucleotides differences in the WSP2 and RCF2 samples of mosquitoes, it was determined that all four samples are *Cx. erraticus*. The PCR products amplified from mosquito DNA with primers ITS4/5 gave a product of approximately 760 base pairs. The PCR ribosomal gene products sequenced with ITS4/5, when compared to sequences in GenBank, both matched *Culex erraticus* (Miller et al. 1996). The comparison sequence was from GenBank (Appendix E). Sample RCF was closest to *Cx. erraticus* clone EOH2J21, and WSP2 was closest to *Cx. erraticus* clone EOH2J33.

Table 5-3. Seven transmission studies exposing Florida infected *Anolis carolinensis* lizards to *Culex erraticus* mosquitoes for maximum one hour feeding time.

Date	Trans No.	Lizard ID	Trophozoites	Schizonts	Gametocytes
Jul-02	1	RF5RR1	47	23	7
		RF5LF1	18	4	5
		RF4RR1	42	9	3
		RF5RR5	31	17	9
		RF5LR1	28	14	5
Sep-02	2	RF5LR2	41	18	6
		RF4RR2	30	22	11
		RF5LR5	11	13	3
		RR3LR1	29	12	8
		RF2RR2	34	10	4
Jun-03	3	RF3RR1	23	8	7
		RR1LR1	43	19	9
		RF5LR2	37	22	10
		RF2RR2	19	11	5
		LR3RR4	62	35	13
Aug-03	4	RF5RR1	43	17	8
		RF4RR1	29	8	3
		RR3LR4	18	6	3
		LF1RF1	37	11	7
		RF5LR1	52	21	11
Jul-04	5	LF2,3LR2,3	26	20	12
		LF3RR1	44	15	7
Oct-04	6	LF3RR5	51	33	18
		Clubfoot	37	19	11
Jan-05	7	LF5RR1,2,3	129	63	29
		LF2RR1	63	27	15

Figure 5-3. *Culex erraticus* trapped at Rocky Creek Farms is light colored, and its size is larger than the typical species found in Florida. This species has the same patch of scales on mesoepimeron and the flat broad scales on occiput plus basal bands on abdomen.

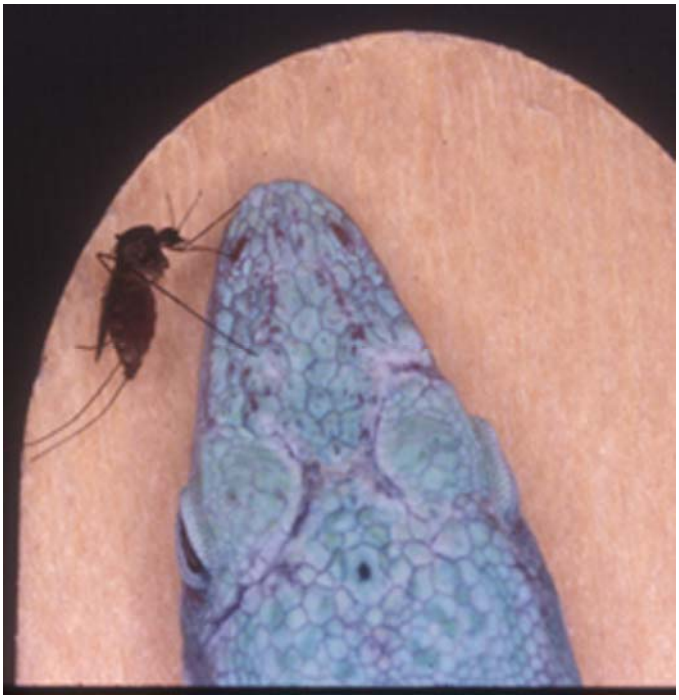


Figure 5-4. *Culex erraticus* feeding directly in the nostril of *Anolis carolinensis* lizard LF5RR123 during transmission study number seven in a dark room.

Both GenBank sequences are from the same study and so it is most likely that the sequences submitted for comparison are representative of the same species of mosquito.

In addition, *A. carolinensis* lizard LF5RR123 and one mosquito sample from the Wade Smith property (WSP) were positive for the *P. floridense* parasite. The three

lizards that were dissected, LF5RR123 (infected Florida lizard), LR4RR4 (noninfected, Texas lizard), LR4RR5 (noninfected, Texas lizard) were all positive for the *P. floridense* parasite. The LF5RR123 Florida lizard was naturally infected from the wild, and the mosquito that fed on LF5RR123 lizard tested positive for the *P. floridense*. Figure 5-5 and Figure 5-6 shows a trophozoite and schizonts of *P. floridense* in RBC of *A. carolinensis* lizard LR4RR4 and LR4RR5, respectively.

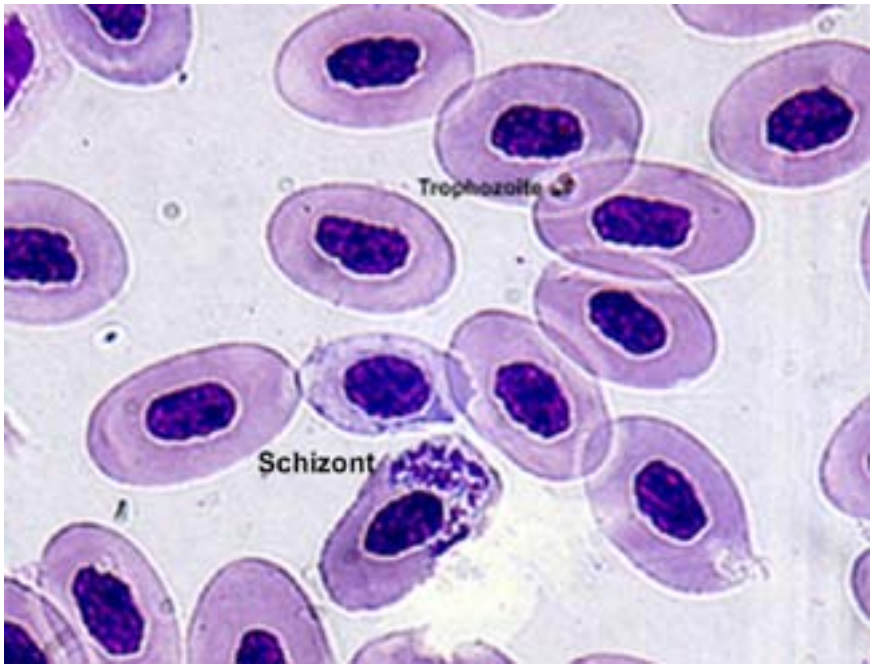


Figure 5-5. Gametocyte and Trophozoite in the RBC of Lizard LR4RR4 in transmission study number 7.

