ASSOCIATIVE LEARNING CAPABILITIES OF ADULT *Culex quinquefasciatus* SAY AND OTHER MOSQUITOES

A Dissertation

by

MICHELLE RENÉE SANFORD

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2010

Major Subject: Entomology

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Approved by:

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ABSTRACT

Associative Learning Capabilities of Adult *Culex*

quinquefasciatus Say and Other Mosquitoes.

(May 2010)

Michelle Renée Sanford, B.S.; M.S., University of California, Riverside

Chair of Advisory Committee: Dr. Jeffery K. Tomberlin

The association of olfactory information with a resource is broadly known as olfactory-based associative learning. From an ecological perspective, associative learning can reduce search time for resources and fine tune responses to changing biotic and abiotic factors in a variable environment, which in mosquitoes has implications for pathogen transmission and vector control strategies. The purpose of this dissertation was to examine the ability for olfactory-based associative learning across the major life history domains of mosquitoes.

Six different experiments comprise this dissertation. The first was to evaluate the response of mosquitoes following conditioning to 5, 10 or 50% sucrose concentrations with individual level mosquito conditioning and testing and introduction of statistical analysis with binary logistic regression. Mosquitoes did not respond in a dose dependent manner with respect to positive response to target odors following conditioning. This effect appears to be related to the mosquitoes' prior exposure to sugar as those exposed to 10% sucrose before conditioning did not prefer 50% sucrose but significantly fewer

chose 5% sucrose. In an evaluation of host associated odors and second blood meal choice by females using a dual-choice olfactometer no significant effects were observed. The lack of significance may have been due to insufficient sample sizes, problems with odor collection or physiological state of mosquitoes. Effects of predatory mosquitofish on larval development and female oviposition choice were evaluated by rearing in separated habitats under three different treatments followed by an oviposition choice assay. Females did not prefer their natal habitat or avoid predators but chose substrate that had contained mosquitofish fed conspecific larvae. Mosquitofish affected larval development with acceleration in treatments with mosquitofish fed Tetramin® and delayed pupation in treatments with mosquitofish fed conspecific larvae. Mosquito memory length was evaluated by conditioning and testing at six time intervals from colony and field populations at two ages. Younger mosquitoes showed higher levels of positive response after conditioning at all time intervals except the longest (24h). Finally the olfactory-based associative learning ability of *Anopheles cracens* was evaluated. Significant evidence for learning was observed in males but not females at a memory length interval of 24h.

DEDICATION

“There's nothing more exciting than science. You get all the fun of sitting down, being quiet, writing down numbers, paying attention. Science has it all.”

-Principal Seymour Skinner, The Simpsons

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TABLE OF CONTENTS

Page

LIST OF FIGURES

FIGURE Page

FIGURE Page

FIGURE Page

LIST OF TABLES

TABLE Page

CHAPTER I

INTRODUCTION

Learning is a difficult concept to define, especially in insects. Almost every author who describes learning uses a different definition. In his often-cited book on animal learning, Thorpe (1963) describes learning as "that process which manifests itself by adaptive changes in individual behavior as a result of experience." Whether a learned behavior can be considered adaptive is often debated, as is the fact that this definition disregards any temporal factor; yet, it remains a valid definition of learning that is not restricted to insects. In a more specific interpretation of learning, with respect to insects, Papaj and Prokopy (1989) provide three guidelines for defining learning in phytophagous insects that attempt to define limits and include important aspects of learning specific to short-lived animals. They proposed that learning be defined by: "1) the individual's behavior changes in a repeatable way as a consequence of experience, 2) behavior changes gradually with continued experience, and 3) the change in behavior accompanying experience wanes in the absence of continued experience of the same type or as a consequence of a novel experience or trauma." Otherwise, there are many other differing definitions for learning in the literature (e.g. Kimble 1961, Tully 1984, Stephens 1993), and it is a highly debated topic among behaviorists and ethologists (Vet et al. 1995).

This dissertation follows the style of the Journal of Medical Entomology.

The most widely-accepted aspects of learning in insects are habituation, sensitization, operant conditioning, and associative learning (e.g. Alloway 1972, Dethier 1976, Papaj and Prokopy 1989, Bernays 1995). Habituation involves the waning of a response to a stimulus after repeated exposure and involves changes in neurotransmitter release at synaptic pathways (Bernays 1995); thus, the animal fails to respond to the stimulus over time. Sensitization is considered the opposite of habituation and involves heightened responsiveness to a stimulus (Bernays 1995). In experiments that require repeated exposure to a stimulus, there should be control for habituation and sensitization (Alloway 1972).

Operant conditioning has been described as a rewarded change in behavior over time. For example, the repeated experience with a particular flower type by Lepidoptera and Hymenoptera which has a “rewarding” nectar source decreases the amount of time the insect requires to extract the nectar from it on subsequent visits (Papaj and Prokopy 1989). Associative learning, also known as classical conditioning, is probably most widely known from the classical experiments of Pavlov (1927) in which he trained dogs by ringing a bell prior to offering them food. Associative learning involves the coupling of an Unconditioned Stimulus (US), in the case of Pavlov’s work this would have been the food, which results in an Unconditioned Response (UR), a dog salivating in response to the food, with a Conditioned Stimulus (CS), the bell (Pavlov 1927). Through a course of training by exposure of the CS with the US, the animal will display the UR with exposure to the CS (Gould 1993). Thus, the dogs salivated (UR) with the ringing of the bell (CS).

Much of what we know about associative learning in insects comes from work with fruit flies in the genus *Drosophila* (Diptera: Drosophilidae; Quinn et al. 1974), the honeybee *Apis mellifera* Linnaeus (Hymentoptera: Apidae; VonFrisch 1956, Bitterman et al. 1983), the tobacco hornworm *Manduca sexta* (Linnaeus) (Lepidoptera: Sphingidae; Daly and Smith 2000), and parasitic wasps such as *Microplitis croceipes* Cresson (Hymenoptera: Braconidae; Lewis and Tumlinson 1988). A majority of the work published on associative learning in *Drosophila* is aversion learning in which the fly is trained to avoid an electrified area either in a maze or in a flight tunnel in conjunction with an odor (e.g., Quinn et al. 1974, Tully 1984, Tully and Quinn 1985). The availability of the *Drosophila* genome and the wide variety of mutants have made work on the genetic basis for behavior and learning in *Drosophila* possible (e.g., Tully 1984, Tully and Quinn 1985, Zars et al. 2000, Suh et al. 2004). In the honeybee, *Apis mellifera*, the proboscis extension reflex has been a valuable tool in determining associative learning and much of the work with bees has been at higher levels of the central nervous system (Meller and Davis 1996). Work in honeybees has led to the discovery of the function of the antennal lobes and mushroom bodies in associative learning involving odors (Erber et al. 1980). Recent work in *Manduca* has led to simultaneous mapping of antennal lobe neural networks in the brain while training to odors (Daly et al. 2004). Studies in parasitoid wasp learning have explored the importance of both olfactory and visual cues in foraging and the associative learning capabilities of these wasps (e.g., Lewis and Tumlinson 1988, Turlings et al. 1993, Wackers et al. 2002). Meiners et al. (2002) showed that this wasp is capable of learning and discriminating among

compounds that have very similar molecular structures. This work led to research on the potential to utilize these wasps as biological sensors, after training, to specific compounds that they would not normally associate with in nature (e.g., Tertuliano et al. 2004, Tomberlin et al. 2005).

The four insect groups just discussed represent not only vastly different life history strategies but the importance of associative learning in each may also differ. Drosophilidae are not aggressive foragers (Meller and Davis 1996) as opposed to honeybees and moths which spend large amounts of time and energy searching for food (bees) and mates (moths). Thus, it would be expected that associative learning may be a more relevant ecological strategy for highly active foragers. Parasitoid wasps also represent another life history strategy where they must aggressively forage for hosts in which to deposit offspring. This is a similar life history strategy to a mosquito, which, regardless of searching for a sugar meal or a blood meal, must actively forage as a required phase of its life history. It would be expected, therefore, that associative learning would be an advantageous life history strategy for mosquitoes as well.

Associative learning in mosquitoes has been proposed as a mechanism to reduce search time for hosts and as a possible explanation for site fidelity or home range, which could have highly significant implications for the epidemiology of mosquito-borne diseases (McCall and Kelly 2002). A few authors have experimentally demonstrated preferential blood feeding on specific hosts which the mosquito has been previously attracted to and fed upon, but which are not necessarily the most abundant host available (Hii et al. 1991, Mwandawiro et al. 2000). Mwandawiro et al. (2000) used the capture-

mark-release-recapture technique to show that mosquitoes attracted to either a cow or a pig and allowed to feed, were more likely to return to that animal at the next gonotrophic cycle. The fact that this effect was not observed in the F_1 progeny of these individuals indicates associative learning as the mechanism for this behavior. The response of individual *Culex* species, however, was not universal and some species were more likely to display this preference than others. More evidence has been presented to support the observance of site fidelity among certain mosquito populations. Site fidelity has been reported by several authors (Ribbands 1949, Renshaw et al. 1994, McCall et al. 2001); but, it is best evidenced experimentally by the work of Charlwood et al. (1988) with *Anopheles farauti* in Papua New Guinea. Charlwood et al. (1988) also utilized a capture-mark-release-recapture by collecting mosquitoes in one village, marking them, and releasing them into another village approximately 4 km from the initial village. They found that the mosquitoes originally collected in one village were recaptured more frequently in their village of origin.

Recent work by Alonso et al. (2003) suggests that associative learning is not possible in *Aedes aegypti* L.; but, the methods used to condition the insects may have been hampered by the lack of controls for habituation and inadequate coupling of CS with US. Tomberlin et al. (2006) have presented evidence of associative learning in *Culex quinquefasciatus* Say in short range experiments involving sugar and blood. They have shown that this mosquito will learn to associate a resource with an odor not typically encountered in nature (vanilla or strawberry). In another study associating sugar-meals with olfactory cues, *Cx. pipiens pipiens* biotype *molestus* Forskal were

found to be innately attracted to flower odors and experience paired with a sugar-meal increased this attractive response (Jhumur et al. 2006). The authors suggested that this was a form of olfactory conditioning.

Learning in mosquitoes is not limited to olfactory cues associated with food resources; females must also search out habitats in which to lay eggs and this is another area in which learning research has just begun. In *Aedes aegypti* L., Kaur et al. (2003) found that, after rearing larvae with a novel chemical, the mosquito repellent Mozaway™, the resulting females laid eggs in water that contained the chemical in a similar amount to clean water. The authors suggest that rearing with the repellent chemical caused a reduction in deterrence, but this is a difficult behavior to test. More convincing work that suggests larval exposure to chemicals induces adult preference at oviposition comes from work with *Cx*. *quinquefaciatus*. McCall and Eaton (2001) reared larvae in water containing normally attractive chemicals at concentrations so high they deter females at oviposition. They then tested the resulting females to these concentrations for oviposition and found that the mosquitoes preferentially laid eggs in water containing these high chemical concentrations. The authors also reared larvae in hay infusion and guinea-pig feces-containing water (regularly used as oviposition substrates for field monitoring programs) and found that, after rearing, the females preferred to lay eggs in these types of habitats.

The preference of adult insects for habitats similar to the habitat experienced as an immature has been observed in many insect groups and was thought to be based on learning of this habitat; however, definitively demonstrating that learning can persist

through metamorphosis in a holometablous insect has proven to be difficult (Barron 2001). The chemical legacy hypothesis, which states that there is some trace of the larval habitat that can persist in the pupa, was put forth as an alternate explanation for the observed preference of adults for habitats similar to their natal habitat (Corbet 1985). Another theory that has been suggested to account for adult preference for natal habitat, which falls under the larger more general category of natal habitat preference induction (NHPI), is that early adult imprinting may occur as the adult emerges from the larval habitat (Davis and Stamps 2004). No one has yet adopted a theory as to how NHPI occurs in mosquitoes or if it occurs under field conditions.

Alonso and Schuck-Paim (2006) outlined several reasons they feel that learning in mosquitoes has yet to be definitively observed. They describe six different “ghosts” that hamper the study of learning in mosquitoes as they see it. The first is the “spatial distribution ghost”, which is described as a problem with the design of experiments such that many field studies lack adequate controls for the distribution of resources in the habitat. This makes it difficult to link the mosquitoes’ return to a habitat as spatial learning rather than merely returning to a favorable habitat. The second “ghost” is the “environmental conditions ghost” and it is an extension of the first as it also deals with the lack of an ability to adequately replicate and control for environmental conditions in field studies. The third “ghost” is the “genetic background ghost” and they describe this problem as being one of not taking into account genetic variation in fixed behaviors in a population. It could be argued that the authors are oversimplifying this particular point by separating the genotype from the behavior which is part of the same concept.

Behavior is the phenotypic expression of the genotype and learning is phenotypic plasticity. The genotype of the individual is going to dictate whether they can learn just as much as if it were to dictate whether site fidelity is fixed. Perhaps a chromosomal inversion mapping technique would serve to alleviate this problem, as it would be a better indicator of population level genetic change and adaptation. The fourth "ghost" is the "ontogenetic ghost" which is described as the difficulty in determining the differences that become apparent with age or what might amount to physiological changes with age and experience. This makes it confusing as to why it is not named as in the next "ghost", the "physiological ghost". The "physiological ghost" is associated with basic factors that revolve around neurological response and how animals respond to responses over time such as habituation and sensitization. The final "ghost" is the "bad teacher ghost" and this refers to problems in experimental design for controlled experiments that do not have adequate controls for the response of the animal. This might include, for example, a lack of controls for habituation to an odor or a proper protocol for presenting a stimulus in a context that the animal will be able to relate to the stimuli.

The goal of this dissertation research was to expand on the work of Tomberlin et al. (2006) by examining several different aspects of associative learning in *Cx. quinquefasciatus* and expanding those methods to other mosquito species in different genera. This research has touched lightly on some really big areas that could each receive much more detailed treatment as individual research foci. Hopefully it will serve as a starting point to more detailed lines of research in each area of study.

Four overarching research objectives comprise this dissertation. The first was to examine ways in which olfactory cues are associated with food resources including both sugar rewards and blood rewards. The second objective was to examine the effect of predator cues on larval *Cx. quinquefasciatis* development and the potential for association of natal habitat cues with future oviposition. In the third objective the length of memory was examined by conditioning *Cx. quinquefasciatus* and testing at six different time intervals from <5 min to 24h. In the fourth and final objective the ability of *An. cracens* to associate olfactory information with a sugar-meal was examined in Thailand.

CHAPTER II

IMPROVING METHODS USED IN TESTING OLFACTORY-BASED ASSOCIATIVE LEARNING PROPERTIES IN *Culex quinquefasciatus* SAY (DIPTERA: CULICIDAE) TO A SUGAR-MEAL

Introduction

Associative learning has been suggested as a means for insects and other arthropods to optimize their search for feeding and oviposition resources by linking high quality resources with the olfactory and visual cues associated with them (Lewis and Tumlinson 1988, Egas et al. 2003, Behmer et al. 2005, Cnaani et al. 2006). Insect learning is a large field with many specialties, which perhaps reflects the wide diversity of life history traits found in the Insecta. Much of the literature on insect learning revolves around social Hymenoptera which rely not only on associative learning but on communication among individuals to transmit information. Learning has long been associated with the honeybee (VonFrisch 1956). In this study, attention is focused on, learning in Culicidae which relate to honeybees as other Diptera do, in that they actively forage for food resources, yet they differ markedly in the fact that they are non-social insects.

Dipteran learning has been described in at least eight different species of forensically and medically important flies including: *Cynomya cadaverina* Desvoidy (Frings 1941), *Phormia regina* (Meigen) (Nelson 1971), *Musca domestica* L. (Fukushi

1976), *Lucilia cuprina* (Wiedeman) (Fukushi 1989), *Calliphora vicina* Robineau-Desvoidy (Maes and Bijpost 1979), *Aedes aegypti* (L.) (Kaur et al. 2003), *Culex quinquefasciatus* Say (Tomberlin et al. 2006) and *Culex pipiens pipiens* biotype *molestus* Forskal (Jhumur et al. 2006). In these studies, learning was demonstrated in conjunction with visual, contact, olfactory stimuli or a combination of these stimuli with food or oviposition resources. The importance of olfactory information to culicid life history has probably led the emphasis of research in this area toward olfactory-based learning.

Kaur et al. (2003) demonstrated conditioning of *Ae*. *aegypti* with olfactory stimuli and oviposition sites. They found that when the larvae developed in water containing the mosquito repellent, Mozaway^{TM} the resulting females laid more eggs in water filled cups containing the repellent chemical when compared to mosquitoes reared in untreated water. The assay used in this study was somewhat limited by taking observations only of the endpoint of the oviposition process. Thus, it is impossible to separate olfactory response from gustatory or tactile response of the females to the oviposition substrates. However, it does demonstrate conditioning of female mosquitoes to a chemical associated with their larval habitat. Learning may not be universal across sensory domains, as evidenced by the lack of learning of olfactory stimuli with food in *Ae*. *aegypti* (Alonso et al. 2003). However, there is reason to suspect that the conditioning procedure may have lacked an adequate control for non-associative conditioning in this study.

Other mosquito species have been found to associate olfactory stimuli with food and/or blood-meal source. In one of the only field studies to demonstrate associative

learning with a blood-meal, Mwandiwiro et al. (2000) tethered either a pig or cow host and collected *Culex* spp. mosquitoes that came to each host. These mosquitoes were marked, allowed to feed on the host they had initially chosen and then held in captivity until they were ready to seek another blood-meal. When the hosts were tethered again within a net cage and the mosquitoes released inside the cage the authors found that significantly more of the marked mosquitoes returned to the host they had previously fed upon. Further investigation of the resulting F_1 progeny indicated a lack of preference which supports the hypothesis that there was development of an association between the host (presumably odor) and a successful blood-meal in the initially exposed mosquitoes.

Jhumur et al. (2006) found that a higher percentage of *Culex pipiens pipiens* biotype *molestus* Forskal exposed to sugar rewards in conjunction with the odors associated with the flowers of *Silene otites* (L.) Wibel (Caryophyllales: Caryophyllaceae) responded positively to the odors presented without sugar in a wind tunnel experiment. The data showed that experience enhanced the innate attraction of this mosquito species to the floral odors and suggested that the mechanism for this increase was associative learning.

Tomberlin et al. (2006) found evidence that adult female and male *Culex quinquefasciatus* Say are capable of associative learning to novel odors presented in a classical conditioning paradigm with a 10% sucrose solution and that females associated a novel odor with a blood-meal. Each mosquito was individually conditioned and assayed to each odor. It represents, to our knowledge, the first study to clearly

demonstrate appetitive based olfactory associative learning in conjunction with a sugar-meal by a mosquito.