Lizards LR4RR4 and LR4RR5 shows *P. floridense* phanerozoites in histological stains of the liver, kidneys, and pancreas (Figures 5-7, 5-8, 5-9, 5-10).

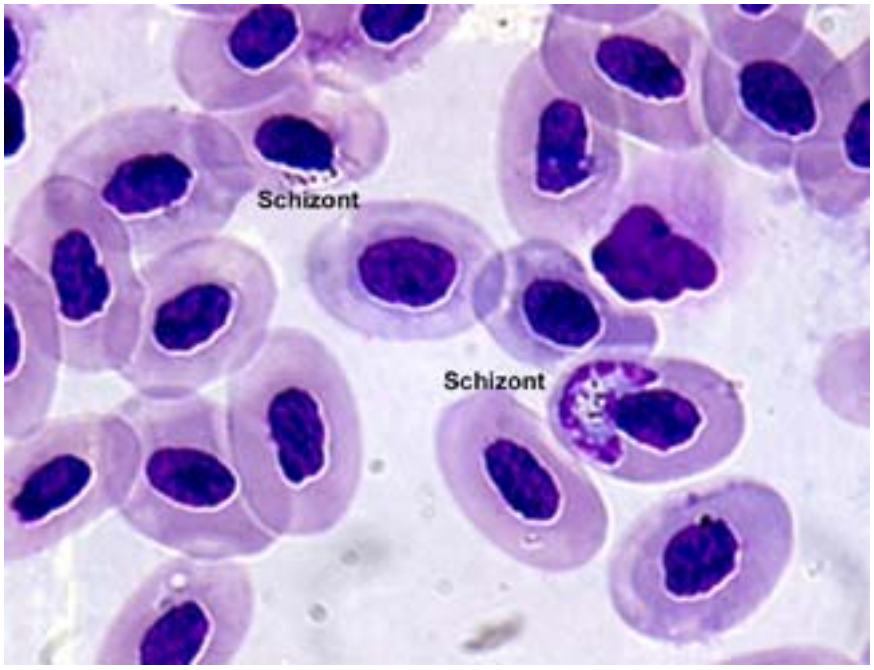


Figure 5-6. Gametocytes in RBC of *A. carolinensis* lizard LR4RR5 in transmission study number 7.

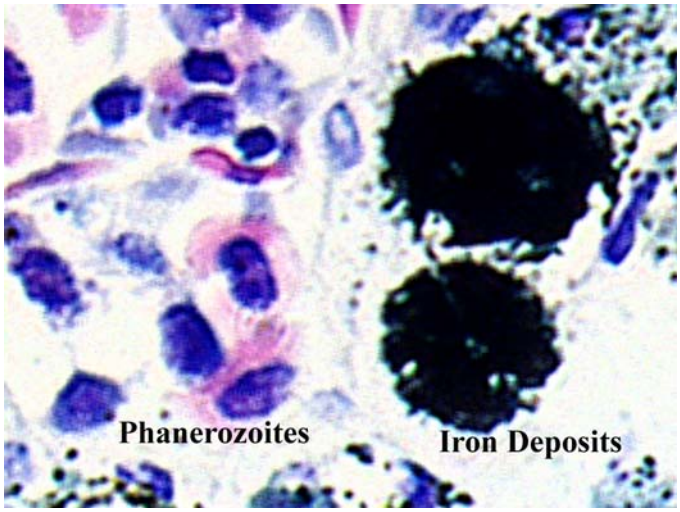


Figure 5-7. Phanerozoites on left and iron deposits on right, found in the liver of *A. carolinensis* lizard LR4RR4.

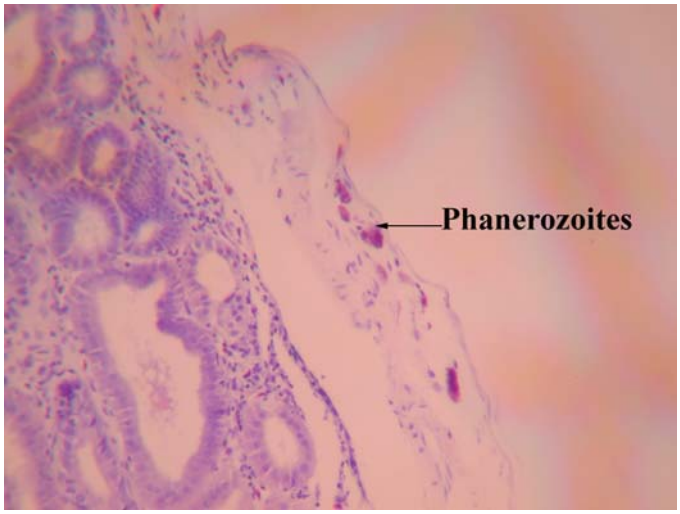


Figure 5-8. Phanerozoites in the kidney of *Anolis carolinensis* lizard LR4RR4.

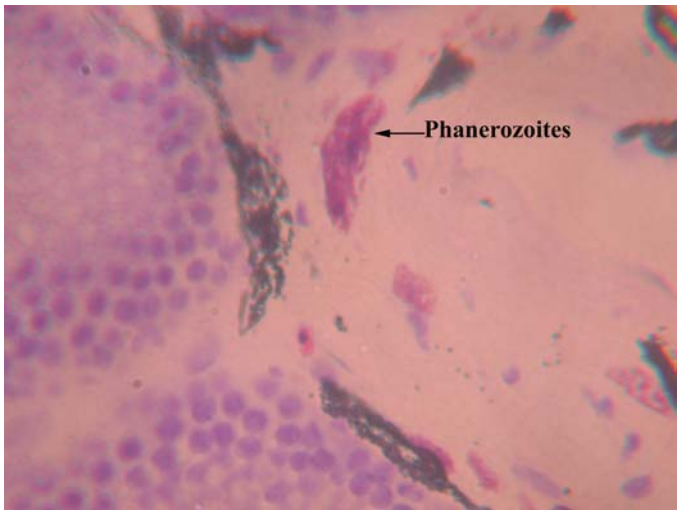


Figure 5-9. Phanerozoites in the kidney of *Anolis carolinensis* lizard LR4RR5.