In this study, the work of Tomberlin et al. (2006) was taken a step further by incorporating methodological improvements on the original learning assay. One of the issues which impairs the ability to compare studies is a lack of standardized protocols for training and testing the insects (Dukas 2008). Thus, attempts were made to improve the conditioning assay and introduce a new method for statistical analysis of these data using binary logistic regression. Under this premise, an answer was sought for the question, would increasing the strength of the unconditioned stimulus offered to the mosquito during training improve the response of conditioned mosquitoes? To answer this question, individual southern house mosquitoes, *Cx. quinquefasciatus* were trained in a classical conditioning paradigm to odors paired with three different reward strengths consisting of 5, 10, and 50% sucrose. This approach was posed as both a methodological question and as an ecological question. The hypothesis was that there would be a higher probability of mosquitoes responding positively to a trained odor when it was paired with the highest level reward, as it would represent a high quality resource in nature and thus, be a more effective training reward for future studies.

Materials and Methods

Insects

Adult *Cx. quinquefasciatus* were obtained from the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), USDA-ARS, Gainesville, FL, USA where they were reared using standardized methods (Gerberg et al. 1994). This

colony was created from material collected in Gainesville, Fl, USA, in 1995. Adult mosquitoes (males and females aged 1-2 days) were held at 27-29°C with 70-85%RH at 16:8 L:D cycle and were offered a 10% sucrose solution prior to overnight shipment to the Insect Biology and Population Management Research Laboratory (IBPMRL), USDA-ARS, Tifton, GA, USA, where experiments were conducted. Shipment of mosquitoes was decided upon because the IBPMRL was not equipped to provide blood-meals for female mosquitoes for rearing onsite and the shipment procedure was used successfully in the past (Tomberlin et al. 2006). Each shipment of newly emerged mosquitoes represented a separate cohort and occurred on a weekly basis for a total of five cohorts during June and July 2006. Upon arrival at the IBPMRL, mosquitoes were transferred to two 12.7 (L) x 12.7 (W) x 7.6 (H) cm Plexiglas® cages with access to distilled water and 10% sucrose solution and held at temperature, humidity and lighting conditions analogous to those that were experienced at the CMAVE. The separation of the mosquito cohort into two cages allowed for alternating starvation and conditioning to maximize data collection.

Adult mosquitoes were starved but had access to distilled water for 24 h prior to conditioning. Mosquitoes were held under conditions resembling those experienced during rearing for the duration of the starvation period prior to conditioning. One hour prior to conditioning, the mosquitoes were moved to the laboratory bench in front of a chemical fume hood to allow acclimation to the new environment. Individual mosquitoes were isolated within clean individual 2-dram glass shell vials (40 mm height x 17 mm diameter) placed under the operating fume hood and allowed to acclimate for a

minimum of 10 min. prior to conditioning. Each glass vial was placed on a small clean piece of office paper (approximately 3.81 cm^2; Fig. 2.1) that allowed for movement and/or rotation of the vial to facilitate equivalence of position and visual cues in the conditioning procedure (observer and pipette were always in the same position relative to the mosquito). This method for offering the pipette differs from the procedure used by Tomberlin et al. (2006) where the pipette was offered to the mosquito wherever the proboscis could be reached.

Conditioning Procedure

Individual male and female mosquitoes were conditioned to either pure vanilla extract (McCormick & Co., Inc., Hunt Valley, MD, USA) or 100% technical grade myrcene (Sigma-Aldrich, Co., St. Louis, MO, USA) in combination with a 5%, 10%, or 50% (w/v) technical grade sucrose (Sigma-Aldrich, Co., St. Louis, MO, USA) solution. The vanilla extract was chosen because it was successfully used in a previous study (Tomberlin et al. 2006) and myrcene was chosen because it is a compound with which the mosquitoes should not have any innate preference and differs completely from vanilla in chemical structure. The initial conditioning procedure was conducted similarly to the methods of Tomberlin et al. (2006). Briefly this method includes filling the first 1-2 cm of a 200 µL calibrated glass micropipette (Drummond Scientific Company, Broomall, PA, USA) with sucrose solution (unconditioned stimulus: US) and coating ~1 cm of the exterior of the distal end with the target odor compound (conditioned stimulus: CS). The mosquito was offered the coated pipette by lifting the edge of the vial

Figure 2.1. A female *Culex quinquefasciatus* waits in the vial between training trials illustrating the use of a small sheet of paper under the vial which allowed for movement and rotation to facilitate introduction of the pipette.

approximately 30° and placing the solution-filled pipette directly onto the mosquito's proboscis and allowing it to feed for 10 s. This procedure was repeated three times with an inter-trial interval of 30 s. All conditioning and testing was conducted under an actively-ventilating fume hood and under laboratory lighting between 1100 and 1800 h (during the mosquito photophase).

Sample sizes for each of the categories created by the experimental design are listed in Table 2.1. The lower number of mosquitoes conditioned to the 5% sucrose unconditioned stimulus (US) reflected difficulties in conditioning females to this sugar concentration especially when vanilla was the target. Females were the most difficult to condition at the 5% sucrose solution, but fewer refused the conditioning protocol at higher concentrations of sucrose (Figure 2.2).

Testing Procedure

Mosquitoes were conditioned using the above procedure and then allowed to wait in the glass vial in the fume hood for a period of time (13.0 ± 9.0 min) while other mosquitoes were being conditioned prior to testing. Each conditioned mosquito was tested with an empty pipette that was either a new unused pipette or blank, a pipette coated with the target odor they were conditioned to (either myrcene or vanilla), or a pipette coated with the non-target odor with which they had not had experience. The mosquito was tested only once and then discarded, thus eliminating any confounding factors associated with testing an individual multiple times as done in (Tomberlin et al. 2006). The pipette was placed under the vial at a distance of approximately one half the diameter of the vial from the mosquito for 15 s to allow for a response.

Data were recorded for each mosquito as either a positive of negative response to a single stimulus test. A positive response was recorded if the mosquito walked toward the pipette and moved the proboscis as if searching for the sucrose solution. A negative response was recorded if the mosquito did not move when exposed to the test pipette or moved away from it.

Table 2.1: Sample sizes (N = 283) for the categories examined in the conditioning of *Culex quinquefasciatus* to two different target odors, both sexes, and three different concentrations of sucrose solution.

Target:	**Sex**	**Sucrose Concentration**	**n**
Vanilla			140
	Female		59
		5%	9
		10%	22
		50%	28
	Male		81
		5%	21
		10%	27
		50%	33
Myrcene			143
	Female		62
		5%	17
		10%	19
		50%	26
	Male		81
		5%	25
		10%	26
		50%	29

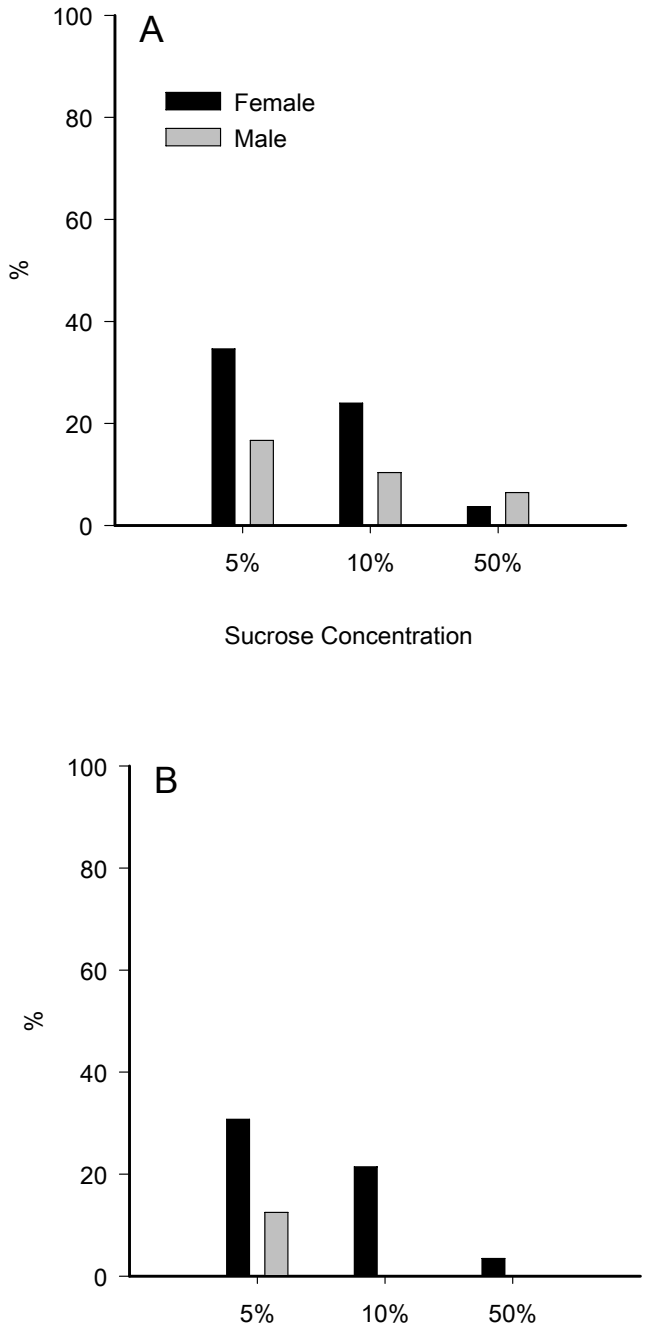

Figure 2.2. Percentage of mosquitoes that refused the conditioning protocol. Fewer mosquitoes refused the conditioning protocol as the concentration of sucrose increased regardless of whether the target odor was myrcene (panel A) or vanilla extract (panel B).

Statistical Analysis

In order to increase the statistical power and examine relationships among the sucrose concentrations a binary logistic regression model (Hardy and Field 1998) was selected over an analysis of variance on percent response (Tomberlin et al. 2006). Binary logistic regression was employed using the backward stepwise variable selection method based on the change in likelihood ratio (entry at $P = 0.05$ and exit at $P = 0.10$; SPSS 2005). The variables of interest included the categorical variables indicating: cohort membership, sex, target to which the mosquito was conditioned, sucrose concentration to which the mosquito was conditioned, and test to which the mosquito was subjected. The amount of time a mosquito waited to be tested was also included in the initial model but was not found to be a significant predictor of response. For the analysis of those variables with more than two categories, the overall effect was tested first then dummy variables were created through binary coding and a reference variable to which the dummy variables were compared; thus, any results obtained from these variables represent the difference between the dummy category and the reference category (DeMaris 1995).

This analysis provided model results in terms of odds ratios which are presented in Table 2.2 as well as predicted probabilities for positive outcome with the model which were plotted for the variables of interest using SigmaPlot (Systat Software 2006). An independent-samples T-test was conducted on the mean predicted probability for each two-category variable and an analysis of variance with Tukey's HSD post hoc test was

conducted for each three-category variable (SPSS 2005). Statistical significance for all tests was observed at $\alpha = 0.05$.

Table 2.2: Final binary logistic regression model (model χ^2: 71.743, d.f.: 8, P <0.001) based on backward stepwise variable selection for likelihood ratio of associative learning data in *Culex quinquefasciatus* to three different sucrose concentrations. The model is based on the response variable of positive outcome. See text for detailed description of variables and tests.

Variable	Log Odds (β)	d.f.	*P*	Odds Ratio (e^{β})
Target: Vanilla	0.703	1	0.029*	2.020
Concentration		2	0.070	
Concentration: 5%	-0.708	1	0.082	0.493
Concentration: 10%	-0.776	1	0.040*	0.460
Sex: Female	-1.558	1	<0.001*	0.210
Test		2	<0.001*	
Test: Blank	-2.282	1	<0.001*	0.102
Test: Non-target	-2.022	1	<0.001*	0.132
Constant	0.666	1	0.074	1.947

* indicates significant difference at $\alpha = 0.05$ level.

Results

The final model, for the binary logistic regression analysis is presented in Table 2.2. Cohort membership did not contribute significantly to the prediction of positive response and was the only variable dropped from the model (variable selection ended at the second step). The final model had a -2log-likelihood of 247.087 and the χ^2 for the

final step was 71.743 (d.f. = 8, $P < 0.001$). The *P*-values presented in Table 2.2 are based on the Wald statistic for each individual variable and indicate the ability of each variable to predict a positive response.

In the final model several variables were significant predictors of a positive response (Table 2.2). Each of the predictors in the model was also plotted as predicted probability for each category for a more visual representation of the data. The target odor to which the mosquito was conditioned was a significant predictor, with 2.020 higher odds of a positive response when vanilla was the target odor (Table 2.2). Mean predicted probability was significantly higher for vanilla than for myrcene (t = -4.407, d.f. = 266.790, $P < 0.001$; Fig. 2.3). The mosquito's sex was also a significant predictor of positive response, as females had 0.210 lower odds of responding positively to the target odor (Table 2.2) as evidenced by the significantly higher mean predicted probability of males over females (t = 8.897, d.f. = 265.934, $P < 0.001$; Fig. 2.4).

Perhaps the most important part of the model is the variable of test which indicates that mosquitoes are learning with lower odds of positive response to blank and non-target odors when compared to the target odor (Table 2.2). The test variable was a significant predictor overall ($P < 0.001$). The mean predicted probability for target was significantly higher than for blank or non-target tests (F = 199.256, d.f. = 2, 280, $P < 0.001$; Fig. 2.5).

The sucrose solution concentration to which the mosquito was conditioned was not a significant predictor of positive response overall, despite significant differences in odds between the 10% and 50% sucrose solutions (Table 2.2) and mean predicted

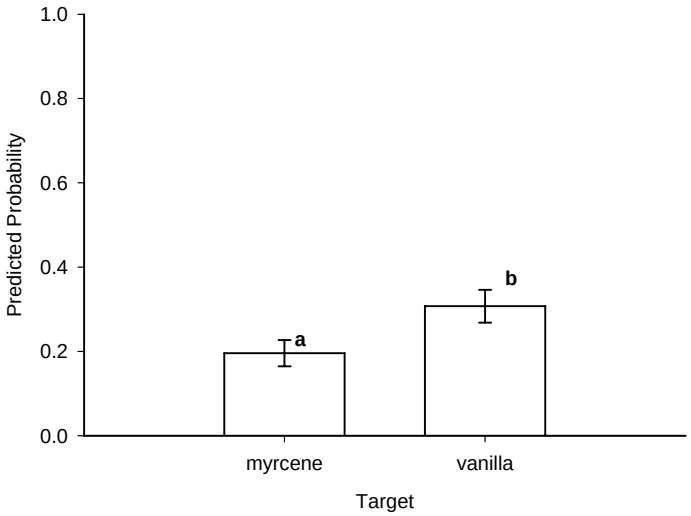

Figure 2.3. Mean (+/- 95% confidence interval) predicted probability of positive response to conditioning of *Culex quinquefasciatus* to pure vanilla extract or technical grade myrcene. Different letters indicate significant differences between groups as determined by independent samples T-test at $\alpha = 0.5$.

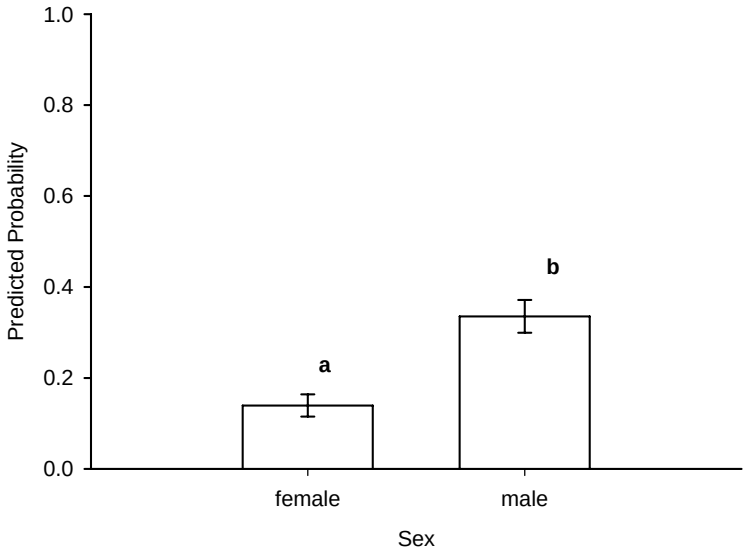

Figure 2.4. Mean (+/- 95% confidence interval) predicted probability of positive response to conditioning of male and female *Culex quinquefasciatus.* Different letters indicate significant differences between groups as determined by independent samples T-test at $\alpha = 0.5$.

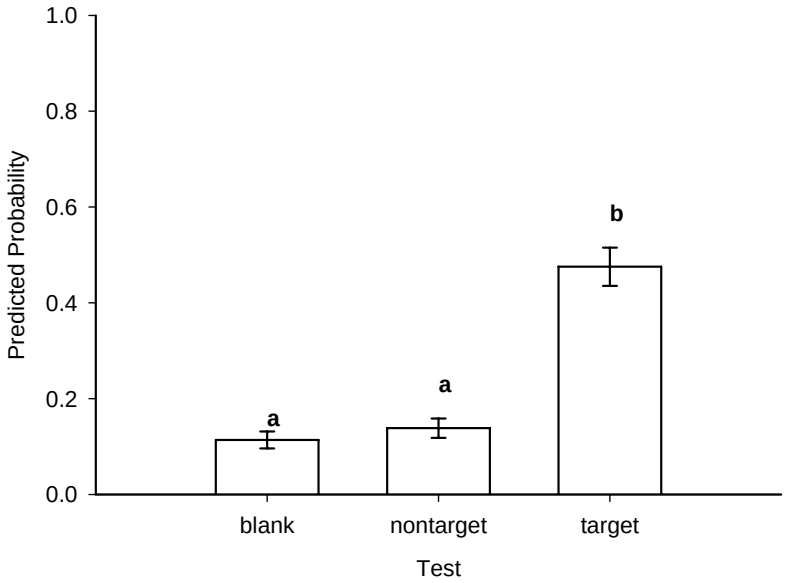

Figure 2.5. Mean (+/- 95% confidence interval) predicted probability of positive response to conditioning of *Culex quinquefasciatus* when tested to a blank pipette, a pipette coated with an unknown odor or a pipette coated with the target (conditioning) odor. Different letters indicate significant differences between groups as determined by independent samples T-test at $\alpha = 0.5$.

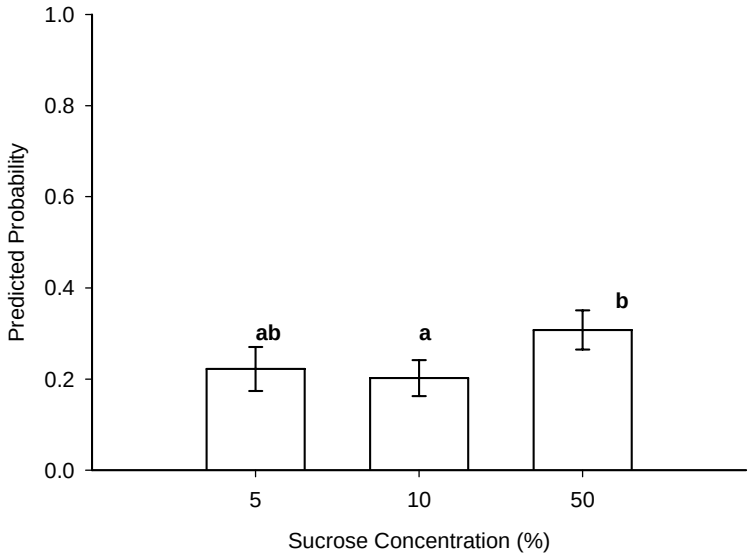

Figure 2.6. Mean (+/- 95% confidence interval) predicted probability of positive response to conditioning of *Culex quinquefasciatus* to three different sucrose concentrations. Different letters indicate significant differences between groups as determined by Tukey's HSD following analysis of variance at $\alpha = 0.5$.

probabilities (Tukey's HSD $P = 0.022$; Fig. 2.6). There were, however, observable trends in the percentage of mosquitoes responding positively to a test when they were conditioned to the different sucrose concentrations. A higher percentage of female mosquitoes responded to the target when trained with 50% sucrose than when trained with the other lower sucrose concentrations regardless of the target odor (Fig. 2.7B and Fig. 2.8B). The percentage of males responding positively to the target odor was similar across sucrose concentrations and target odors, with the exception of those trained to vanilla extract as the target with 5% sucrose (Fig. 2.7A and Fig. 2.8A). Acceptance of the conditioning assay does not infer that these mosquitoes were successfully trained, only that they accepted feeding for the three trials constituting the conditioning assay. In general, there was a higher percentage of positive responses by male mosquitoes than females regardless of target odor (Fig. 2.7 and Fig. 2.8).