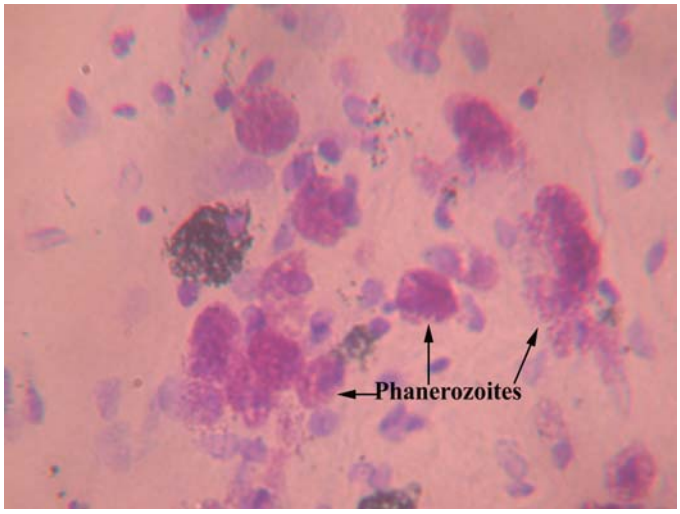


Figure 5-10. Phanerozoites in the Pancreas of *Anolis carolinensis* lizard LR4RR5.

Discussion

The average infection rate of the 113 wild *A. carolinensis* lizards used in this study was 23.9%, which was approximately one out of every four lizards. Klein (1985) used 154 *A. carolinensis* lizards with an average infection rate of 26.6%, resulting in one out of every three lizards being infected.

Garnham (1966) reported that *A. carolinensis* lizards do not normally experience harsh symptoms from *P. floridense*. The immunity in the lizards seems to be higher in experimental infections, and if the lizard becomes anemic, it is often due to the number of times the lizard is sampled for blood by toe clipping or nicking of the tail.

In this study, there was a high mortality rate in the lizard colony. It is felt this was mostly due to not knowing the age of the lizards when they were brought into the laboratory from the wild, and in combination with changing the lizards' environment and established territorial boundaries. The wild lizards may have brought other related conditions with them that may have contaminated the colony, resulting in uncertain deaths within the lizard population. The *A. carolinensis* lizards acquired from Texas through Carolina Biological also had a high mortality rate on arrival because of stress from two days of being in a cardboard box without water and food and acclimating to the laboratory environment.

Some of the Florida lizards that died in colony may have had a relapse of the *P. floridense* in the wild before being caught. They may have been on the down side of the high parasitemia rate and could not recover in the laboratory (Huff and Marchbank 1953). It was also noted that laboratory *A. carolinensis* lizards, when stressed either by handling, toe clipping and/or possibly age, developed a round black circle behind their eyes. Usually these lizards would lose their appetite, rest on the bottom of the cage, and then

soon die. Several important differences are noted in this discussion by comparing the seven unsuccessful transmissions with Klein's study (1985).

The feeding trials performed in this study were in screen cages that measured 30.5 cm x 30.5 cm x 30.5 cm. This standard mosquito cage was used for two reasons: the availability of space to put multiple lizards restrained on large tongue depressors, and to provide sufficient area for the mosquitoes to fly around and make a deliberate choice to land and probe on the host. Klein (1985) restrained each lizard on a small tongue depressor with surgical tape. However, the container that was used for host feeding was 4.5 cm x 15 cm. Klein placed multiple laboratory reared *Cx. erraticus* mosquitoes in the plastic cylinder with one *A. carolinensis* lizard and left them for 24 hours to feed. In this study, the Institutional Animal Care and Use Committee set a one-hour exposure of the lizards to the mosquitoes because they felt it bordered on cruelty to restrain an "animal" so it had no chance to exhibit the fight or flight response. Also, Klein fed his adult *Cx. erraticus* on baby chickens, and in this study, animal feeding other than lizard feeding for transmission was prohibited. Therefore, bovine blood treated with sodium citrate and antibiotics may have contributed to the overall effect of the mosquito colony by not providing the necessary proteins and cholesterols needed for egg development. The antibiotic may have played an important role in the development of *P. floridense* in *Cx. erraticus*.

This study did not have approval to feed the *Cx. erraticus* mosquitoes on baby chicks, but the USDA did have a protocol for feeding mosquitoes on chickens. A USDA chicken was placed feet first into a cage of 150 *Cx. erraticus* for one hour with one *Cx. erraticus* taking a partial meal. This mosquito was removed from the cage and placed in

a separate ovipositioning cage with 10% sugar solution and an oviposition container. The mosquito was dead the next morning.

Major factors contributing to the failure to produce transmission of *P. floridense* to *A. carolinensis* lizards were due to the number of *Cx. erraticus* that took complete blood meals, the number of gametocytes present in the lizards, and one hour time limit for feeding of the mosquitoes.

Two of the *Cx. erraticus* that took complete blood meals died during ovipositing of egg raft. Two *Cx. erraticus* only took partial blood meals and the other eight took complete blood meals with only one testing positive through DNA analysis for the *P. floridense*. The gametocyte stage found in the red blood cells of the host, is the stage in the life cycle of the *Plasmodium* parasite that is taken up by the mosquito and develops into the infective stage known as the sporozoites.

The value that is considered a low, medium or high parasitemia varies from author to author, but it is apparent that the higher the number of gametocytes per 10,000 RBC, greater the chance of infection taking place (D. Forester, personal communication).

The size of the cage used in the transmission studies may have played an important part in *Cx. erraticus* finding the lizards within the one-hour time limit they were exposed. In Klein's 1985 study, the number of *Cx. erraticus* trapped in 924 lizard-baited traps totaled 19, with seven of those feeding in the trap and 11 feeding in the laboratory. The mosquitoes used in this study were from the wild and, without knowing the age of the mosquito and how many gonotrophic cycles it had experienced before being trapped; it was difficult to determine which factors played a role in laboratory feedings.

Establishing a year round colony of *Cx. erraticus* would have made several differences in this feeding study. First, the convenience of having readily available *Cx. erraticus* mosquitoes to place with infected *A. carolinensis* during all levels of parasitemia would provide additional information on the transmission success based on the numbers of trophozoites, schizonts and gametocytes found in each host during infection. Huff (1941) and Jordan (1964) found that *P. floridense* was more prevalent in *A. carolinensis* in the fall because the parasite was transmitted in late summer and early fall months with a significant decline in December. Another factor in establishing a laboratory colony of *Cx. erraticus* would be that that age of the mosquitoes would be known which is important to understanding how many days post emergence when the adult female feeds. At the same time, knowing that the mosquitoes did not feed on other hosts prior to the transmission study with *A. carolinensis* would rule out transmission from another vector such as *Culex territans* Walker (Young and Perkins 1984).