Discussion

The application of binary logistic regression in this study represents a novel application to the study of associative learning in mosquitoes and improves upon the methods developed for research in this area. Of the studies that have explicitly set out to investigate appetitive, olfactory based associative learning in adult mosquitoes (Alonso et al., 2003; Mwandawiro et al., 2000; Tomberlin et al., 2006), the current study is the first to analyze the response of an individually-conditioned mosquito to a single stimulus. The methods of Tomberlin et al. (2006) were modified to allow for the collection of independent data points that can be analyzed using a more encompassing and informative analysis. The main issue with the results collected in Tomberlin et al.

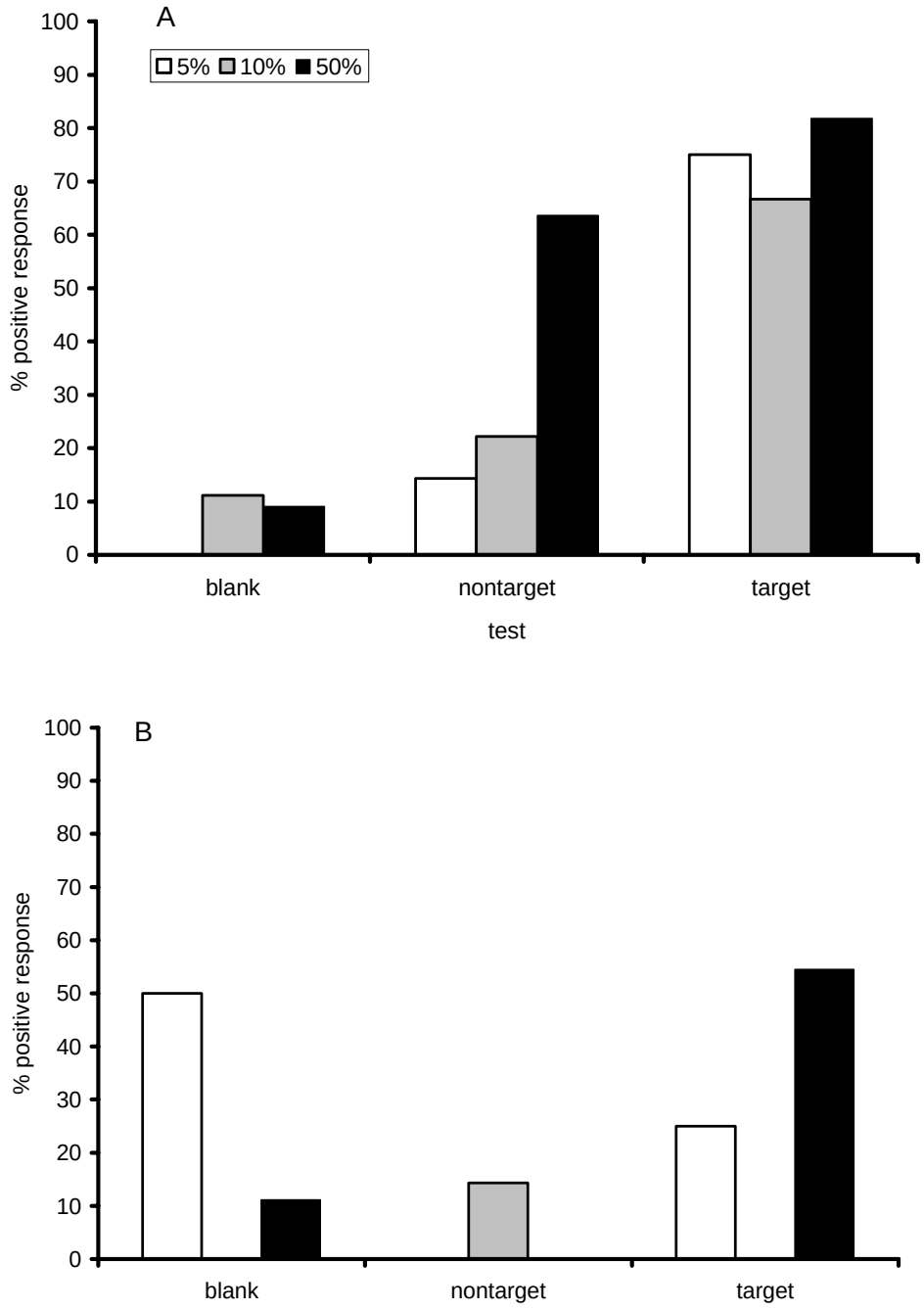

Figure 2.7. Percent positive response of mosquitoes trained to three different sucrose concentrations (5% white bars; 10% gray bars; 50% black bars) when the target odor was myrcene for males (panel A) and females (panel B). Each mosquito was trained to the sucrose-odor combination and subjected to only one test.

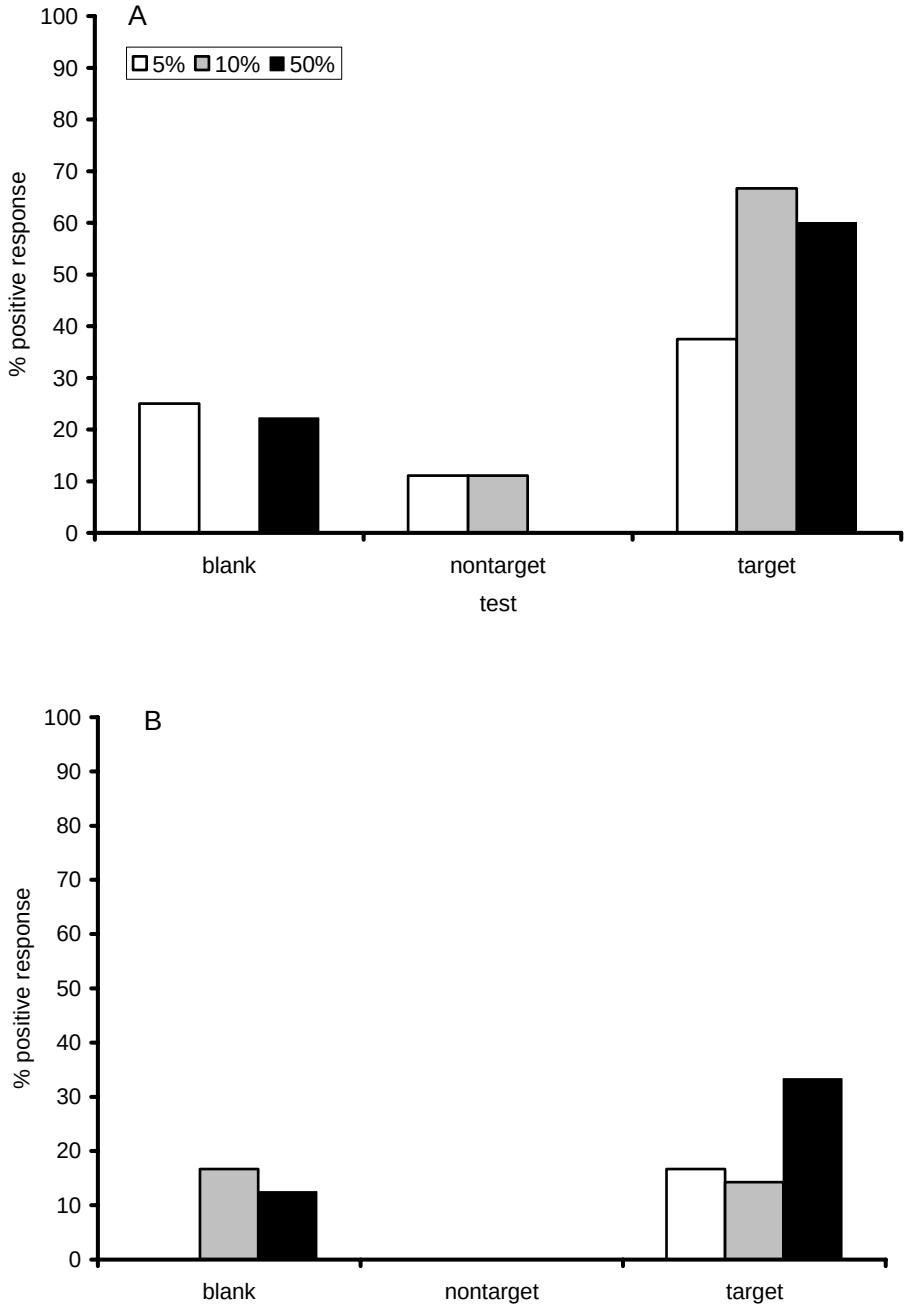

Figure 2.8. Percent positive response of mosquitoes trained to three different sucrose concentrations (5% white bars; 10% gray bars; 50% black bars) when the target odor was vanilla extract for males (panel A) and females (panel B). Each mosquito was trained to the sucrose-odor combination and subjected to only one test.

(2006) was the lack of independence between test results, as a single mosquito was tested to all three test options in the same order each time. Not only does this approach complicate data analysis, but it may also complicate the behavioral response of each mosquito. The presentation of a non-target odor prior to a target odor each time may have sensitized the mosquito to any odor presented subsequently, or it may have broken any habituation associated with the target odor left in the conditioning chamber. In either case, it may have artificially inflated the positive results obtained from trained mosquitoes. Nevertheless, learning by *Cx. quinquefasciatus* was demonstrated by Tomberlin et al. (2006) and our study supports the results presented therein.

There are several interesting results from this experiment that merit discussion including the significant difference in odds between the myrcene and vanilla targets, the significant odds difference between males and females, and the lack of difference among sucrose concentrations. Each of these results has implications for both experimental design and olfactory learning in mosquitoes and will be discussed in order.

The significant differences observed between both the odds and predicted probability between positive response in mosquitoes conditioned to vanilla and those conditioned to myrcene raises some interesting questions. Preliminary data collected on starvation and learning conducted concurrently suggest that *Cx. quinquefasciatus* may have an innate attraction to the vanilla extract (Lewis et al. unpublished data); thus, conditioning may have only increased the innate attraction through learning, as was found by Jhumur et al. (2006). It is also possible that the various component ingredients in the vanilla extract may have attracted mosquitoes as well. A potential solution to this

issue could be to collect data with the pure compound vanillin so as to reduce the potential confounding effects of an odor blend.

One possible explanation for the significantly lower odds in the positive response of female mosquitoes may be due to inherent life history differences between male and female mosquitoes. Females rely heavily on olfactory cues when searching for hosts and oviposition sites in addition to the sugar-seeking behaviors they share in common with males (Clements 1999). Therefore, one would expect that they would be more sensitive to a wider range of odors than males and may have higher thresholds for the development of habituation in response to prolonged odor exposure. The closed glass vial may not provide as much air circulation as might be required to evacuate the odors adequately and thereby, prevent habituation in females, thus resulting in fewer positive responses in females compared to males. Tomberlin et al (2006) found that more male mosquitoes responded positively to a vanilla target odor after training to strawberry extract which may suggest some innate attraction to vanilla by male *Cx. quinquefasciatus*. It is also possible that the sensitivity of the females may be so high that small differences in the concentration of the odors may not replicate those associated with the sugar meal at the time of training. Nevertheless, there is evidence that female *Cx. quinquefasciatus* learn odors in association with a sugar meal; but, future studies should examine the possibility that females may have inherent learning differences as compared to males.

The component of the experiment on sucrose concentration yielded results contrary to the hypothesis. It was predicted that there would be higher odds and hence

higher predicted probabilities of positive response by mosquitoes to odors linked to high sucrose concentration which might induce a more consistent positive response to the target odors and hence, better training. Sucrose concentration was not a significant predictor of positive response in the binary logistic regression model. The main factor responsible for this was the lack of difference in odds between the 5% sucrose concentration and either of the other higher concentrations. Female mosquitoes were more difficult to condition to this concentration of sucrose and often refused the pipette with solution after one or two trials resulting in lower sample size for this category (Table 2.1 and Fig. 2.3). It may be that this low sucrose concentration was not readily accepted by females; however, this concentration is often used in colony maintenance (e.g., Awono-Ambene et al. 2001, Nasci et al. 2001, Fradin and Day 2002, Kim et al. 2003). The colony used in this experiment was maintained on a 10% sucrose concentration and was exposed to this concentration prior to conditioning which might suggest that this exposure could have affected their responsiveness to the 5% sucrose solution and may have affected their ability to be trained to the 10% sucrose solution by depressing positive responses to conditioning (US pre-exposure effect; Randich & LoLordo, 1979). A lower mean predicted probability for 10% sucrose concentration was observed (Fig. 2.5), and these data suggest that the significant difference observed between the mean predicted probabilities of 10% and 50% sucrose concentrations may not be meaningful.

A possible approach to overcome an effect of previous exposure to a stimulus cue might be to conduct experiments prior to exposure to sucrose by newly-eclosed

mosquitoes. Newly-eclosed naïve wild-type mosquitoes, 24-48 hours old, had a 60% positive response to target odors of vanilla and myrcene (20% positive response to non-target odor) when trained with a 10% sucrose solution in a pilot study (Sanford et al. unpublished). Thus, the ability to learn odors in adult mosquitoes is developed as early as the timing of the first sugar meal obtained and might be a more precise methodology for future study on sucrose concentration. Future experiments should examine the potential for an US pre-exposure effect by conditioning mosquitoes without prior exposure to the 10% sucrose solution.

Another possible explanation for the lack of an effect due to sucrose concentration may be that mosquitoes do not sample multiple habitats but take sugar from a source equivalent to or of a higher quality than what they had encountered previously. Cnaani et al. (2006) found that bumble bees, *Bombus impatiens*, (Cresson) would make repeated foraging flights from nectar sources that contained a high sucrose concentration over a low concentration. They suggested that this behavior may be because sampling based on sucrose concentration may be a quick and reliable indicator of resource quality. Mosquitoes are also much smaller animals with a much higher surface to volume ratio and presumably make foraging bouts that are separated by longer periods of time. Thus, resource sampling may not be a strategy that is utilized by mosquitoes and rapid engorgement occurs at any available sugar source that will fulfill necessary energy and water requirements. This explanation illustrates some of the difficulty in comparing learning properties across taxa. It may be that sucrose concentration on the first foraging effort creates a threshold for discrimination or pre-

conditions the mosquito to sucrose concentration in future foraging bouts. If this occurs, it may a part of the mechanism that keeps local populations returning to a "home range", and it may be a means to exploit this behavioral characteristic and deploy certain chemicals or agents near emergence sites if one knows they will return to collect sucrose from the same source repeatedly. Further research into the potential for sucrose discrimination thresholds and learning are warranted.

From a purely methodological perspective, learning occurred with all three concentrations of sucrose, which gave cause not to reject the hypothesis that a high concentration of sucrose would result in higher probability of positive response or better training. This result is similar to that obtained by Wäckers et al. (2006) for the solitary parasitoid *Microplitis croceipes* (Cresson) in which they found there was no difference in learning between the solution concentrations of 0.25 M or 1 M sucrose. They found that the higher sucrose concentration was a better feeding stimulant but did not result in higher learning rates despite what has been observed in the bumble bee, *Bombus terretris* L. (Laloi et al. 1999) and the honey bee, *Apis mellifera* L. (Bitterman et al. 1983). The data presented here suggest that a better outcome might be obtained in a study that utilizes a sucrose concentration higher than the one at which a colony is maintained, as evidenced by more mosquitoes accepting the conditioning protocol at higher sucrose concentrations. This does not mean that the mosquitoes were more likely to respond positively, however, as was evidenced by the lack of significant differences observed among sucrose concentrations; but, a higher sample size will allow for more powerful statistical analyses.

This study provides for several experimental and analytical improvements to the methods used for studying appetitive olfactory associative learning in mosquitoes. It also lays the foundation for future experiments examining basic classical conditioning concepts developed for vertebrate systems on mosquitoes and other invertebrates. Significant evidence was not found to indicate that sucrose concentration affected mosquito response to target stimuli, which is similar to the results found in *M. croceipes* (Wackers et al. 2006) but not those for honeybees (Bitterman et al. 1983) or bumblebees (Laloi et al. 1999). We were also able to confirm the results of Tomberlin et al. (2006) by showing that both male and female mosquitoes learn odors associated with a sugar source despite potential complications in the data collection methods. Further refinement of the methods developed for associative learning in mosquitoes can only enhance the understanding of insect learning and allow for comparison of results across studies and hence, across taxa.

Associative learning in mosquitoes may be a means to explain some of the behavioral characteristics observed in the field, such as the evidence for "home ranges" or shifts in host preference in certain local populations. Information about associative learning could be used in the modification and enhancement of models that predict disease outbreaks to more precisely apply control and prevention measures. The study of associative learning in mosquitoes as it relates to carbohydrate seeking behavior could lead to the development of baiting technologies that might be used to introduce chemical or genetic controls into mosquito populations.

CHAPTER III

EFFECT OF PRIOR 10% SUCROSE EXPOSURE ON SUCROSE CONCENTRATION PREFERENCE IN *Culex quinquefasciatus* SAY

Introduction

Food-rewarded learning is among the most common types of associative learning studies performed, perhaps largely due to the extensive literature available on honey bee (*Apis mellifera* L.; Hymenoptera: Apidae) learning (Bitterman et al. 1983, Menzel and Muller 1996, Giurfa 2007). Among the many factors that are important to insect learning, the quality of the unconditioned stimulus may have a significant effect on what is learned. The concentration of sucrose appears to be an important factor in learning of colored targets in the honey bee, where it was found that a 50% sucrose concentration target was approached faster and departed from after a longer period of time when compared to a 20% sucrose concentration target (Loo and Bitterman 1992). In learning experiments with the bumble bee (*Bombus impatiens* (Cresson) Hymenoptera: Apidae), Cnaani et al. (2006) found that, when offered artificial flowers filled with 40% or 13% sucrose concentrations, bees sampled both and chose to return to the higher concentration flowers. Pankiw et al. (2001) found that exposure to different sucrose concentrations changed the apparent perception of sucrose concentration by altering the threshold for response in the honey bee. This study also illustrated how important the

quality of the unconditioned stimulus and specifically, sugar concentration might be in the experience of the individual animal.