Young and Perkins (1984) did show that a high percentage of *L. vexator* infected with *P. floridense* reared in the laboratory develop oocysts, but the life span of the adult sand fly in the laboratory is generally shorter than the sporogonic development of *P. floridense*, which can range from 11 to 14 days in mosquitoes (Klein et al. 1987). Jordan (1964) reported that *Cx. territans* could possibly be a vector of *P. floridense* because this mosquito has a preference for poikilotherms. Jordan had minimal success with showing oocysts development in *Cx. territans* and Klein et al. (1987) had no success in infecting this mosquito in the laboratory with *P. floridense*. *Culex territans* is known to feed on reptiles, but this mosquito is found in its highest prevalence in the spring when the parasitemia in the green anole lizards is at the lowest point in most populated areas.

In this study, examinations of 260 salivary glands and midguts of wild *Cx. erraticus* mosquitoes were negative for sporozoites in the salivary gland and oocysts on the midgut of the mosquito. Therefore, it would be advantageous to establish *Cx. erraticus* as a natural vector by sampling populations of mosquitoes and examining the salivary glands and midguts for *P. floridense* sporozoites and oocysts, respectively.

At the end of this study, a possible *Cx. erraticus* subspecies was thought to be found. This may bring into question whether species specific strains of parasites could possibly be found within two genetically different populations of *Cx. erraticus.* The ribosomal DNA analysis of the two specimens of *Cx. erraticus* showed that they were the same species of mosquito that may be phenotypically different because of environmental factors, such as breeding habitats and, most importantly, host sources for blood meals. Also, the ribosomal DNA analysis does show a difference in two base pairs between the two mosquitoes that could be interpreted as enough difference to warrant a closer look at the genetics of both populations of mosquitoes to determine if they are subspecies (Appendix E).

The histological tissues from both noninfected Texas lizards LR4RR4 and LR4RR5 contain phanerozoites in the kidneys, liver, pancreas, lungs and heart tissues 60 days after the transmission study. However, close examination of the blood smears for both Texas *A. carolinensis* lizards LR4RR4 and LR4RR5 showed chronic infections of young asexual stages of *P. floridense* in the red blood cells prior to the transmission study. The *Plasmodium* DNA analysis was correct in showing positive infections of the *P. floridense* from the blood sample of the Florida infected lizard LF5RR123 and the blood samples of

the two Texas lizards LR4RR4 and LR4RR5, therefore ruling out positive transmission taking place in the laboratory during this study.

Telford (1996, 1998) reported that phanerozoites of *P. sasai* were found in the Japanese grass lizard *Takydromus tachydromoides* tissues and he showed that these stages in the life cycle of *P. sasai* were evident in the tissues during the first month of experimental post-inoculation. The Texas *A. carolinensis* lizards LR4RR4 and LR4RR5 were both positive for phanerozoites in the liver, kidneys, pancreas and heart tissues. This is the first report of *A. carolinensis* having phanerozoites, which indicate why this reptilian malaria continues to cycle inside this lizard. Garnham (1966), reported that *A. carolinensis* lizards often times are asymptomatic to this infection, indicating a high immunity to this malaria causing this small, green beautiful lizard to live with the parasite instead of dying from it.

Future research to establish *Cx. erraticus* as a natural vector would include a large scale trapping study to examine the salivary glands and midguts from all wild mosquitoes specifically *Cx. erraticus*. Performing DNA analysis on mosquitoes will help establish host preference from previous blood meals.

Tracking the development of *P. floridense* in *A. carolinensis* every 12 hours after injecting naturally occurring sporozoites from the salivary glands of *Cx. erraticus* and by the bite of *Cx. erraticus* into *A. carolinensis* lizards. Tracking the development of *P. floridense* in *A. carolinensis* will also provide valuable information on the establishment of phanerozoites in various tissues of *A. carolinensis*.

A catch and release program of *A. carolinensis* lizards starting at the Torreya State Park west of Tallahassee, Florida to Vera Cruz, Mexico is necessary in order to update

the occurrence of the *P. floridense*. Toe clipping *A. carolinensis* lizards to retrieve blood samples for the presence of *P. floridense*, and using a global positioning system to geographically identify the movement of malaria infected *A. carolinensis* is important for distribution of the plasmodium organism.

Recovery of the DNA from the Giemsa stained slides of Texas *A. carolinensis* lizards LR4RR4 and LR4RR5 is important for species identification purposes to the Florida *A. carolinensis* lizards to see if they are the same or different. Also, recovery of the DNA from the Giemsa stained slides will be used to compare the Florida strain *P. floridense* to the *P. floridense* found in the supposedly Texas lizards to see if the plasmodium organism is different. If they are different, then the Texas lizards LR4RR4 and LR4RR5 that was believed to be positive prior to the transmission study may possibly be established in Texas.

CHAPTER 6
CONCLUSION

Culex erraticus was found in high populations at the University of Florida Equine Horse Teaching Unit in Alachua County, Florida, and at the James Park property and Wade Smith property in Marion County, Florida. Sampling two sites without submersed and floating aquatic vegetation, and four sites with aquatic vegetation, supported published information that *Cx. erraticus* favors the latter type habitat and is considered to be one of the most common mosquitoes found in fresh water habitats with aquatic vegetation. Several studies suggest that if the water surface has a dense matting of aquatic vegetation, the population numbers of *Cx. erraticus* decreases and the population numbers of other genera of mosquitoes such as *Anopheles* increases, and this was apparent when comparing the total numbers of *Cx. erraticus* trapped at Hatchet Creek and the numbers of *Cx. erraticus* trapped at the James Park and Wade Smith properties in Marion County, Florida.

Although the Hatchet Creek trapping site was ideal, the amount of aquatic vegetation and the canopy of trees in combination with the shallow depth lowered the temperature of the water by nearly three degrees, which could cause *Cx. erraticus* to move its population according to published data. Past research has shown that *Cx. erraticus* prefers shallow grassy margins around ponds, lakes and other types of fresh water habitats.

In Klein's 1983 to 1985 study, he was successful in rearing *Cx. erraticus* past the third generation in the laboratory, but never in high numbers. Klein occasionally had to

trap wild mosquitoes to produce new egg rafts in order to keep the colony ongoing (personal communication).

In this study, three generations of adults were achieved before the production of egg rafts ceased. The diets used in this nutrition study were based on success with diet regimes including Tetramin® Fish Food, powdered milk and brewer's yeast. Previous diets used in successful rearing campaigns for *Melanoconion* mosquitoes were mostly different between species. Also, past researchers found it difficult to rear *Melanoconion* mosquitoes under laboratory conditions with artificial light.