Little is known about the perception and foraging behavior of mosquitoes with respect to sugar-feeding. It is a required life history requirement for almost all mosquito species and for both sexes (Foster 1995); yet, very little is known about how mosquitoes forage for sugar, i.e., whether it is based on quality or volume or if it is really merely opportunistic. There are a examples in the literature of mosquitoes as pollinators (Knab 1907, Thein 1969, Brantjes and Leemans 1976, Gorham 1976); but, there has been little recent work in this area (Kevan et al. 1993) and no documentation of what the flowers offer the mosquito in terms of nectar resources or how they attract the mosquitoes to pollinate.

At the conclusion of the previous study, where the ability to learn in conjunction with different sucrose concentrations was examined, the question was raised as to whether the exposure of the mosquitoes to a 10% sucrose concentration before conditioning may have affected their preference or rejection of the different sucrose concentrations and thus influenced the experiment. In essence did the mosquitoes' previous experience with 10% sucrose prior to conditioning influence their preference for or acceptance of other sucrose concentrations? The study described herein was intended to address this question.

Materials and Methods

Mosquitoes

The mosquitoes used in this study were from the laboratory colony maintained at the Mosquito Research Laboratory at Texas A&M University, College Station, TX, USA which was originally obtained from the *Culex quinquefasciatus* Say colony at the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), USDA-ARS, Gainesville, FL, USA. The original source of the colony was material collected in Gainesville, FL, USA, in 1995. All rearing and experiments were carried out in a Rheem Environmental walk-in growth chamber (Ashville, NC, USA) at 25-27°C and approximately 50-70% relative humidity with a 14:10 L:D cycle.

The mosquitoes were reared at a standard density of two egg rafts per liter of deionized water in white metal enamel pans (20.5 cm width x 33.5 length x 4.5 cm depth) and fed a diet consisting of a ground Tetramin® Tropical Flakes (Tetra Holding (US) Inc., Blacksburg, VA, USA) slurry at a ratio of 3 parts ground Tetramin® to 1 part deionized water. Pupae were collected from multiple rearing pans on the second day of pupation to ensure both males and females were collected and mixed before placing 100 pupae into approximately 100 mL of deionized water in each of four small Plexiglass emergence cages (19.5 x 19.5 x 19.5cm) for each replicate of the experiment. Each emergence cage was provided with a square piece of clean flat cotton (approximately 3 x 3cm) soaked in 10% sucrose solution and placed in a plastic weighboat (VWR Hexagonal Antistatic Polystyrene Weighing Dish, 50 mL, VWR, West Chester, PA, USA). At three to five days of adult mosquito age, the sugar solution was removed from

each cage for 24 hours to prepare the mosquitoes that had emerged for the experiment. This was the same protocol used in the previous study to prepare the mosquitoes for conditioning.

Testing

The sucrose solutions used in the experiment were 5, 10 and 50% sucrose by weight created with reagent grade sucrose (Aldrich®, Sigma-Aldrich, Inc., St. Louis, MO, USA) and deionized water as in the previous study of learning and sucrose concentration (Chapter II). The solutions were tested by offering the mosquitoes the solutions in pairs on clean flat cotton squares as previously described and each solution was dyed with either red (Strawberry Red Color, Royallee Brand, Bangkok, Thailand) or green (Apple Green Color, Royallee Brand, Bangkok, Thailand) food coloring. The colors were alternated between the solutions so as to determine if there was a preference for one food coloring or the other. Food coloring has been successfully used to reintroduce bacteria and evaluate feeding in mosquitoes without affecting feeding success (Lindh et al. 2006). The concentration of dye was two drops in 5 mL of deionized water, and 2 mL of the solution was soaked into each cotton pad (Fig. 3.1). The experiment was replicated three times for each concentration, pairing of 5 versus 10% sucrose and 10 versus 50% sucrose.

A preliminary run of the study indicated that the mosquitoes preferred the red food coloring under the lighting of the growth chamber, so all experiments were conducted with the growth chamber lights turned off. The sucrose soaked cotton was

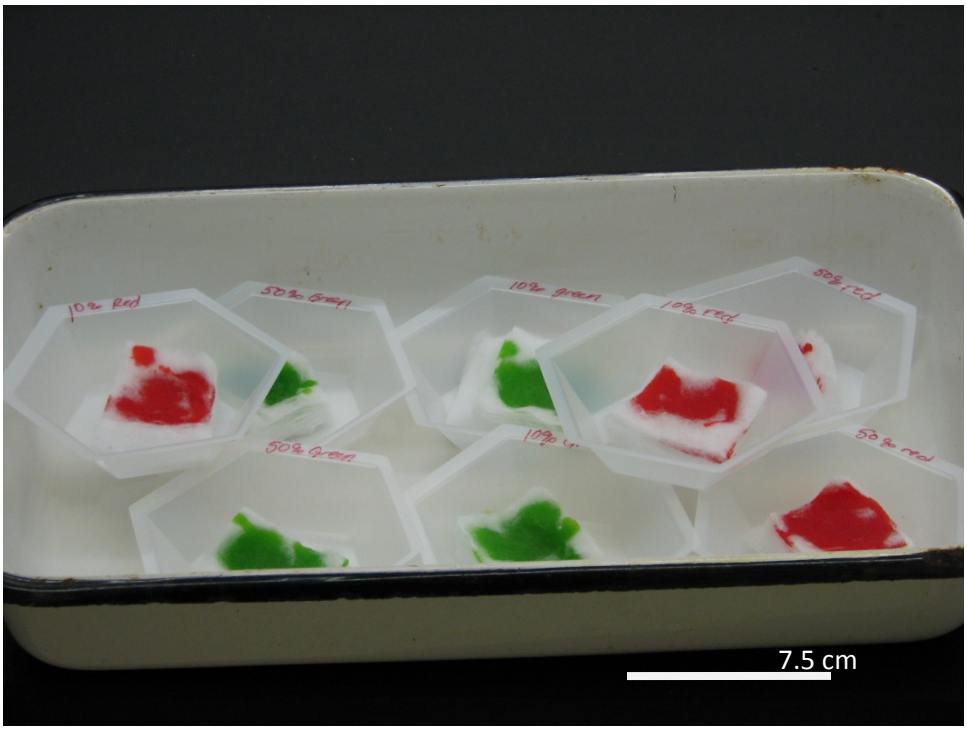

Figure 3.1. Plastic weigh dishes containing small cotton squares soaked in colored sugar solutions. The sugar solutions were paired as 5 vs. 10% or 10 vs. 50% in alternating red and green colored solutions.

placed in each cage in the dark with the aid of a red filtered flashlight for two hours during the beginning of the scotophase (18:30 – 20:30) to allow the mosquitoes to feed. Upon completion of the two hours, the cages were placed in the freezer (-20°C) overnight to kill and preserve the mosquitoes for determination of their sugar-meal choice.

The frozen mosquitoes were processed quickly to ensure that the colors could be determined easily without the need of a microscope (Fig. 3.2A-B). The number of males and females as well as the meal they chose as indicated by color, the number of non-feeding mosquitoes, and the number of mixed meals was recorded for each cage. Mixed meals were rare but when they occurred the color of the sugar-meal was brown (Fig. 3.3).

Statistics

Non-fed individuals were considered non-responders and not considered in the analysis although their numbers were recorded (Table 3.1). Mixed meals were relatively rare (Table 3.1) and were dropped from the analysis, which allowed for parametric analysis. The data were analyzed separately for each concentration pair experiment (5 vs. 10% or 10 vs. 50%) with a full factorial analysis of variance (ANOVA) using the general linear model (GLM) procedure in SPSS 16.0 (SPSS 2007). The factors of interest were the sex of the mosquito, the concentration of the sucrose solution, the color of the solution, and the interactions between each of these factors.

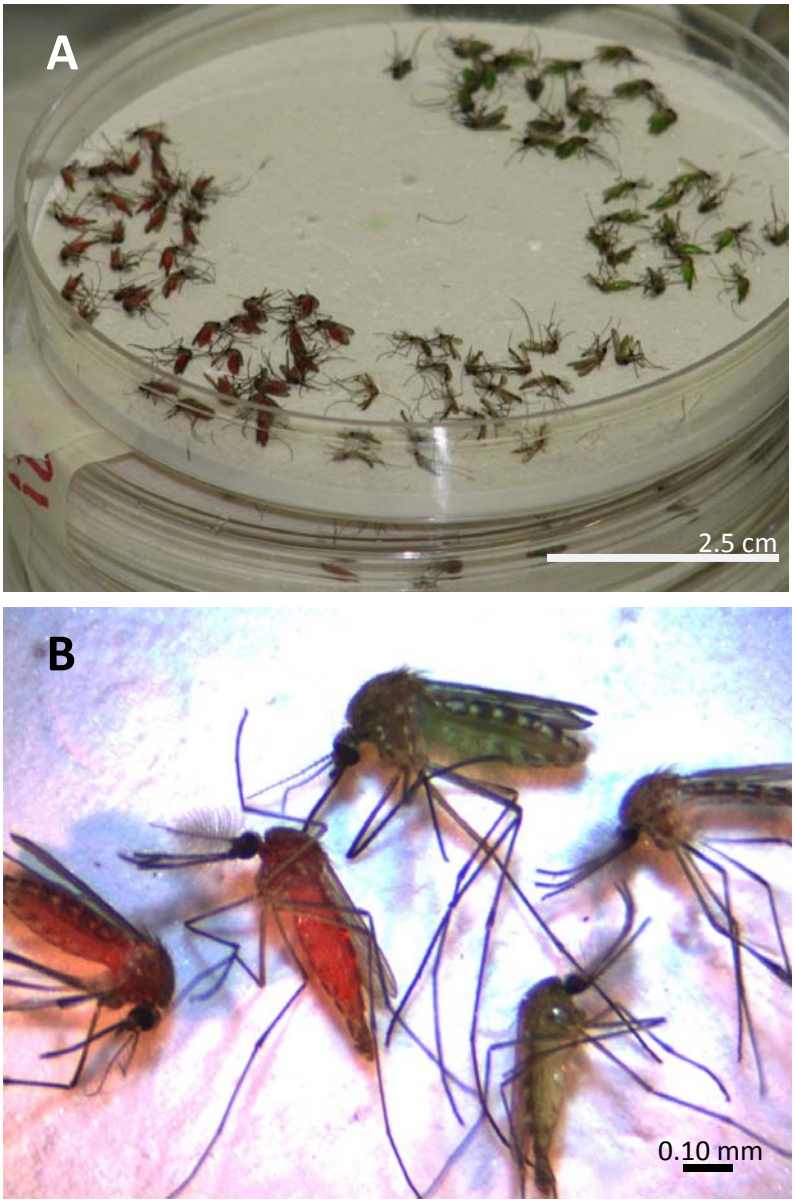

Figure 3.2. Mosquitoes were separated and counted as to which sugar concentration they chose based on dye color. This was most easily accomplished by processing the mosquitoes quickly after removing them from the freezer (A). Panel B illustrates how well the color could be seen in the mosquito gut (image taken with Meiji Techno model EMZ-8TR microscope (Meiji Techno America, Santa Clara, CA, USA) fitted with an Infinity 1-3C digital camera (Lumenera Corporation, Ontario, Canada) at 7X magnification).

Figure 3.3. A female mosquito with a mixed meal (both red and green colored solutions) appearing brown in the gut (image taken with Meiji Techno model EMZ-8TR microscope (Meiji Techno America, Santa Clara, CA, USA) fitted with an Infinity 1-3C digital camera (Lumenera Corporation, Ontario, Canada) at 7X magnification).

Table 3.1: The percentage of total mosquitoes selecting the 5, 10, or 50% sucrose concentrations, mixed meals and non-feeding individuals by sex and color during an experiment on sucrose concentration preference following exposure to 10% sucrose. For the 5 vs. 10% pairing experiment N = 1083 with a ratio of males to females = 610:530 and for the 10% vs. 50% pairing experiment N = 1140 with male to female ratio = 602:481.

Pairing	Sex	Color	Concentration	%
5 vs. 10%	Female	Red	5%	4.6
	Male	Red	5%	8.2
	Female	Green	5%	3.9
	Male	Green	5%	7.5
	Female	Red	10%	13.5
	Male	Red	10%	9.6
	Female	Green	10%	9.6
	Male	Green	10%	10.4
	Female	Mixed	N/A	4.6
	Male	Mixed	N/A	1.1
	Female	None	N/A	6.7
	Male	None	N/A	16.8
10 vs. 50%	Female	Red	10%	9.5
	Male	Red	10%	10.2
	Female	Green	10%	8.6
	Male	Green	10%	11.3
	Female	Red	50%	9.6
	Male	Red	50%	10.6
	Female	Green	50%	11.7
	Male	Green	50%	9.3
	Female	Mixed	N/A	2.0
	Male	Mixed	N/A	0.5
	Female	None	N/A	3.0
	Male	None	N/A	13.8

Results

Table 3.1 displays the percentage of total mosquitoes selecting each of the color – concentration combinations by sex for each of the pairing experiments. It shows that a higher percentage of males did not feed as opposed to females. In the 5 vs. 10% pairing experiment, there was a higher percentage of males and females with mixed meals compared to the 10 vs. 50% concentration experiment. The full factorial ANOVA did not indicate any significant effects in the comparison between the 10 and 50% sucrose concentrations for any of the factors of interest (Table 3.2). Figure 3.4A illustrates the mean (+/- SE) number of males and females with either red or green sugar-meals by concentration for the 10 versus 50% comparison. There is no obvious trend in the data, males and females chose both solutions and colors in approximately equivalent amounts (Fig. 3.4A).

Table 3.2: Full factorial ANOVA results for the factors of interest in the comparison of mosquito sucrose concentration choice between 10% and 50% sucrose using dyed solutions following access to 10% sucrose. The model included the color of the sucrose solution, concentration of the solution, sex of the mosquito, and the interactions among these factors.

Factor	**d.f.**	**F Statistic**	***P*-Value**
Color	1	0.072	0.790
Concentration	1	0.214	0.646
Sex	1	0.262	0.612
Color x Concentration	1	0.029	0.865
Color x Sex	1	0.133	0.717
Concentration x Sex	1	1.543	0.221
Color x Concentration x Sex	1	2.065	0.159

* indicates significance observed at the $\alpha = 0.05$ level.

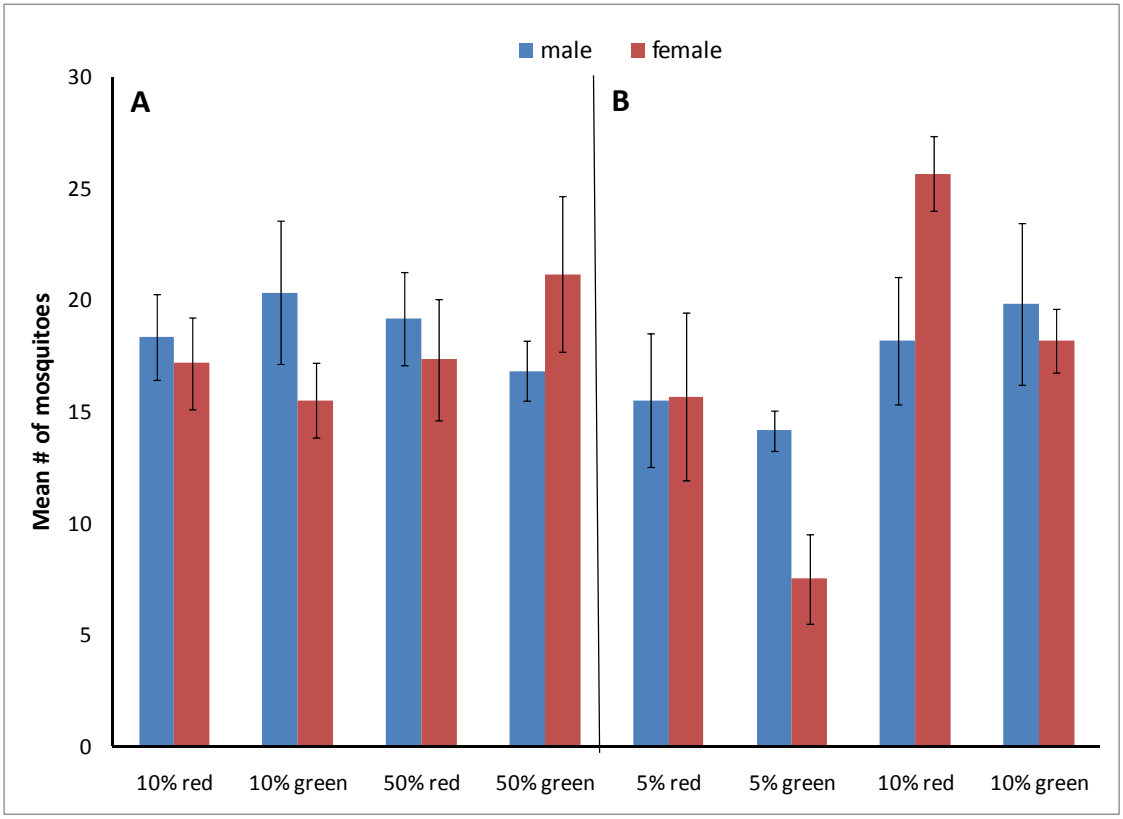

Figure 3.4. Mean (+/- SE) number of male (blue) and female (red) *Culex quinquefasciatus* mosquitoes selecting 10% or 50% (A) and 5% or 10% sucrose solutions (B).

Table 3.3: Full factorial ANOVA results for the factors of interest in the comparison of mosquito sucrose concentration choice between 5% and 10% sucrose using dyed solutions following access to 10% sucrose. The model included the color of the sucrose solution, concentration of the solution, sex of the mosquito, and the interactions among these factors. * indicates significance observed at the $\alpha = 0.05$ level.

Factor	d.f.	F Statistic	*P*-Value
Color	1	4.351	0.043*
Concentration	1	15.564	<0.001*
Sex	1	0.008	0.928
Color x Concentration	1	0.249	0.621
Color x Sex	1	4.738	0035*
Concentration x Sex	1	2.815	0.101
Color x Concentration x Sex	1	0.101	0.753

Results of the full factorial ANOVA for the comparison between 5 and 10% sucrose solutions indicated a significant difference for the factors of color, concentration and the interaction between concentration and sex (Table 3.3). Overall, significantly more males and females chose the higher 10% sucrose solution regardless of color (Fig. 3.4B). From Figure 3.4B, it can also be seen that significantly fewer females chose the green solution when compared with the number choosing red within each concentration strength. There does not appear to be a difference in the mean number of males selecting green or red within each sucrose concentration (Fig. 3.4B). The indication of a significant interaction between sucrose concentration and sex appears to be within the response of females. Significantly more females chose 10% sucrose regardless of color and this effect does not appear to be as pronounced in males (Fig. 3.4B).

Discussion

The results observed for male and female *Cx. quinquefasciatus* sucrose preference for 5%, 10% or 50% sucrose after exposure to 10% sucrose mirror those obtained for learning in conjunction with these same sucrose concentrations. In the previous study there was no difference in learning found between 10% and 50% sucrose concentrations, while a significant difference was found in learning between the 5% and 10% sucrose concentrations (Chapter II). This was contrary to the hypothesis that higher sucrose concentrations would lead to higher probability of response and also lead to higher levels of conditioned responses. In addition, it was found that females were very difficult to condition to 5% sucrose, and the data in this experiment support that

observation as well. It appears that exposure to 10% sucrose may have an effect on sucrose preference or acceptance in *Cx. quinquefasciatus*.

The difference in response to sucrose solutions higher and lower than those experienced during colony maintenance suggest that experience may affect mosquito preference or acceptance of variable sucrose concentrations. The data also suggest that perhaps more sampling is occurring at the lower sucrose concentrations. Though mixed meals were rare in the experiment, overall, the percentage of mixed meals in the 5 versus 10% sucrose paring was approximately double that of the 10 versus 50% pairing experiment (Table 3.1). Sampling resources makes sense from an ecological perspective, if an individual has some knowledge about the habitat and knows that higher quality resources exist. Since so little is known about how mosquitoes forage for sugar in the field it is assumed that they are opportunistic (Yuval 1992).

Pankiw et al. (2001) found that honey bees restricted to feeding on low sucrose concentration solutions had a lower sucrose response threshold than those fed higher sucrose concentrations (concentrations: 10%, 30% and 50% sucrose). It was suggested that experience affected the bee's sucrose response threshold. In the current study the mosquitoes were found to have an equivalent response to a concentration higher than what they had experienced prior and a lower response to a concentration lower than the one they had previously experienced. There is an inherent difficulty in comparing bees to mosquitoes, because bees clearly sample multiple habitats and must remember their location and quality to report back to the hive and express during the waggle dance. As far as is known, mosquitoes do not communicate with each other as to location of

resources in such a direct manner. However, it could be suggested that the mosquitoes had a similar response and were able to sample and distinguish between the low 5% concentration and the higher 10% concentration sucrose within the confines of the test cage. In a small scale test of the mosquitoes' lower limit of sucrose concentration detection of sucrose, it was found that the limit of detection for this species following 10% sucrose concentration is somewhere between 4.3% and 8.6% sucrose (Sanford unpublished data). Thus, the 5% sucrose solution is potentially near the lower end of their detection limit. Further study of the lower level of sucrose response and how it may change with exposure to different sucrose concentrations would aid in determining if perception changes or merely acceptance.

One hypothesis presented in the previous study to account for the lower levels of conditioning achieved with the 5% sucrose concentration was the unconditioned stimulus pre-exposure effect. This effect is described as a reduction in response to the conditioned stimulus if the subjects have been exposed to the unconditioned stimulus before pairing (Randich and LoLordo 1979). The results of this study do not support this hypothesis and suggest a simpler explanation. Either experience with 10% sucrose prior to conditioning altered the mosquitoes' perception of sucrose concentration or it alters their preference and acceptance of different sucrose concentrations. It is possible that exposure to other sucrose concentrations could have a similar effect, and it would be interesting to observe the effect of exposure to 5% sucrose on later preference.

Though the data collected in this experiment may appear to answer questions raised in the previous experiment, there is one piece to the puzzle that remains before the

assumption can be made that experience may alter sucrose preference or acceptance. The one major component that is still needed is the response of the naïve mosquitoes to the different sucrose concentrations. This was not conducted as part of this study and would have to be done as the first sugar-meal the mosquitoes experience. These data would have to be collected before one can confidently suggest that sucrose experience alters perception because the results observed may be the result of an innate preference for different sucrose concentrations. This was not evaluated by the current experimental design.

CHAPTER IV

THE IMPACT OF A SUCESSFUL FIRST BLOOD-MEAL ON FEMALE HOST CHOICE AT THE SECOND BLOOD-MEAL

Introduction

The ability of a female mosquito to be a successful vector depends on her ability to secure more than one blood-meal in her lifetime. Mosquito-borne pathogens require this time in the mosquito to either replicate and disseminate into the salivary glands for reintroduction into a host as in most viruses (Chamberlain and Sudia 1961) or time to complete development, replicate and disseminate as in the case of pathogens such as malaria (Beier 1998). This time required for this to occur in the mosquito is referred to as the extrinisic incubation period and is different for each pathogenic organism. The vector capacity model (Garret-Jones 1964) was developed for the prediction of malaria transmission and has since been applied to the study of other mosquito-borne pathogens This model depends on the assumption that mosquitoes must take more than one blood-meal to be an effective vector. Recently McCall and Kelly (2002) suggested that because learning seems to be a quality found among many insect groups, it would be expected that mosquitoes be capable of this ability as well. They further suggested that, if mosquitoes are capable of learning about their hosts then this may change our assumptions about their behavior and hence, our models of vector capacity and our understanding of vector-borne disease transmission in general.

Within the Culicidae we find many examples of vector-host associations that often imply that there is a tight association between the vector mosquito and what it will accept for a host (e.g., Tempelis 1975). Some species might be considered host specialists, for example *Uranotaenia lateralis* Ludlow appears to feed exclusively on mudskippers (Gobiidae) in Malaysia (Tempelis 1970); but, these associations show up in the literature rarely due to a lack of studies. The best most well studied mosquito- host associations are those affiliated with major disease vectors. The *Culex pipiens* complex, which vectors both viruses and nematodes in its worldwide distribution, is one of the most well known (Foster and Walker 2002) . Though it is largely considered an avian feeding group, there are numerous examples in the literature showing the *Cx. pipiens* complex to be quite plastic in host feeding preference depending on location. In this regard members of this complex can be found switching between avian and mammalian hosts (Edman 1974, Tempelis 1975, Apperson et al. 2002, Molaei et al. 2006, Savage et al. 2007); and thus, they might be considered more generalist in host preference when this occurs. *Culex quinquefasciatus* Say is a member of this complex that has apparent host preference plasticity (Elizando-Quiroga et al. 2006, Molaei et al. 2007).

Learning about hosts by a generalist feeder could reduce search time and fine tune responses in a changing environment where host availability can shift over time (McCall and Kelly 2002). There are several examples in the literature on *Anopheles* spp. which suggest memory of a "home range" that is presumed to be associated with the odors of the hosts in a certain location (e.g., *An. funestus* Giles; (Ribbands 1949); *An. farauti* Laveran; (Charlwood et al. 1988); *An. arabiensis* Patton; (McCall et al. 2001)).

In *An. balabacencis* Baisas, Hii et al. (1991) found that a higher percentage of mosquitoes that had taken blood from either a buffalo or a human returned to the same host-type in a mark-release-recapture study. Mwandawiro et al. (2000) used a similar method and found that more *Culex* spp. mosquitoes that had successfully fed on either a pig or a cow returned to the same host-type at the next opportunity. They were able to carry this study a step further and captured the returning mosquitoes, reared the subsequent eggs, and tested the innate preference of these individuals. The next generation lacked the preference exhibited by the parental generation; and this strongly supported that conditioning had occurred. In more artificial laboratory settings, Tomberlin et al. (2006) found that female *Cx. quinquefasciatus* could be conditioned to a novel odor in association with a blood-meal. They used a small scale conditioning method that was limited to very short range attraction and perhaps, not the level of attraction that might be more indicative of the behavioral sequence that must occur for a mosquito to fly a distance to return to a particular host-associated odor in the field.

Given the ability of *Cx. quinquefasciatus* to exhibit host shifts from birds to mammals, the question asked in this study was whether this was dependent or potentially susceptible to manipulation based on the first blood-meal experience of a female; i.e. a female takes a successful blood-meal from a chicken or a human, will she seek out the odors associated with that host at the next host-seeking event? Based on the literature suggesting host experience impacts subsequent host seeking behavior it was predicted that females would choose odors associated with the host they had first encountered.

Materials and Methods

Mosquitoes

The *Culex quinquefasciatus* colony used in the study was established from material obtained from the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), USDA-ARS, Gainesville, FL, USA, which had been in culture at the Mosquito Research Laboratory at Texas A&M University, College Station, TX, USA for approximately 13 generations. The original source of the mosquitoes was material collected in Gainesville, FL, USA, in 1995. Mosquitoes used in the study were from the F_{13} (run one) and F_{14} (run two) generations. All mosquito rearing and experimentation was conducted in a Rheem Environmental walk-in growth chamber (Ashville, NC, USA) at 25-27°C and approximately 70% relative humidity with a 14:10 L:D cycle.

Larvae were reared at a standard density consisting of two egg rafts per liter of deionized water in enamel pans (20.5 cm width x 33.5 length x 4.5 cm depth). Pupae were collected on the second day of pupation (to ensure both males and females were collected) and mixed from eight different pans (from sixteen different females). Pupae were isolated into eight different adult emergence cages during each run of the experiment. Each emergence cage contained 100 pupae per cage (assuming a 50:50 sex ratio). A 10% sucrose-water-soaked cotton pad was provided for the emerging adult mosquitoes.

Due to the potential for confounding effects associated with the mosquito rearing person also being the source of the human blood-meal and associated odors, plastic cages were used to minimize airflow through the cage and potential odor exposure. Two

types of cages were used including the Horsfall small colony cage (Figure 4.1A) which is a small plexiglass cage (19.5 x 19.5 x 19.5cm) with one vent area of five – 1.5cm diameter circles arranged in a 6.5cm circle in the back of the cage (used during the first run of the experiment) and modified plastic food containers (Figure 4.1B; 1890 mL, HEB, San Antonio, TX, USA) which had a 13.5 x 7.5 cm hole cut out of the lid for venting and a 9.5 x 8 cm access hole in one side (used during the second run of the experiment). The access holes were covered in a clean sleeve of stockinette. All the cages were cleaned with Liqui-Nox® Critical-Cleaning detergent (Alconox, Inc., White Plains, NY, USA) and handled with gloved hands after cleaning and during the experiment. The cages were all also modified by creating a sachet of activated charcoal (Aqua-Tech, Marineland Aquarium Products, Moorpark, CA, USA) sandwiched by a layer of cheesecloth (Cheesecloth Wipes™, VWR, West Chester, PA, USA) and taped over the opening of the cage (over the vent window and the access port with associated sleeve). This was done in an attempt to minimize human odor exposure and to reduce airflow.

Blood-feeding

At 5 d post-emergence the cages of mosquitoes were randomly assigned to receive either a chicken blood-meal or a human blood-meal. This was accomplished by mechanically aspirating all of the mosquitoes from the cage and placing them into clean mosquito feeding cages (Fig.4.2) that were then either taped to the chicken restrained in a new black knee-high stocking (Americal Corporation, Henderson, NC, USA) as per the

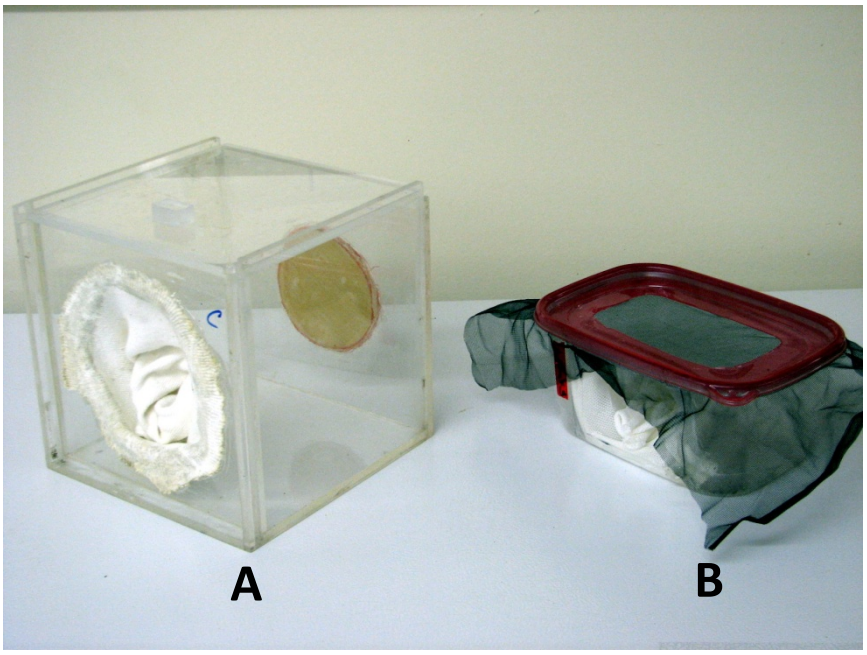

Figure 4.1. Image of the Horsfall small colony cage (A) and the plastic food container cage (B) used to hold *Culex quinquefasciatus* adults before and after blood-feeding on either the human or chicken host.

Figure 4.2. Image of the mosquito blood-feeding cage. The mosquitoes are introduced through the stoppered hole and mosquitoes can feed through the screen. When feeding is complete the metal bottom slides off to release the mosquitoes.

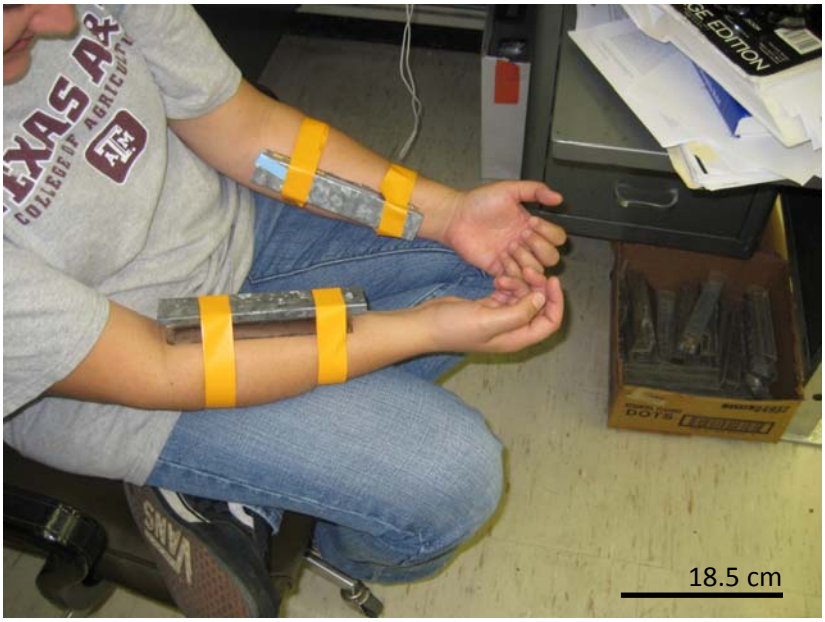

Figure 4.3. An image of the human volunteer during the blood-feeding process. During run one of the experiment the mosquitoes were taped to the arms for a total of one hour. For run two the mosquitoes were taped to the lower legs to allow the arms to heal.

Animal Use Protocol (TAMU AUP # 2007-162) or taped to the human volunteer (Fig. 4.3; the TAMU Institutional Review Board was consulted but a permit was not deemed necessary) for one hour. Due the short duration of time (10 d) between run one and run two, the first group of mosquitoes was fed on the human's arms and the second group on the human's lower legs. Mosquitoes fed in these two areas were kept separate and associated with odor collection at these sites specifically throughout the experiment. The one hour feeding time was selected based on the total amount of time allowed by the AUP; and it became apparent that the mosquitoes completed feeding well before conclusion of the time allotted (personal observation), though they continued to probe the skin for the remaining time. At the completion of the feeding period the mosquitoes were reintroduced to their respective cages by dismantling the feeding cage inside the larger colony cage.

At 5-6 d post-blood-feeding, deionized water-filled petri dishes were placed in each adult holding cage to collect egg rafts. The number of egg rafts was noted to assess how many females took successful blood-meals.

Olfactometer

To evaluate the choice of female mosquitoes at the second blood-meal opportunity, a dual-choice olfactometer was used. The olfactometer was composed of three stacked sheets of solid Teflon (each approximately 2.7 cm thick) covered by a removable sheet of glass. The overall dimensions of the olfactometer are displayed in Figure 4.4. The intake ports at the end of each arm of the olfactometer measured approximately 1.3 cm each, and the outflow port measured approximately 1.7 cm in

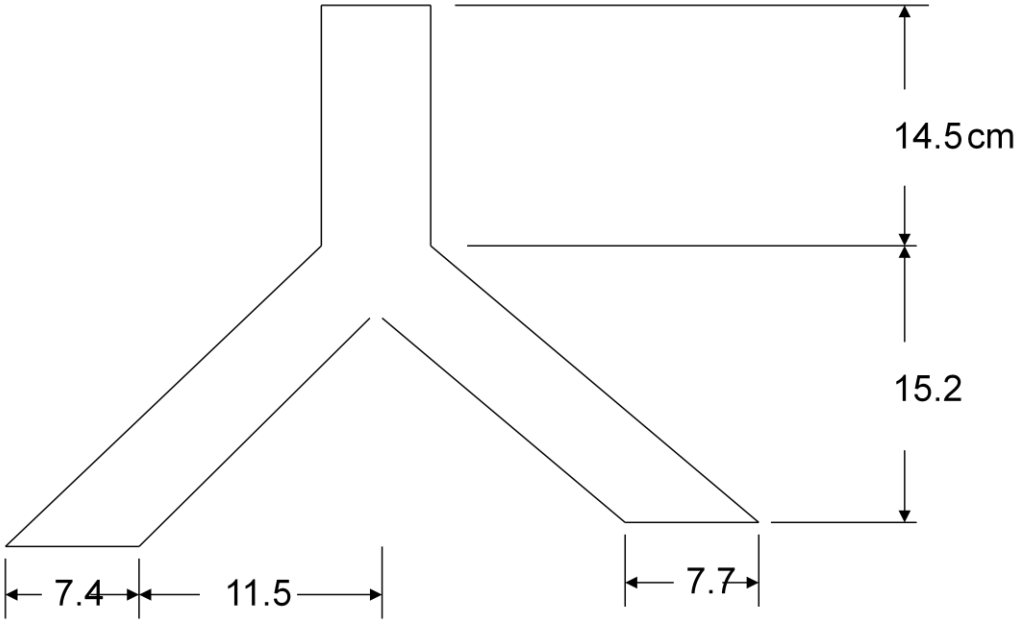

Figure 4.4. A schematic drawing of the dual-choice olfactometer indicating the length of each portion of the apparatus in centimeters. Schematic drawing by Glen Rains.