Klein's *Cx. erraticus* colony was reared in an insectary illuminated with a combination of natural daylight and fluorescent lighting. In this study, Environmental Health and Safety required that the mosquitoes be reared in a designated an approved biological safety 2 facility, with all floor drains covered with special covers and air conditioning vents covered with fine mesh screen so that wild mosquitoes could not escape if accidentally released. The wild *Cx. erraticus* had to be kept in a growth chamber with controlled artificial lighting and relative humidity.

Failure of the colony was due to the larval diet not satisfying the requirements of *Cx. erraticus.* The highest mortality occurred in the larval stages, and it was evident in the number of adults that died during eclosion that the adult fitness was compromised because of the lack of some type of nutrients, whether plant or animal, that was not received in the early stages of development. The five *Melanoconion* species reared in colony started out with failure of larvae to survive. Of the 250 species of *Melanoconion* approximately 10 have been colonized and less than five are stable with ongoing generations that are self produced.

Trapping of *Cx. erraticus* has been successful using various lights traps in the past, therefore using modified lights traps similar to Klein (1985), Burkett (1998) and Hoel (2005) were important for use in this study.

Klein (1985) trapped *Cx. erraticus* along Hatchet Creek with CDC light traps, and Burkett (1998) modified the CDC light trap with blue and green diodes in combination with CO_2 and reported a sizeable increase in the numbers of *Cx. erraticus* trapped in various locations throughout central and northern Florida. Recently Hoel (2005) also used modified CDC light traps with blue diodes in combination with CO_2 and reported in his study that large numbers of *Cx. erraticus* were trapped, especially at the University of Florida Horse Teaching Unit. Using the modified CDC light traps with blue and green diodes in combination with CO_2 and lizard extract produced significantly higher numbers of *Cx. erraticus* in various locations in Alachua and Marion counties, Florida.

Anolis carolinensis lizards test positive in the wild for *P. floridense* saurian malaria, which is also found in several other species of lizards including *Sc. undulatus*. Klein (1985) used live *A. carolinensis* lizards in baited traps to see which species of mosquitoes were attracted to the lizards in the wild. Klein (1985) set out a total of 924 lizard baited traps during April to October and collected 19 *Culex erraticus* and 43 *Cx. territans*. He also set out along Hatchet Creek 43 CDC light traps during the same months and trapped 741 *Cx. erraticus* and two *Cx. territans*. In this study, the Institutional Animal Care and Use Committee prohibited the use of traps with live *A. carolinensis* lizards. Therefore, washing *A. carolinensis* lizards that died in colony with filtered hexane was important in order to remove the compounds from the external

surface of the skin to see if compounds exists that *Cx. erraticus* might use as an attractant for locating the host.

When the lizard extract was placed in the modified CDC light trap with CO_2 and compared to modified CDC light traps with CO_2 and hexane, the lizard extract outperformed the other traps with a mean of 840.23 *Cx. erraticus* trapped daily in comparison to 344.63 for CO_2 and 159.07 for hexane. The residual components found in the lizard extract were certainly a positive influence on *Cx. erraticus* making a deliberate attempt to locate the host when the host was actually absent. The 30-day trapping study was randomized, and every fourth day the traps were repositioned along with the treatment and no significance were found in the numbers of *Cx. erraticus* trapped at the three site positions around the Wade Smith property.

Gas chromatography (GC) was used to evaluate the crude lizard extract to see if there is a different profile of peaks appearing in the extract that would possibly indicate the presence of components that may be utilized by *Cx. erraticus* as an attractant, but not to be identified in this study. The results of the GC were very small peaks that were difficult to decipher because of several factors. The crude extract may have had contaminated components in it from the washing technique, loss of compounds in the washing through the charcoal filters, and/or improper handling of sample.

Several researchers reported that the compounds found on the outside of anole and gecko lizards were different for male and female lizards. Male and female gecko skin lipids were hexane-extracted and it was shown that several fatty acids were common to both sexes. It was also shown that some and some steroids cholesterol based were unique to males only and long-chain methyl ketones were unique to females only. The skin

components and compounds found in the *Anolis* spp. were not identified and the reader is advised not to make a decision to classify the unknowns as cholesterol based. The identity of the properties found in the *A. carolinensis* hexane-extract in this study are not known, therefore no comment can be made at this time on the compounds. The 30-day trapping study was used to evaluate the effects of the lizard extract's performance as found in the olfactometer in the laboratory.

The pie-shaped 10-port olfactometer was used to determine if *Cx. erraticus* mosquitoes could detect the presence of *A. carolinensis*. In the first trials, consisting of 15 replications of a paired *t*-test partitioned off with two ports in each replication, the mosquito could not locate the presence of the lizard. However, the olfactometer lights and cylinder CO_2 was used to enhance the attractancy within the model. In several papers near the end of the study, it was noted that *Cx. erraticus* were trapped in CDC light traps in greater numbers from 6:00 pm till 9:00 pm, remaining the same until 12:00 am then sharply declining thereafter. The lizard/no-lizard pair *t*-test was run again in the olfactometer, this time with no CO_2 and with the lights out. The results were in favor of the mosquito locating the lizard with overwhelming significance. The last transmission study was performed in a screen cage with the lights on, but no feeding took place. The lights were turned off and eight *Cx. erraticus* mosquitoes took complete blood meals. It is not known if Klein (1985) observed *Cx. erraticus* feeding in the daytime because the small container holding the lizard and mosquitoes were left for 24 hours, giving more than ample time for feeding at night.

The position of the lizards in the olfactometer made it impossible for the mosquitoes to use visual cues to locate the hosts, and the cylinder CO_2 was eliminated to

allow *Cx. erraticus* to use sensory perceptions, such as odors from the skin and CO_2 components to locate the lizard. When the fiber optic lights, CO_2 and attractant artificial host were eliminated from the system, *Cx. erraticus* made deliberate landings on the sensors registering significantly more bite-seconds at the ports where *A. carolinensis* were located.

The lizard extract was evaluated in five different dosages (5, 10, 15, 20 and 25 µl) to evaluate the amount that could be considered an attractant by *Cx. erraticus*. The five dosages were compared in 10 replications of an open chamber randomized study with 160 *Cx. erraticus* mosquitoes in each replication. With the lights out and the cylinder CO_2 eliminated from the system, *Cx. erraticus* chose the 10 µl as the dosage to receive the highest number of contacts. The 10 µl was then compared against hexane standard to see which one *Cx. erraticus* would favor, and it was established that the 10 µl was the ideal dosage that *Cx. erraticus* would choose to land on and probe. Therefore, the 10 µl was taken to the field and evaluated, as reported in the chapter three trapping studies, and found to be more significant in attracting mosquitoes than just the CDC light traps with CO_2 and hexane.

How *Cx. erraticus* locates the small green anole near dark is still undetermined because this mosquito is thought by many researchers to be a dusk and nocturnal feeder. Visual cues, skin odors and movement are possibly all connected in host location because it is known that small lizards only respire 1.5 ml/hour of CO_2. What is interesting is that in this study the flow rate of CO_2 was 250 ml/min. In the wild, it appears that the *Cx. erraticus* mosquito would make a choice to find the host at a very close distance, where the skin odors are perhaps strong (as experienced in the extract) and movement can be

ascertained by the mosquito, because the minute amount of respired CO_2 by the lizard would be lost within the environmentally produced CO_2, unless the mosquito is very sensitive to the vapors emanating from the lizard breath, which is extremely minimal.

Anolis carolinensis is infected with *P. floridense* in the wild, and it has been reported that several mosquitoes could possibly transmit the parasitic organism to the lizard. The frequency at which the saurian malaria is transmitted is approximately one out of four lizards. But, it is also known that an infection must be prevalent in an area within the hosts at a high enough level that the parasite is acquired by the vector during a blood meal and successfully develops the sexual stage in the vector and then transmits the infective sporozoites to another host that is capable of developing the asexual cycle of the parasite. Another important objective in this study was to determine if *Cx. erraticus* has a preference for malaria infected lizards to noninfected lizards.

Florida lizards infected with *P. floridense* and Texas noninfected lizards purchased through Carolina Biological were used in the olfactometer to measure whether *Cx. erraticus* mosquitoes preferred lizards infected with malaria to noninfected lizards. The data suggested that there was no significant difference shown by the mosquitoes. However, the Florida infected lizards did receive slightly higher bite-seconds than the Texas noninfected lizards. It was also found after the last transmission study that the lizards acquired from Texas had a chronic infection prior to the transmission study.

Because the Texas lizards were found positive prior to the last transmission study, it is questionable if all of the Texas lizards used in the olfactometer were also noninfected. The two Texas lizards used in the last transmission study were not used in the olfactometer, but other Texas lizards were.

The focus of the transmission part of this study was to follow the development of the exoerythrocytic stage of *P. floridense* in *A. carolinensis* lizards starting at 12 hours after feeding by infected *Cx. erraticus* mosquitoes. Transmission of *P. floridense* by bite of *Cx. erraticus* was not successful because the parasitemia was light to moderate in most lizards and the two Texas lizards used in the last transmission study were found to be infected prior to the transmission study. Following the development of the *P. floridense* was also restricted because the Institutional Animal Care and Use Committee prohibited the euthanization of more than 10 lizards.

Euthanizing the green anole lizards every 12 hours would have allowed important information about how this host adapts to living with a potentially harmful parasite in the reptile community and why it establishes a high tolerance to *P. floridense*. This process was not achievable in this study because of developing issues with the Institutional Animal Care and Use Committee prohibiting the euthanization of large numbers of green anole lizards for exploratory research. What was shown in this study is that *Cx. erraticus* will deliberately feed on *A. carolinensis* and it is a nocturnal feeder. Klein (1985) showed under laboratory conditions that *Cx. erraticus* is a competent vector of *P. floridense* and it readily feeds on *A. carolinensis* lizards.

Culex erraticus has mostly been known in past literature as an opportunistic feeder with an affinity for avian hosts and is only a nuisance to man by occasional biting. *Culex erraticus* has tested positive for eastern equine encephalitis, St. Louis encephalitis, *Dirofilaria immitis* (dog heart worm), and recently has tested positive for West Nile virus. After reviewing vast numbers of papers on trapping, host feeding patterns and previous blood meal analysis of mosquitoes, it is this author's opinion that *Cx. erraticus*

is an aggressive, opportunistic feeder, and will take a blood meal from mammals, birds and reptiles.

APPENDIX A
MEAN PERCENT OF LIFE STAGES

Summary of mean percent for *Culex erraticus* life stages

Egg to Larvae, Pupae, Adults, Males, Females

Diet/Gen	Mean % E-L	Mean % E-P	Mean % E-A	Mean % E-M	Mean % E-F
FF1	87.6	40.7	13.6	8.1	5.5
FF2	83.8	39.2	11.1	6.5	4.5
FF3	70.7	28.7	6.3	3.4	2.9
FFBY1	92.6	32.2	10.3	5.9	4.0
FFBY2	90.3	55.3	23.2	13.3	10.0
FFBY3	60.6	63.2	27.8	78.7	60.2
FFPM1	91.4	40.9	8.1	4.9	3.2
FFPM2	86.0	45.1	7.3	4.6	2.6
FFPMBY1	87.8	39.6	9.6	5.6	2.9
FFPMBY2	86.3	55.0	18.1	9.0	9.1
FFPMBY3	89.7	58.3	22.5	12.5	10.1