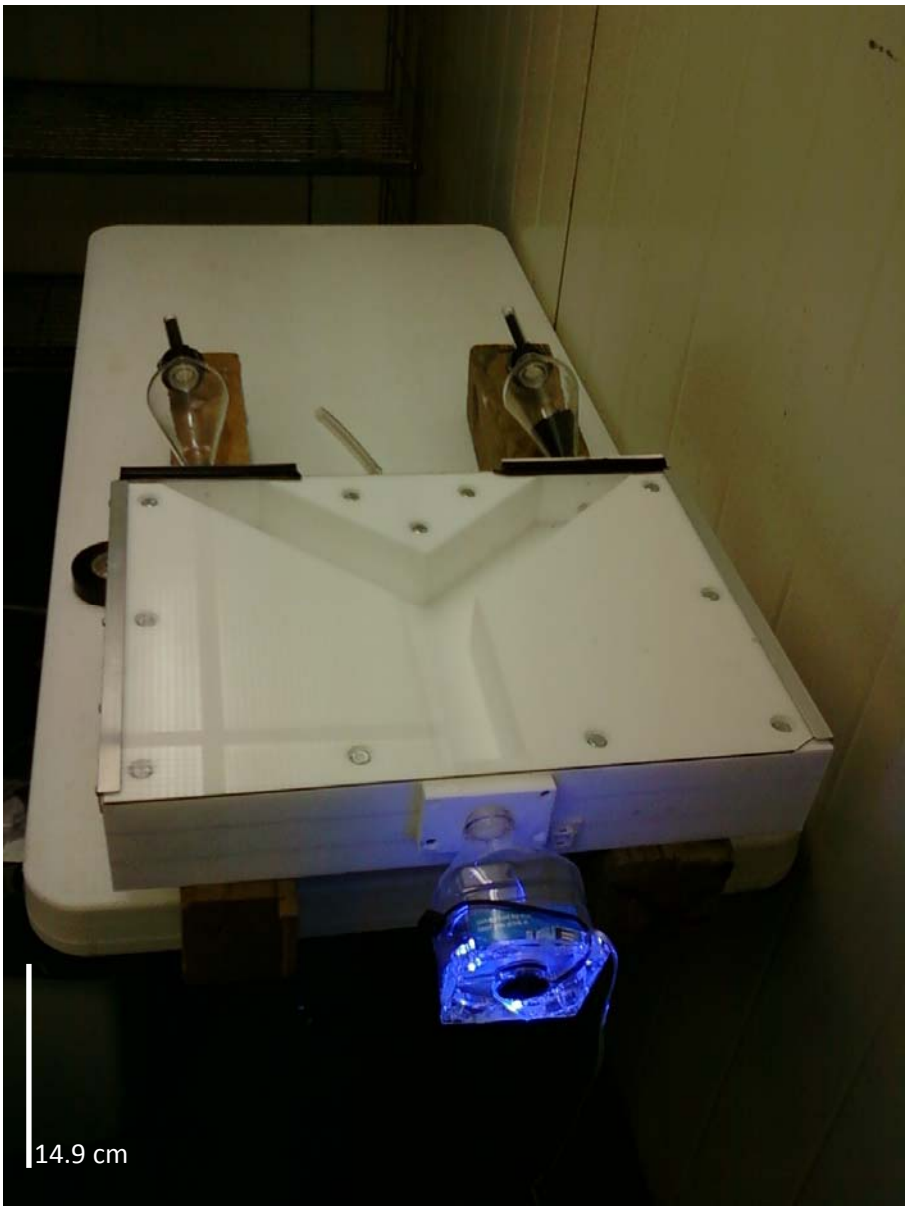

Figure 4.5. An image of the complete dual-choice olfactometer setup for this experiment. Air was pulled through the computer cooling fan mounted in the water bottle top. Odor sources were held n the two 250-mL glass separator funnels which were fitted with activated charcoal to filter the air before it entered the olfactometer. More details of the airflow and dimensions are provided in the text. The blue LED's were disabled once the experiment started as it was found that the mosquitoes were flying toward them.

diameter. There was an access port cut in the bottom of the olfactometer neck that was approximately 1.7 cm in diameter to allow for introduction of insects into the apparatus. Airflow was pulled through the olfactometer with an 80 mm three speed computer case cooling fan (Blue LED Tricool, Antec, Inc., Fremont, CA, USA) glued into the top 13 cm of a 1.5 liter square water bottle (FIJI Water, Los Angeles, CA, USA) and powered with a variable voltage AC adapter (3-12 V DC 1000mAmp AC Adapter, Enercell, RadioShack Corporation, Fort Worth, TX, USA). The blue LED's on the cooling fan were disabled in the preliminary portion of the experiment because it appeared that the mosquitoes were attracted to them. The olfactometer experiments were also run within the walk-in growth chamber so humidity was on average 70%, and air was filtered with activated charcoal columns consisting of a 1.1 cm inner diameter glass tube filled with activated charcoal (Aqua-Tech, Marineland Aquarium Products, Moorpark, CA, USA) and plugged with clean cotton. The columns were attached via new rubber funnel adaptors to 250 mL glass separator funnels (Kimax, Kimble Chase Life Science and Research Products, LLC, Vineland, NJ, USA) which were used to hold the odor source materials for the experiment. The separator funnels were selected because they could be easily washed and placed in the oven to ensure removal of odors and because they allow for a straight path of airflow through the olfactometer (Fig. 4.5). Vinyl electrical tape (Temflex^{TM}, 3M, St. Paul, MN, USA) was used occasionally to attach glass pieces and seal gaps in the apparatus and was replaced whenever odor contamination was likely to have occurred.

Airflow through the olfactometer at the access port was measured using an anemometer (Testo 435-1, Testo, Inc., Sparta, NJ, USA) to be 0.5 m/sec (based on a 90 second average). Preliminary runs were conducted with colony mosquitoes to ensure that upwind flight was elicited by this wind speed as this was the maximum achievable speed with the apparatus as it was setup. The period of maximum *Culex* spp. host-seeking activity in nature was also taken into consideration and experiments were conducted late in the mosquito photophase and during the mosquito scotophase (1730 – 0430 h). To simulate low light conditions and to reduce the potential for the mosquitoes to follow the overhead fluorescent light bulbs while in the olfactometer, a small fluorescent light fixture (18 inch (46 cm) Lithonia Lighting, Conyers, GA, USA connected to an AC power cord with a 15 watt Natural Sunshine^{TM} light bulb, Philips Lighting Company, Markham, ON, Canada) was placed at the intake end of the olfactometer during testing. This provided even lighting to both arms of the olfactometer and a gradient of light intensity as the mosquito moved from the release point to the intake port in either arm. Light measurements were taken with a FieldScout® quantum light meter (Spectrum® Technologies, Inc., Plainfield, IL, USA) and ranged from 1 – 4 μmol/m^2/sec (~74 – 296 lux) from the access port to the end of each intake arm.

Each odor sample was collected on a new black knee-high stocking (Americal Corporation, Henderson, NC, USA) which was selected as a method for collecting odors as it has been used successfully in another experiment (Mboera et al. 1998) and because it was presumed that it might collect odors specifically where the mosquitoes had fed and reduce variation caused by odors associated with different areas of the

human/chicken. Odors were collected from the chicken by collecting the stocking the chicken wore during blood-feeding and placing it immediately into the freezer (at -20°C). The human volunteer wore the stocking on either the arms or lower legs depending on where the mosquitoes had fed for one hour and these were also frozen to maintain consistency with the chicken odor samples.

Testing

On days three to four post-oviposition, the mosquitoes from each cage were placed into individual clean glass shell vials (2-dram, 40 mm height x 17 mm diameter) with a plug of clean cotton and a small amount of deionized water. Each mosquito-containing vial was marked as to which blood-meal the mosquito had received and given an individual number based on the cage they were taken from. Within each treatment group (based on blood-meal) 24 female mosquitoes were randomly-selected for inclusion into each of one of four different testing groups. These groups consisted of a control for the odor of the new stocking (new) and the airflow restriction potentially created by the presence of the stocking in the separator funnel (blank), a control for the odor of the new stocking versus the odor of the chicken, a control for the odor of the new stocking versus the odor of the human, and the test of two odors consisting of the human versus the chicken.

The olfactometer was cleaned with 80% ethanol before new odors were introduced to the olfactometer and on every third replicate when the odor sides were also alternated to reduce residual ethanol odors in the olfactometer and to make sure that bias for one side of the olfactometer was detected and corrected. Each mosquito was

observed in the olfactometer for 90 seconds (based on preliminary data), and the initial choice of the mosquito was recorded as well as where they were and how much time they spent in each area of the olfactometer. This was accomplished with the use of the freely-available behavioral analysis software JWatcher™ (Blumstein et al. 2000). Only three behavioral states were used to indicate the mosquito's presence in either the neck, arm one or arm two of the olfactometer. Once programmed into the software, the user can use the computer keyboard to keep track of the behavioral state and duration of each state. Thus, data were generated on the duration of time or residence time spent in each location of the olfactometer.

Statistics

Non-responding mosquitoes were removed from the data set prior to analysis. This was based on whether they chose to enter one of the arms of the olfactometer; if they did not, they were considered a "non-responder". Data on mosquito initial choice were analyzed using a Binomial test for a 0.50 test proportion with significance observed at the $\alpha = 0.05$ level. The JWatcher™ software provides some basic calculations such as the proportion of time spent in each observed behavioral state, and these data were moved over into SPSS 16.0 (SPSS 2007) for further analysis. Analysis of residence time proportions was initially transformed with the arcsine-square root transformation, but it failed to meet the assumption of normality for parametric analysis. A Friedman test was used to compare the proportion of time spent in the neck and the two arms of the olfactometer, and a Wilcoxson-Signed-Rank test was used to compare the proportion of time spent in each arm of the olfactometer (McDonald 2009). Significance was also

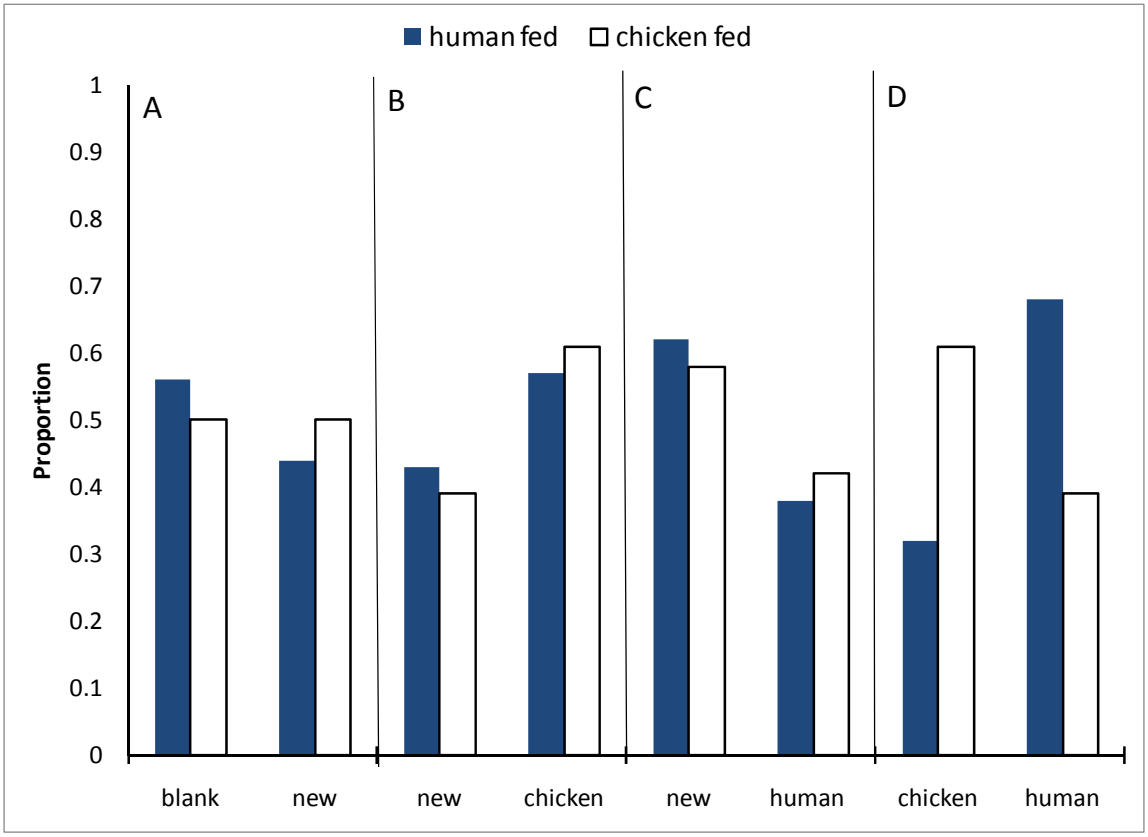

Figure 4.6. The proportion of mosquitoes selecting the different arms of the dual-choice olfactometer on their initial choice for each test group. Panel A displays the proportion of mosquitoes choosing either the blank (indicates an empty separation funnel) versus a new black stocking. Panel B displays the proportion of mosquitoes choosing either a new stocking or a stocking worn by a chicken for one hour. Panel C displays the proportion of mosquitoes choosing either a new stocking or a stocking worn by a human for one hour. And Panel D displays the proportion of mosquitoes choosing either a stocking worn by a chicken or a human for one hour. Binomial tests were conducted within each treatment group in each panel of the figure. The letters indicate which comparisons were made and the lack of significant results.

observed at the $\alpha = 0.05$ level for these tests. All tests were conducted only within each treatment group. Some data manipulations and figures were created and managed in Microsoft Excel 2007 (Microsoft, Corp. Redmond, WA, USA).

Results

Initial Choice

Figure 4.6 illustrates the proportion of mosquitoes selecting the different arms of the olfactometer for the four different tests within each treatment group. There were no significant differences observed using the Binomial test for each of the four test groups. Proportions of mosquitoes entering the blank and new stocking arms of the olfactometer were close to 50:50 (Fig. 4.6A). A slightly higher proportion of mosquitoes from both the chicken-fed and human-fed treatments chose the olfactometer arm containing chicken odor (Fig. 4.6B), while the opposite trend was observed when both treatments were offered a new stocking versus human odor (Fig. 4.6C). The most discernible trend was observed in the test of the human odor versus chicken odor in which a slightly higher proportion of mosquitoes chose the odor of the host they had previously fed upon (Fig. 4.6D).

Residence Time

Figure 4.7 illustrates the mean proportion of total time spent in each location of the olfactometer for each test group in both the human-fed and chicken-fed treatment groups. The results of the Friedman tests, that analyzed the proportion of time spent in each area of the olfactometer including the neck, did not indicate significant differences; so, the neck was dropped from further analysis. Wilcoxson-Signed-Rank tests did not

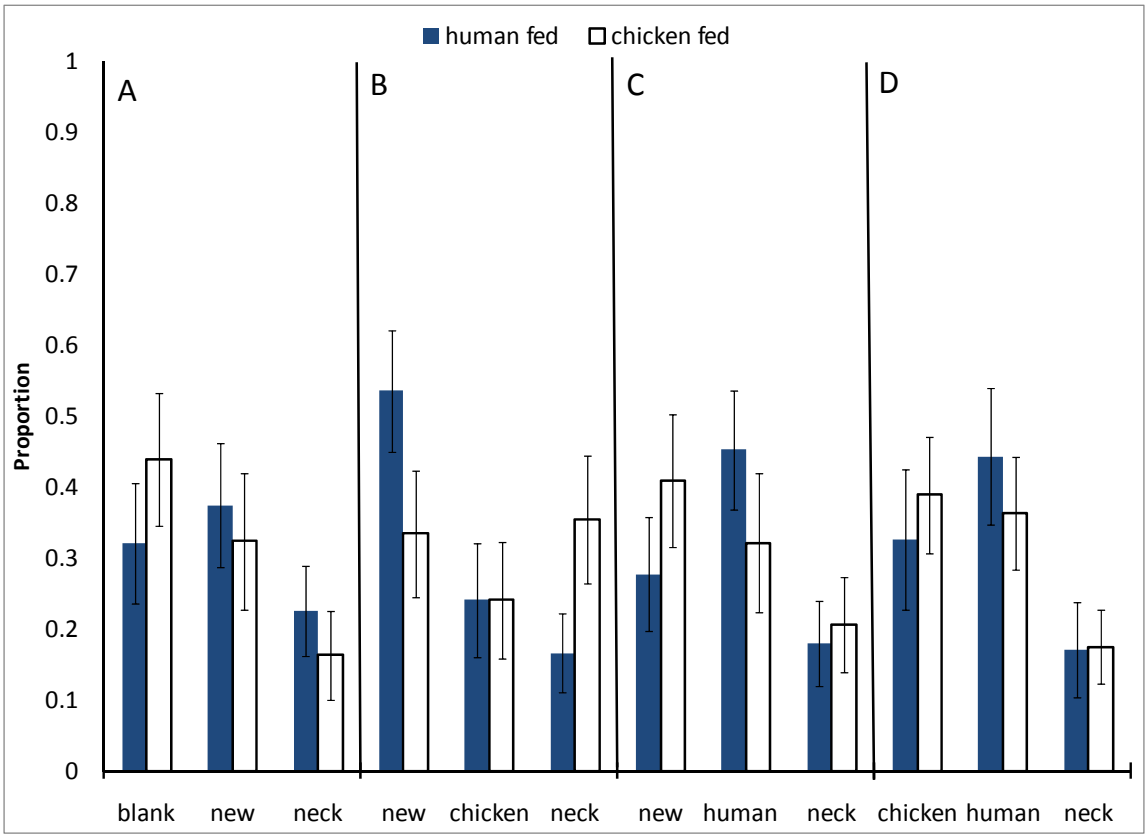

Figure 4.7. The mean proportion (+/- SE) of the total time spent in each location of the dual-choice olfactometer for each test and treatment group. Panel A displays the proportion of time mosquitoes spent in the blank (indicates an empty separation funnel) or new black stocking arms and the neck of the olfactometer. Panel B displays the proportion of time that mosquitoes spent in the new stocking or stocking worn by a chicken arms and neck of the olfactometer. Panel C displays the proportion of time that mosquitoes spent in the new stocking or stocking worn by a human arms and neck of the olfactometer. And Panel D displays the proportion of time that mosquitoes spent in the stocking worn by a chicken arm or a human arm and neck of the olfactometer. No significant differences were observed with Friedman or Wilcoxon-Signed Rank Tests.

indicate a significant difference in proportion of time spent in either of the olfactometer arms for any of the tests regardless of treatment group. The mean proportions of mosquitoes in the blank and new arms of the olfactometer were approximately equal with less time spent in the neck of the olfactometer (Fig. 4.7A). In the new stocking versus the chicken odor test, the mosquitoes from the human-fed treatment spent more time in the new arm of the olfactometer (Fig. 4.7B), which is the opposite of their initial response (Fig. 4.6B). The same trend can be observed in the human-fed treatment group offered a choice of a new stocking or human odor; more time was spent in the human odor arm of the olfactometer (Fig. 4.7C), but the initial response indicated a higher proportion chose the new stocking (Fig. 4.6C). Chicken-fed mosquitoes did not show really discernable trends in the mean proportion of time spent in each arm of the olfactometer (Fig. 4.7B-D). Contrary to the trend observed in the initial response data, the mean proportion of time spent in each arm of the olfactometer did not show a definable trend (Fig. 4.7D).

Discussion

Though it is quite tempting to suggest that the trends observed in the initial response test of chicken versus human odor suggest something about preference after exposure to host odor at the first blood-meal, there are many other reasons to suggest that more work needs to be conducted before a conclusion is drawn. The lack of significant effect observed for the tests of a new stocking to either of the other known attractive odors suggests a flaw in the experimental design. It is suggested that this is the major reason that a conclusion should not be drawn at this time.

A lack of attraction to the odor of the known attractants could be the result of inadequate sample sizes. In this study, sample sizes were targeted for 24 mosquitoes per test group; but, after dropping out the non-responders, sample sizes ranged from 18 to 23. Conducting a power analysis for a Binomial test with the ability to detect just a 10% deviation from 50% at $\alpha = 0.05$ and 80% power would require 210 samples (McDonald 2009). This would require 210 mosquitoes within each test group and 840 mosquitoes total for each treatment group. This would prove difficult for several reasons. Mortality is present at every step in the process of preparing mosquitoes for an experiment such as this; so, a very large number of mosquitoes would be required to be at the same age and state at the same time. If that requirement is met, feeding groups of 100 mosquitoes at a time on a chicken or human is difficult enough for the host; but, numbers as high as 840 would be impossible to do simultaneously. There are two ways one could increase replicate number, i.e., by increasing the number of mosquitoes fed at one time by using multiple hosts or by increasing the number of times the experiment is conducted using the same hosts. Either way this introduces new potential for variation and error.

Another potential reason for a lack of significant attraction to known host odors could be in the collection and presentation of odors. Although stockings were used in a previous experiment successfully, they were purposefully worn by the human so as to collect as much odor as possible (Mboera et al. 1998). It is possible that the odor collection times in this study were not long enough to impregnate the stockings with enough odor to attract the mosquitoes. The storage time in the freezer (also used as a storage method by Mboera et al (1998)) may have reduced the effectiveness of the

material to release odors. The human volunteer for this experiment is a person regularly sought out by mosquitoes and took efforts to use the same soaps and clothing detergent during the experiment to reduce potential variation. It is possible, however, that not enough attractive odor was impregnated onto the stockings.

In 2006, Allan et al. (2006) published a dual-choice olfactometer study using this same *Cx. quinquefasciatus* colony (a few years younger and not reared in Texas). They found that this species was attracted to a human arm and the feathers removed from a chicken at an equally attractive level, but the level of attraction was low at around 25-30%. They also determined a whole living chicken was significantly more attractive than feathers, a human arm, carbon dioxide or even a combination of feathers and carbon dioxide. This supports my study in that this species, and more specifically this colony, is attracted to both humans and chickens. However, it suggests that there is something more than just odor required for significant attraction.

Though there are major questions regarding whether the assay functioned well enough to observe conditioned behavior, there is always the possibility that, despite appropriate efforts, there is no observable conditioning. In *Drosophila*, Mery and Kawecki (2002) demonstrated that traits associated with associative learning ability can be selected for in laboratory culture in as little as 15 generations. The colony used in this study had been in culture for 14 years in Florida prior to the 13 generations it had been in culture in Texas, which is certainly sufficient time to select for traits associated with laboratory culture conditions rather than field conditions. The best way to remedy this

would be to use field-collected colonies; but, this is difficult because the pathogen status of wild mosquitoes is unknown.

Since it was not possible to reject the null hypothesis, the possibility that there is no effect must also be considered. It is possible that conditioning of host-associated odors is not possible and that females are merely opportunistic and take blood from any available host. This would explain the pattern observed in generalist blood-feeding species based solely on the available data on blood-meal identification (e.g., Elizando-Quiroga et al. 2006, Molaei et al. 2007). Specialist blood-feeding species may be restricted by genetic predetermination of host preference, as has been suggested in the highly-anthropophilic *An. gambiae* (Costanini et al. 1999). However, it can be argued that these generalizations apply at the largest scale of observable behavior and that conditioning could occur at the very local level and at the very smallest scales (e.g. within a village) and still have an impact on focal pathogen transmission dynamics.

One other point that deserves discussion is the difference observed in the data between the initial choice of the mosquito and the residence time indicated. In some instances these data indicated completely opposite trends. One might assume that, in nature, a mosquito or any other insect seeking a resource is going to approach the more attractive substance first to reduce search time and the inherent risk and energy expenditure associated with it. However, at what point does the insect give up on a resource it cannot access? In this experiment, the mosquitoes typically made a choice within the first ten seconds of introduction to the olfactometer (personal observation). There are not significant results to suggest that this was anything but random; but, it

does lead to an interesting question of how the type of data recorded and the length of data collection in a behavior experiment have the potential to alter the results.

The impact of first blood-meal host odors remains an interesting question, because the potential impact of experience shaping host preference could have significant implications for vector control. The vector capacity equation relies on the assumption that mosquitoes feed heterogeneously among hosts. However, if hosts derive preference from experience and feed on hosts with the lowest defenses (which are probably those who are infected with a disease), this could increase disease transmission (McCall and Kelly 2002). One aspect of vector-disease management that could be significantly enhanced by shaping of host preference is zooprophylaxis. Zooprophylaxis is the purposeful presentation of alternative hosts that do not support pathogen propagation or development, as in trap-crop systems in the control of agricultural pests, so, if the mosquitoes prefer to return to hosts they had previously fed on, the introduction of alternative hosts into an area would potentially increase their encounter rate and reduce disease transmission . It has been suggested for the management of the malaria vector *An. arabiensis* (WHO 1982) to introduce cattle to areas of transmission, but, this approach had met with mixed results. It has also been suggested in the management of Japanese Encephalitis by increasing the number of cattle as alternative hosts for *Cx. tritaeniorhynchus* which was supported by the research conducted by Mwandawiro et al. (2000) in Thailand. Thus, the importance of experience in shaping host preference could have significant impacts on not only how we understand disease transmission, but also on how we manage it and control vector species.

CHAPTER V

THE NON-CONSUMPTIVE EFFECTS OF *Gambusia affinis* (BAIRD & GIRARD) (CYPRINODONTIFORMES: POECILIIDAE) ON *Culex quinquefasciatus* SAY (DIPTERA: CULICIDAE) DEVELOPMENT AND ADULT OVIPOSITION DECISIONS

Introduction

Predators can have significant impacts on prey populations that are not limited to direct consumption. Non-consumptive effects (NCE), also known as trait-mediated interactions (TMI), or nonlethal effects, are those effects that a predator has on a prey species that are related to reductions in fitness due only to the mere presence of the predator (Abrams 2008). Many NCE are due to changes in prey behavior that reduce foraging success and hence, delay development, reduce adult size, and overall reproductive output (Lima and Dill 1990, Lima 1998, Abrams 2008, Preisser et al. 2009). Induction of NCE requires that prey species are readily able to detect and respond to predator cues and has been best documented in aquatic systems (Lima and Steury 2005).

Immature mosquitoes (Diptera: Culicidae) lend themselves well to studies of simple aquatic food webs, and NCE for a number of different species to both vertebrate and invertebrate predators have been documented (e.g., *Culex pipiens* L., *Aedes aegypti* (L.) and *Notonecta undulata* Say (Hemiptera: Notonectidae) (Sih 1986), *Cx. tarsalis*

Coquillet and *Gambusia affinis* (Baird and Girard) (Cyprinodontiformes: Poeciliidae) (Blaustein and Karban 1990), *Aedes triseriatus* Say and *Toxoryhnchites rutilus* (Diptera: Culicidae) (Juliano and Gravel 2002), *Ae. albopictus* (Skuse) and *Mesocyclops pehpeiensis* (Hu) (Copepoda: Cyclopoida) (Dieng et al. 2003) and *Culiseta longiareolata* (Macquart), *Cx. pipiens* and *Anax imperator* Leach (Odonata: Aeshnidae) (Stav et al. 2005)). Larval mosquitoes may reduce movement and foraging (Juliano and Gravel 2002) and seek out low risk areas of the habitat to avoid interactions with predators (Sih 1986). Mosquito response to the threat of predation appears to be both dependent upon concentration of predator cues (Kesavaraju et al. 2007) and correspondent to resource availability (Beketov and Leiss 2007). Results from studies examining predator cues on prey development have demonstrated both accelerated time to pupation, as in *Cx. tarsalis* reared in enclosures with the mosquitofish *G. affinis* (Blaustein and Karban 1990) and delayed time to pupation in *Cs. longiareolata*, but not *Cx. pipiens* reared with the cues of the dragonfly, *A. imperator* (Stav et al. 2005). A reduction in adult size congruent with the lengthened development time in *Cs. longiareolata* was not observed in *Cx. pipiens* (Stav et al. 2005).

The ability to adaptively respond to the threat of predation is not limited to immature mosquitoes. Adult female mosquito avoidance of oviposition sites containing predators or predator cues has also been documented. In both *Culiseta* spp. and *Culex* spp., the female lays her entire egg complement in a single oviposition site as an egg raft; thus, the ability to detect and avoid predators has a significant impact on the next generation (Angelon and Petranka 2002).

There is a growing amount of literature demonstrating that some mosquito species can detect the presence of predators and avoid colonization of these habitats (Petranka and Fakhoury 1991, Blaustein and Kotler 1993, Angelon and Petranka 2002, Kiflawi et al. 2003b, Eitam and Blaustein 2004, Blaustein et al. 2005). *Culiseta longiareolata* is one of the best studied species in terms of its ability to avoid both vertebrate and invertebrate predators during oviposition (Blaustein and Kotler 1993, Stav et al. 1999, Stav et al. 2000, Eitam et al. 2002, Spencer et al. 2002, Kiflawi et al. 2003a, b, Blaustein et al. 2004, Eitam and Blaustein 2004, Arav and Blaustein 2006, Silberbush and Blaustein 2008). In *Culex* spp., the ability to avoid oviposition sites with fish predators is variable. Oviposition avoidance response was recorded in *Cs. longiareolata* and the co-occurring species, *Cx. laticinctus* Edwards when presented with predator cues of *Notonecta maculata* Fabricius (Hemiptera: Notonectidae) (Kiflawi et al. 2003a), but was lacking when this *Culex* spp. was presented with predator cues of *Anisops sardea* Herrich-Schaeffer (Hemiptera: Notonectidae) (Eitam et al. 2002). *Culex tarsalis* avoided fish exudates, while *Cx. quinquefasciatus* Say did not distinguish between oviposition sites containing mosquitofish exudates and those without in a laboratory cage assay (Van Dam and Walton 2008). A follow-up field study confirmed these results by demonstrating that *Cx. tarsalis* avoided oviposition sites containing caged *G. affinis* while *Cx. quinquefasciatus* did not avoid ovipositing in mosquitofish-containing sites (Walton 2009). Other studies reported reduced oviposition by *Culex* spp. in conjunction with predator cues but lack specific taxonomic resolution (Chesson 1984, Blaustein et al. 2005) or actual egg raft data (Angelon and Petranka 2002).

Another aspect of *Cx. quinquefasciatus* oviposition that is particularly interesting is their apparent ability to learn about their natal habitat and preferentially oviposit in sites containing the same chemical cues. McCall and Eaton (2001) found that rearing *Cx. quinquefasciatus* in water containing skatole at concentrations repellent to ovipositing naïve females rendered the resulting females more attracted to the normally-repellent water containing skatole over known attractants for oviposition. A similar finding has been noted in *Ae. aegypti* reared in water containing the insect repellent Mozaway® (Kaur et al. 2003). Natal habitat preference induction is a phenomenon that has been observed in other insects and is suggested as a means to reduce search time for appropriate oviposition sites and as a factor in sympatric speciation (Davis and Stamps 2004, Stamps and Davis 2006). Learning cues associated with the natal habitat would benefit mosquitoes, as search for oviposition sites presents significant environmental exposure and predation risks to the female.

Non-consumptive effects of predators on *Culex* spp. during development, coupled with inconsistency in *Culex* spp. response to predators at oviposition, suggests variability overall in predation response. Could this variability be explained in part by a learning process such that larval experience shapes the response to predation in adults? In this study, the intersection between these three areas of study was investigated by examining the NCE of the mosquitofish *G. affinis* on the larval development, time to pupation, and size at metamorphosis and the oviposition preference of the resulting adult female *Cx. quinquefasciatus* to evaluate whether they preferred habitats most like their natal habitat and if naïve individuals avoided fish exudates at oviposition. It was

hypothesized that the presence of mosquitofish would affect overall *Cx. quinquefasciatus* development, particularly in the presence of consumed conspecifics, and resulting females would preferentially oviposit in substrates most like their natal habitat and substrates with mosquitofish cues would be avoided by naïve mosquitoes.

Materials and Methods

Insects

Wild *Culex* spp. egg rafts and larvae were collected using a standard coastal hay infusion (Reiter 1986) in Snook, Texas, USA on 12-13 November 2007. Larvae and adults were identified as *Culex quinquefasciatus* in the F_1 generation and the subsequent F_2 generation was used for the experiment. Both pre-experiment rearing and experimental rearing were conducted in a Rheem Environmental walk-in growth chamber (Ashville, NC, USA) at 25-27°C set at approximately 80% RH and a 14:10 L:D cycle.

Experimental Rearing

The goal of this experiment was to rear mosquito larvae in close proximity to the cues that predatory mosquitofish may produce, while not allowing the mosquitofish, *Gambusia affinis,* to feed on the mosquitoes directly. Mosquito larvae were reared in aluminum turkey roasting pans (Handi-Foil, Wheeling, IL, USA) measuring 29.8 x 23.8 cm and 5.9 cm deep separated in the middle by a 6.5 cm tall screen barrier (0.8 x 0.8 cm mesh, tan color; Fig. 5.1). This barrier was a double layer of screen hot-glued (Crafty Magic Melt® general purpose glue, Adhesive Technologies, Inc., Hampton, NH, USA)

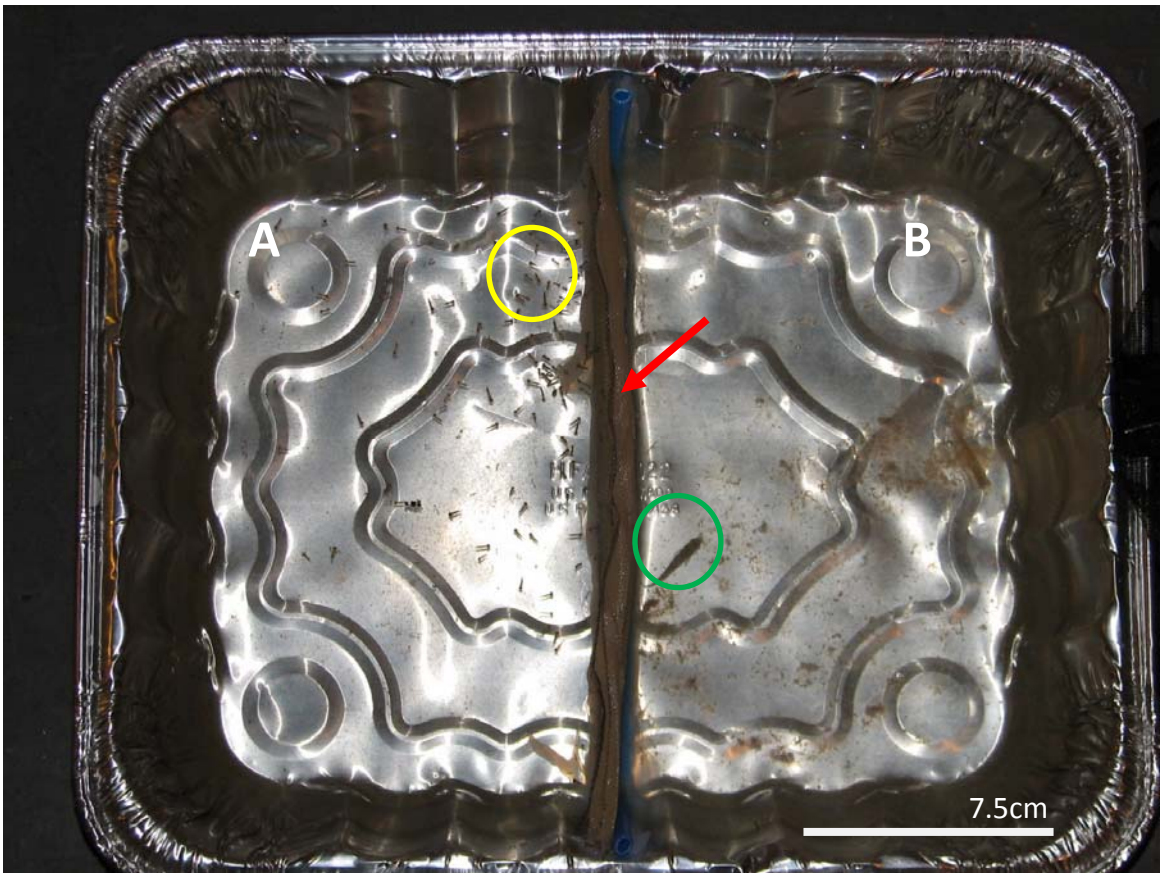

Figure 5.1. Picture of a pan separating mosquito larvae from mosquitofish used in rearing treatments. *Culex quinquefasciatus* larvae (yellow circle) were reared on one side of the pan (A) and separated by a double layer of fine mesh screen (red arrow) from the mosquitofish (*Gambusia affinis*) predators (green circle; B) if present in the rearing treatment.

to a frame consisting of 0.5 cm diameter polystyrene tubing attached to the bottom and sides of the pan with clear GE Silicone I® Kitchen & Bath 100% silicone sealant (GE Sealants & Adhesives, Huntersville, NC, USA). The silicone sealant was allowed to cure for seven days after application to pans prior to setting up the treatments. Initially, it was noted that first instar larvae could pass through both screen layers, so an additional layer of screen was added by cutting two rectangular windows (approximately 4 in height by 6 cm in width) in a 1.2 L square plastic food container (Rubbermaid® TakeAlongs, Atlanta, GA, USA) on opposite sides of the containers approximately 1-1.5 cm from the bottom and screening them with additional screen. This "hatching cage" was used until the second instar was reached, and larvae could no longer get past the main screen barrier.

Two treatments consisted of mosquitofish (Carolina Biological Supply, Burlington, NC, USA and maintained under Texas A&M University Animal Use Protocol #2007-174) housed on one side of the separated pan (Fig. 5.1B) and the third treatment, without mosquitofish on either side of the pan, served as the control.. The control treatment consisted of a single *Cx. quinquefasciatus* egg raft reared on one side of the barrier. The two mosquitofish treatments each contained three mosquitofish (density based on accepted stocking rates for mosquito larval control Walton (2007)) that differed in terms of the diet fed to the mosquitofish. One of the mosquitofish treatments consisted of three mosquitofish maintained on a Tetramin® tropical flakes (Tetra Holding (US) Inc., Blacksburg, VA, USA) diet fed *ad libitum* on one side of the barrier, while the other mosquitofish-containing treatment consisted of three

mosquitofish maintained on 20-30 3rd and 4th instar *Cx. quinquefasciatus* larvae per day derived from the laboratory colony and reared separately. A diet of conspecifics was chosen to maximize any potential cues from the injured or dying larvae and/or the mosquitofish frass upon consuming the conspecific larvae (Kesavaraju et al. 2007). Each mosquitofish treatment pan had a single *Cx. quinquefasciatus* egg raft (approximately 150-200 eggs; (McCann et al. 2009)) reared on the other side of the barrier as in the control (Fig. 5.1A). Mosquito larvae were maintained on a ground Tetramin® to deionized water slurry diet fed *ad libitum* (evaluated daily by visual observation of the absence of food particles at the bottom of the pan) at a ratio of three parts food to one part water. A total of eighteen pans were prepared and six were assigned to each of three treatments for a total of six replicates per treatment randomly numbered and placed on the shelves of the walk-in incubator. A sample consisting of five larvae was taken every other day from each pan until pupation occurred for size and development evaluation, Sampled specimens were preserved in 80% ethanol.