Larvae to Pupae, Adults, Males, Females

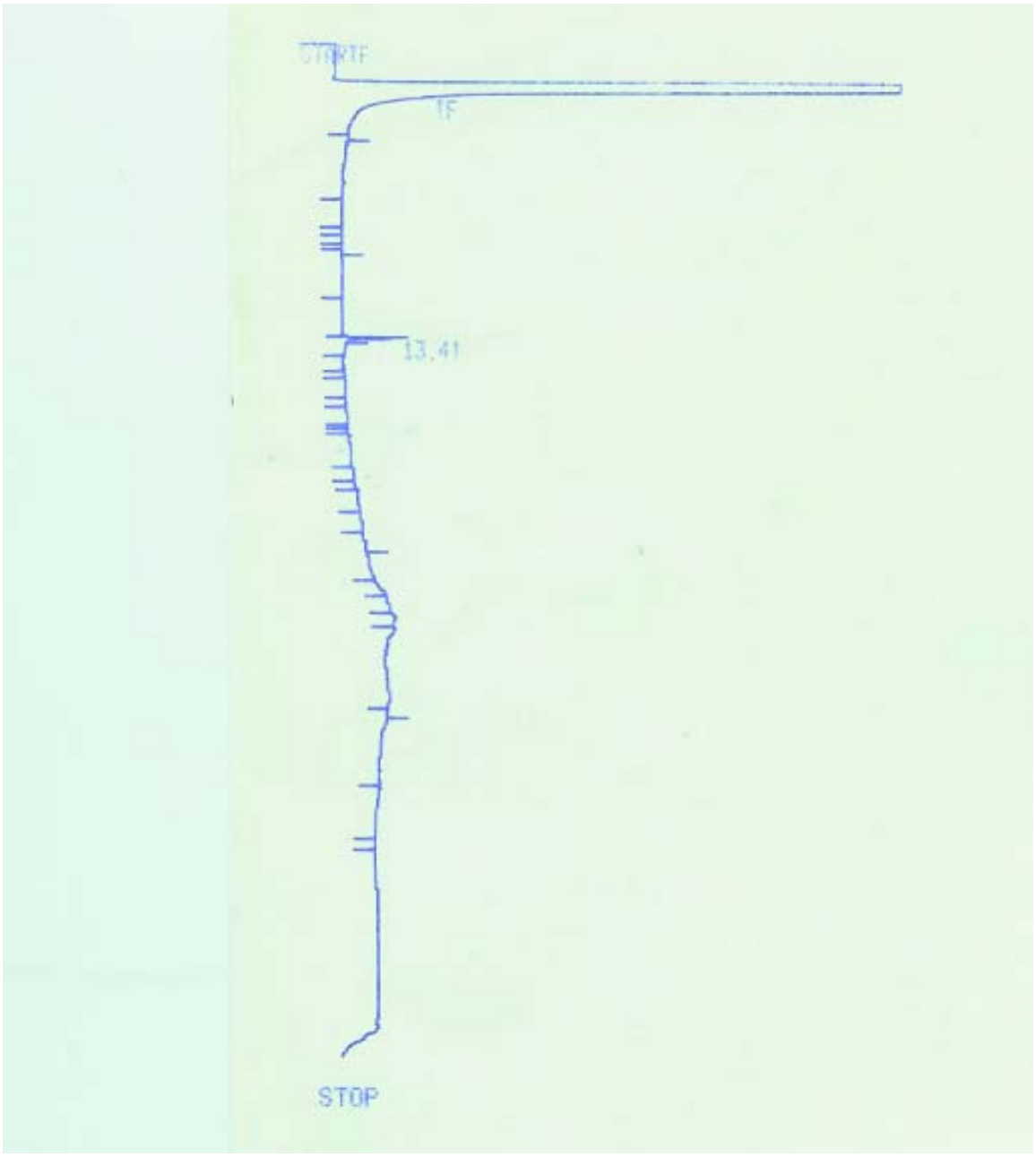
Diet/Gen	Mean % L-P	Mean % L-A	Mean % L-M	Mean % L-F
FF1	47.3	33.1	9.3	12.1
FF2	46.9	26.5	8.2	12.0
FF3	41.1	25.5	5.5	9.4
FFBY1	34.7	19.0	6.4	11.2
FFBY2	61.1	29.1	14.6	18.8
FFBY3	65.7	28.8	16.3	19.2
FFPM1	45.3	16.8	5.4	8.3
FFPM2	52.3	19.4	5.3	4.9
FFPMBY1	44.8	20.1	6.2	12.6
FFPMBY2	64.2	27.4	10.7	18.4
FFPMBY3	64.5	44.6	13.4	16.5

APPENDIX A
MEAN PERCENT OF LIFE STAGES CONTINUED

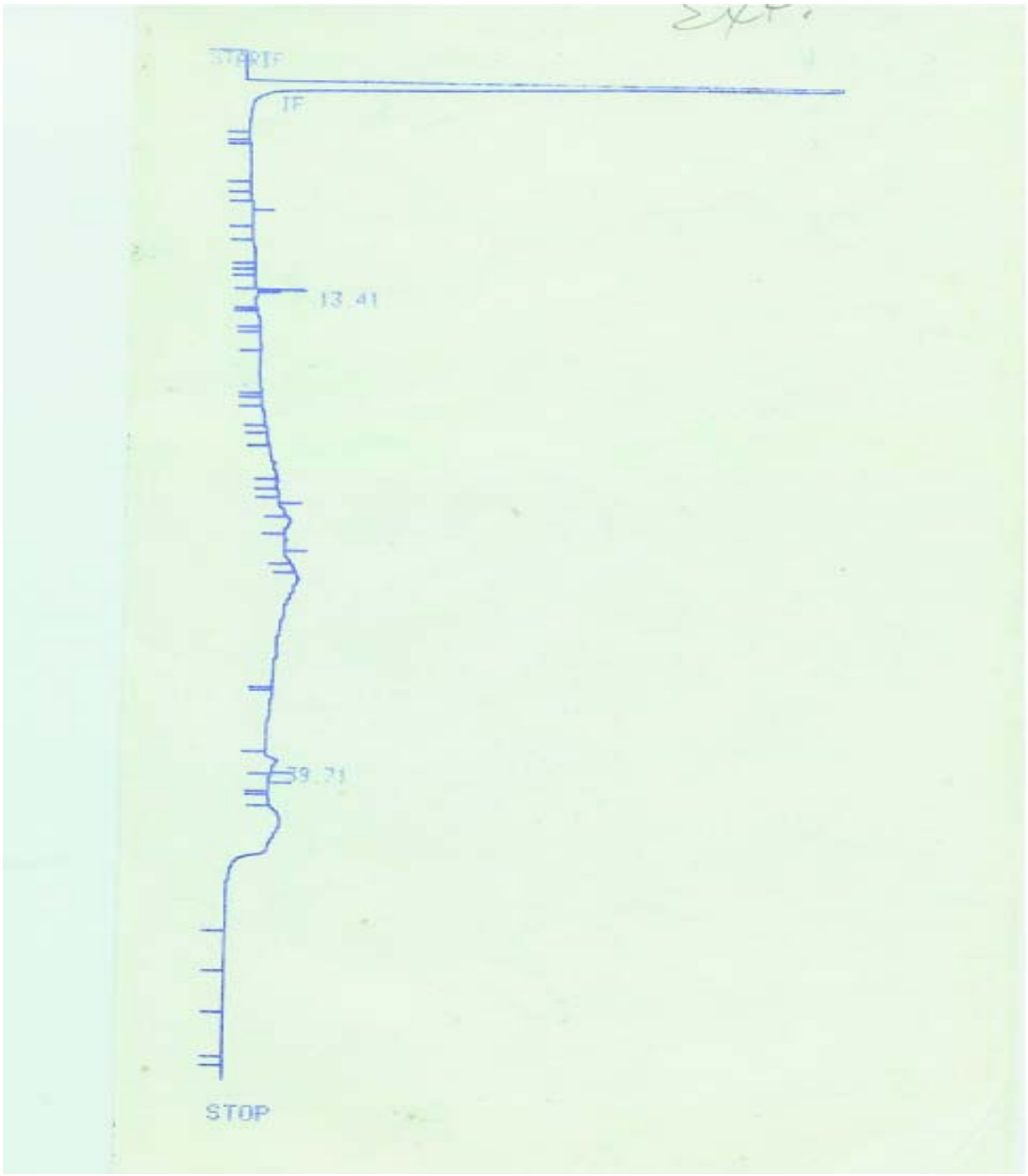
Summary data of mean percent for *Culex erraticus* life stages

Pupae to Adults, Males,Females			
Diet/Gen	**Mean % P-A**	**Mean % P-M**	**Mean % P-F**
FF1	61.5	19.1	12.6
FF2	58.0	18.0	12.0
FF3	59.4	13.0	9.4
FFBY1	53.8	18.5	11.2
FFBY2	50.4	24.1	18.8
FFBY3	43.4	24.3	19.2
FFPM1	37.3	20.4	8.3
FFPM2	32.1	54.5	4.9
FFPMBY1	59.1	20.4	12.6
FFPMBY2	43.8	16.8	18.4
FFPMBY3	9.5	20.1	16.5

APPENDIX B
GAS CHROMATOGRAPHY LIZARD EXTRACT 0.2ML



APPENDIX C
GAS CHROMATOGRAPHY LIZARD EXTRACT 0.4ML



APPENDIX D
WADE SMITH PROPERTY *CULEX ERRATICUS*

WSP2 (ITS4/5 primers) sequence comparison in GenBank. "Query" is the sequence submitted for comparison and "Sbjct" is the sequence in GenBank that it is being compared to.

gi|988225|gb|U33021.1|CEU33021 Culex erraticus clone EOH2J33 putative 5.8S rRNA gene, complete
sequence, 18S rRNA and 28S rRNA genes, partial sequence,
and internal transcribed spacers 1 and 2
Length=1006

Score = 434 bits (219), Expect = 5e-119
Identities = 236/242 (97%), Gaps = 2/242 (0%)
Strand=Plus/Minus

```
Query  2    AATTTAGGGGGTAGTCACACATTATTTGAGGCCTACTGGGGTACTACTANTGATGGTCGG  61
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  999  AATTTAGGGGGTAGTCACACATTATTTGAGGCCTACTGGGGTACTACTACTGATGGTCGG  940

Query  62   TACTGATGGGGCACACAAAACGGTATGGAAGCCAAGTCCAAGCCACTGGGGTGTACTCAT  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  939  TACTGATGGGGCACACAAAACGGTATGGAAGCCAAGTCCAAGCCACTGGGGTGTACTCAT  880

Query  122  CAATCGCGTGCAGCACAGCACGGGGGCTGCTGCGCGCGTCTGACTATCTTGAACGTTTTA  181
            |||||||||||||  ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  879  CAATCGCGTGCAG--CAGCACGGGGGCTGCTGCGCGCGTCTGACTATCTTGAACGTTTTA  822

Query  182  CCGCCACTGAAGGCAGGAAAACGTCACTACNCANACGCGGCGGGGTGGACCGCGCGCGNA  241
            |||||||||||||||||||||||||||||| || ||||||||||||||||||||||| |
Sbjct  821  CCGCCACTGAAGGCAGGAAAACGTCACTACGCATACGCGGCGGGGTGGACCGCGCGCGTA  762

Query  242  TA  243
            ||
Sbjct  761  TA  760
```

APPENDIX E

ROCKY CREEK FARM *CULEX ERRATICUS*

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RCF2

>gi|988224|gb|U33020.1|CEU33020   Culex erraticus clone EOH2J21 putative 5.8S rRNA gene, complete
           sequence, 18S rRNA and 28S rRNA genes, partial sequence,
           and internal transcribed spacers 1 and 2
          Length = 1008

 Score =  228 bits (115), Expect = 2e-56
 Identities = 173/185 (93%), Gaps = 8/185 (4%)
 Strand = Plus / Minus


Query: 533 cacagcccatccgacgcgcgagcgggatgctgcgcgtgtgttgcgcattcgaa-cggcac 591
            ||||||||||||||||||||||||||||||||||  ||||||||||||||||| ||||||
Sbjct: 469 cacagcccatccgacgcgcgagcgggatgctgcg--tgtgttgcgcattcgaaacggcac 412


Query: 592 cgatga--ggcacccttcgagcaaacactcgcactcgctcctt-cctaccgcgtggaccg 648
            ||||||  ||||||||||||||||||||||||||||||||| | ||||||||||||||||
Sbjct: 411 cgatgaacggcacccttcgagcaaacactcgcactcgctccctacctaccgcgtggaccg 352


Query: 649 tcgg-tggtggcctggccgaaaatccgccgtaaaacacactcctgct-acgtggttgggt 706
            |||| ||||||||||| |||||||||| ||||||||||||||||||| ||||| ||||||
Sbjct: 351 tcgggtggtggcctgggcgaaaatccggcgtaaaacacactcctgctaacgtgtttgggt 292


Query: 707 tcggc 711
            |||||
Sbjct: 291 tcggc 287
```

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BIOGRAPHICAL SKETCH

Byron Richard Coon was born in Akron, Ohio, and moved to Ocala, Florida, in 1952. He is the fourth child of five children, born to James R. Park, Sr. and Mary Lee Park. He finished high school in Ocala, Florida, and went on to college to study in the field of medicine. In his third year of college he was drafted into the United States Navy where he served four years during the Viet Nam War. After serving in the military he entered Florida State University and received his degree in elementary education. While attending Florida State University he worked for the State of Florida where he vested 22 years in the Florida state retirement system. Mr. Coon enrolled in the University of Florida and received his bachelor's degree in entomology in 1998 and master' degree in entomology in 2000 and will finish his doctorate in entomology in December 2005. He was the recipient of the National Association of College Teachers in Agriculture graduate student teacher award in July 2000. He has taught chemistry for health care, analytical chemistry with quantitative analysis, undergraduate and graduate medical and veterinary entomology, principals of entomology, insect field biology and undergraduate and graduate life science courses for general education requirements. He is a certified science facilitator with the Florida Game and Fresh Water Commission and performs workshops for pre-service and in service teachers in the Florida school system. He is an instructor in the Department of Entomology and Nematology at the University of Florida. He and Vicki have been married for 35 years and have a daughter Amanda, married with 4 children and son Matthew, who has one daughter.