At initial setup, the mosquitofish treatment pans were filled with 1500 mL of deionized water and 200 mL of water from a stock aquarium, where the mosquitofish were maintained before the experiment, (depth of water in the pans was approximately 3.5 cm and maintained by adding deionized water as needed during rearing) so that the ionic concentration did not shock the newly-introduced mosquitofish. The control pans received 1700 mL of deionized water only. Mosquitofish were introduced to their respective treatment pans and started their diet 2 d before the mosquito egg rafts were introduced into the pans. Egg rafts were placed in each treatment on 28 November 2007

and hatching was noted on 29 November 2007. Before the mosquitoes were added to the pans it was noted that the mosquitofish could jump out of the pans so a piece of black mosquito netting was placed over the top of the mosquitofish side of the pan and a sheet of glass over the mosquito side of the pan to prevent mosquitofish escape and accidental introduction into the predator-free side of the pan. As the experiment progressed mosquitofish occasionally died and they were promptly removed and replenished from a stock aquarium in order to maintain three mosquitofish per mosquitofish-containing treatment during the experiment.

As mosquito pupae began to appear in the treatments (approximately 8 d after egg hatch), they were collected daily and placed in a 10 mL glass beaker containing water from the rearing treatment so that, if contact with the natal habitat was needed to create an association for the adults, it could be made (Davis and Stamps 2004). Pupae from each pan were maintained in separate cages where emerging adults had access to a cotton wick soaked in 10% sucrose solution. Pupal exuvia and any dead pupae remaining in the beaker after adult emergence were collected daily from each cage and preserved in 80% ethanol for measurement and enumeration. At 10 d post-emergence a restrained chicken (*Gallus gallus* L.; Texas A&M University Animal Use Protocol # 2007-162) was placed in each cage to allow females to take a bloodmeal.

At 20 d post-hatch, any remaining mosquito larvae and pupae were removed from the pans and preserved in 80% ethanol, and the water from each pan was frozen (approximately -10 °C) to preserve any cues that may have been present during the rearing process for the oviposition assay. The water from each pan was frozen in clear

plastic cups (9 oz. (266mL), Solo®, Urbana, IL, USA) in 100 mL aliquots covered in aluminum foil (HEB, San Antonio, TX, USA). The frozen rearing water was thawed during the day of the oviposition assay so that it would be at rearing chamber temperatures (25-27°C) during the oviposition assay conducted during the mosquito scotophase.

Oviposition Assay

At 4 d post-bloodmeal, the mosquitoes were offered the oviposition assay consisting of water from their natal habitat, water from the other rearing treatments, and a deionized water control. Water from treatments that were not the natal habitat of the cage was randomly-selected from the different treatment pans. The four cups were placed randomly in the four corners of each oviposition cage in the evening and the number of egg rafts in each cup was counted the following morning.

Measurements

Measurements of preserved specimens were evaluated to the nearest 0.01 mm by placing them under a Meiji Techno model EMZ-8TR microscope (Meiji Techno America, Santa Clara, CA, USA) fitted with a Infinity 1-3C digital camera (Lumenera Corporation, Ontario, Canada). An image of each specimen was taken using the Infinity Analyze Version 4.4 software package (Lumenera Corporation, Ontario, Canada) and measurements were taken in the image using the *Calibrate* and *Caliper* commands in the Infinity software.

Larval headwidths were measured for larval samples collected every other day until pupae were observed to evaluate instar (Deslongchamps and Tourneur 1980). Any

larvae and pupae preserved after the 20th day post-hatch were measured by either headwidth for larvae or cephalothorax length for pupae (Koendraadt 2008). Koendraadt (2008) developed regression equations that relate pupal cephalothorax length to the wing length of adults and hence, adult size of *Ae*. *aegypti*. In this study pupal exuvia were collected which had yet to be associated with adult size in any mosquito species. In order to relate the exuvia to the adult *Cx*. *quinquefasciatus*, 52 pupae (one day into pupation, 33 male, 19 female) from the laboratory colony were chilled, measured using the same methods as previously described, and allowed to emerge. On 1 d post-emergence, the adults were killed and wing length measurements were taken and the associated pupal exuvia was measured as for pupal cephalothorax length. Regression equations were generated to evaluate the correlation between pupal cephalothorax length with adult wing length and pupal exuvia cephalothorax length with adult wing length using SPSS 16.0 (SPSS 2007).

Statistical Analysis

All statistical analyses were conducted using SPSS 16.0 (SPSS 2007), with some data manipulations and figures created and managed in Microsoft Excel 2007 (Microsoft, Corp. Redmond, WA, USA). Significance was determined at the $\alpha = 0.05$ level for all tests. Each type of data required specific data analysis, which is detailed in the following sections.

Larval Headwidth Measurements. Larval headwidths were analyzed with a Repeated Measures Analysis of Variance (RM-ANOVA) design. The repeated measure was headwidth for the four dates that samples were collected, with the non-repeated

factor being treatment. The data failed to meet the assumption of equality of variance, so the Greenhouse-Geisser correction was used to examine the overall test effect and the Tamhane's T2 was used for post-hoc analyses.

Pupation Rate. The number of pupae collected per date did not meet the assumptions of ANOVA in that it violated the assumption of homogeneity of variance. Transformation did not alleviate this problem; thus, a non-parametric approach was employed using a two-way Kruskal-Wallis test on the factors of date of pupation, rearing treatment, and the interaction between the two (Garcia-Granero 2002). Post-hoc analysis consisted of an individual Mann-Whitney U test for each treatment combination on each date based on the significance observed in the interaction term.

After the conclusion of rearing, the remaining larvae were preserved for larval headwidth measurement. These data violated normality despite transformation; thus, a Mann-Whitney U test was used to determine if significant differences existed.

Emergence Success. The proportion of pupae that were unsuccessful in emergence were transformed using the arsine-square root transformation and analyzed with a two-way Kruskal-Wallis ANOVA followed by post-hoc analysis Mann-Whitney U tests, because of violation of the assumption of homogeneity of variance. As in the other data sets where this analysis was used, it allows for a simultaneous test of the effects of data, treatment, and the interaction between these two factors.

Pupal Exuvia. Measurements of pupal exuvia cephalothorax lengths were not normally distributed and violated the assumption of homogeneity of variance despite multiple methods of transformation and weighting to remedy the issue. Thus, a two-way

Kruskal-Wallis was employed to evaluate cephalothorax length by the factors of date of emergence, rearing treatment, and the interaction of the two. This was followed up by analysis with Mann-Whitney U tests of each treatment combination for each date.

Data on the emerged mosquito's sex was not available, since mosquitoes had to be alive for the oviposition portion of the experiment. This created a problem for the regression analysis as dropping the factor of mosquito sex greatly reduced the variance explained by the equation from an R^2 of 0.905 to $R^2 = 0.797$ and the data no longer met the assumption of homogeneity of variance in the residuals. So, the data were analyzed using the K-Means Cluster analysis to create two groups within each treatment group, where these two groups represented males and females. This was done based on the assumption that males emerge first and are smaller than females (Holzapel and Bradshaw 2002). This allowed for further analysis of the size of males and females among the different rearing treatments and of the sex ratio from each rearing treatment. A one-way ANOVA was executed on these data; however, they violated the assumption of equal variance; so, a Brown-Forsythe F-statistic was generated followed by the Tamhane's T2 post hoc analysis.

Oviposition. Data consisting of the number of egg rafts laid in the four different oviposition substrates was transformed by the ln(x+1) transformation to normalize the data prior to ANOVA. Also, because no eggs were laid in the deionized water substrate, it was dropped from the analysis to maintain homogeneity of variance. Post-hoc analysis consisted of Tukey's HSD test.

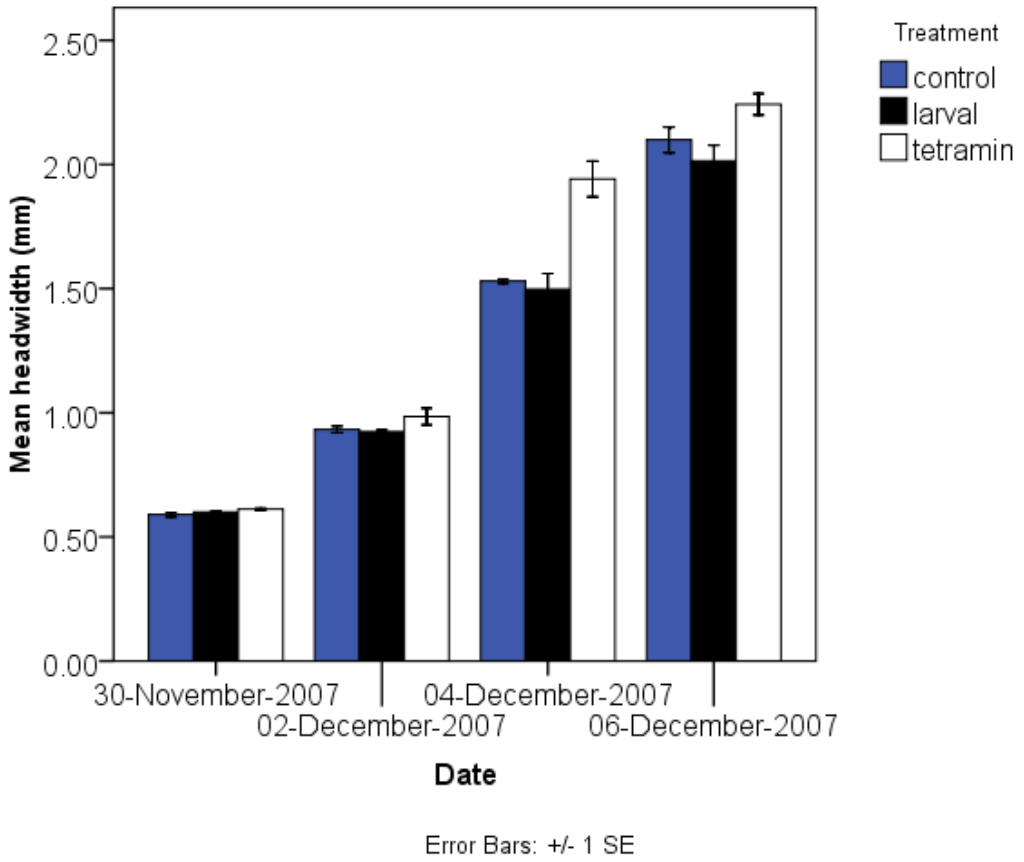

Figure 5.2. Mean (+/- SE) headwidths (mm) of *Culex quinquefasciatus* larvae collected every other day from hatch until pupation in the three rearing treatments. The control treatment consisted of mosquito larvae reared without mosquitofish. The tetramin treatment consisted of mosquito larvae reared in a separated pan with mosquitofish fed Tetramin® flakes on the other side. The larval treatment consisted of mosquito larvae reared in separated pans with mosquitofish fed a diet of 20-30 conspecific 3rd and 4th instar larvae per day. The means separated well into the four larval instars associated with Culicidae.

Results

Larval Development

Figure 5.2 illustrates the mean *Cx. quinquefasciatus* larval headwidths observed each date for each treatment included in this study. Larval headwidths separated well into the four instars observed in Culicidae. Also, as the experiment progressed beyond the second collection date, the difference between the Tetramin treatment and the other treatments increased. By the third collection date, it appears that the larvae in the Tetramin treatments were approximately one instar ahead of the other treatments in development. As would be expected within a developmental series where individuals grow progressively larger with time, the RM-ANOVA results indicated a significant difference within-subjects (Table 5.1). The significant differences of interest were those observed among treatments where a between-subjects difference was observed ($F_{2.010, 177.067} = 941.373$, $P < 0.001$). The Tamhane's T2 results indicated that larval headwidths from the Tetramin rearing treatment were significantly larger than from the other two treatments (Fig. 5.2).

Table 5.1: The results of a repeated measures analysis of variance for *Culex quinquefasciatus* larval headwidths collected on four separate dates during larval development across three different rearing treatments. Data violated the assumption for homogeneity of variance and the Greenhouse-Geisser adjusted F-statistics are presented here.

Factor	F test	df	P value
Within-Subject:			
Date	941.373	2.010	<0.001*
Date X Treatment	9.655	4.019	<0.001*
Between-Subject:			
Treatment	21.082	2	<0.001*

* indicates significant differences observed at the $\alpha < 0.05$ level.

Pupation Rate

Figure 5.3 displays the mean number of pupae collected per date from each rearing treatment for the dates of pupal collection. Pupation occurred in the tetramin treatments first, with the first pupae observed 24 hours before the other treatments. Pupation in the Tetramin treatments was highest on the first two dates of collection and did not have another large peak during the study, with pupation completed by the 19 December collection date. In the other treatments pupae started to appear one day later than in the tetramin treatment. Larval treatments showed a bimodal distribution of pupal abundance, with a peak early in the study followed by a smaller peak about ten days later. Pupation from the control treatment did not have as well defined peaks during the study, with daily pupal abundance remaining fairly constant throughout.

Due to the daily variation in the different treatments during pupation, the assumption of homogeneity of variance was not met despite transformation; so, a two-

way Kruskal-Wallis analysis of variance (KW-ANOVA) test on ranks was performed using SPSS syntax developed by Garcia-Granero (2002). This allowed for a simultaneous evaluation of the number of pupae per date, per treatment and the interaction term. Each individual factor was found to be significant (date $\chi^2 = 78.83$, d.f. = 11, $P < 0.001$; treatment $\chi^2 = 11.328$, d.f. = 2, $P = 0.004$), as was the interaction between date and treatment ($\chi^2 = 65.012$, d.f. = 22, $P < 0.001$). Due to the significant interaction term, a Mann-Whitney U test was conducted for every possible treatment combination for each date (Table 5.2). Significance among treatments for each date was not constant across the entire study, which matches the variability observed in the raw data (Fig. 5.3).

Mosquito larvae remaining after day 20 were collected and preserved from the rearing treatments and their headwidths measured. Larvae were present only in the control and larval rearing treatments. Larvae were present in all larval rearing replicates (six replicates: 138 larvae), but only in half of the control rearing replicates (three replicates: 15 larvae). Figure 5.4 displays the mean headwidth of larvae collected from the control and larval rearing treatments. From this figure, it can be observed that the mean headwidth of larvae from control treatments was significantly larger than that from the larval rearing treatments (Mann-Whitney U: 369.000, Asymptotic Significance: $P < 0.001$) and based on the dataset of headwidth measurements at these larvae appear to be at approximately the second instar.

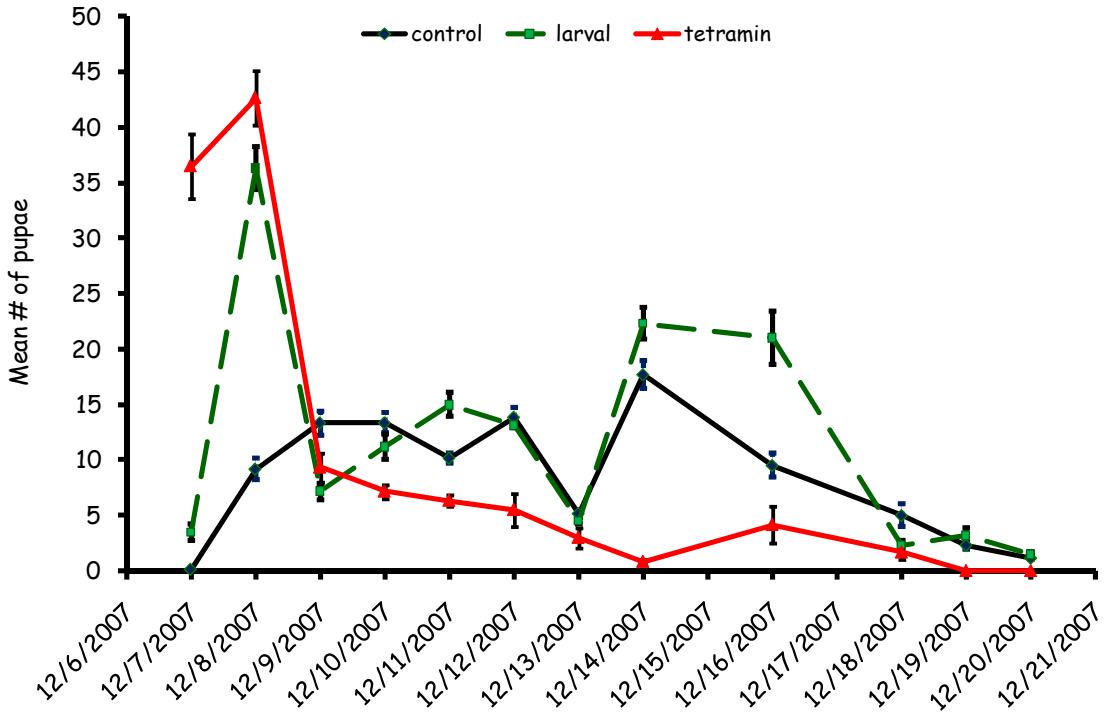

Figure 5.3. Mean (+/- SE) number of *Culex quinquefasciatus* pupae collected per date from each of the three rearing treatments. The control treatment (dark blue) consisted of mosquito larvae reared without mosquitofish. The tetramin treatment (red) consisted of mosquito larvae reared in a separated pan with mosquitofish fed Tetramin flakes on the other side. The larval treatment (dashed green) consisted of mosquito larvae reared in separated pans with mosquitofish fed a diet of 20-30 conspecific 3rd and 4th instar larvae per day.

Emergence Success

Collections of pupae and exuvia allowed me to enumerate the number of pupae that successfully emerged versus those that did not. Figure 5.5 shows the mean proportion of dead pupae collected from each treatment by date. Variation particularly at the end of the study was high and analysis was performed with a two-way KW-ANOVA followed by Mann-Whitney U tests as a post hoc analysis. The KW-ANOVA revealed that the proportion of dead pupae did not differ by date (χ^2 = 12.620, d.f. = 10, P = 0.246) but was significantly different by treatment (χ^2 = 14.236, d.f. = 2, P = 0.001). The interaction of date and treatment was also not significant (χ^2 = 19.484, d.f. = 18, P = 0.363). Mann-Whitney U tests among treatments revealed that significantly more pupae died before emergence in the control treatment than either mosquitofish treatment (control vs. tetramin: Mann-Whitney U = 649.000, Asymptotic Significance, P = 0.001; control vs. larval: Mann-Whitney U = 1030.000, P < 0.001; tetramin vs. larval: Mann-Whitney U = 1093.000, P = 0.757).

Table 5.2: Individual Mann-Whitney U test results for daily pupation of *Cx. quinquefasciatus* for each combination of the three rearing treatments on each date. The overall test of effects using a two-way Kruskal-Wallis test indicated that the interaction between date and treatment was significant. The reported P values represent the Exact Significance results. Sample sizes were equivalent across treatments within dates (n = 6 per treatment).

		Mann-Whitney		
Date	**Treatments**	**U**	**Z score**	***P*-value**
07-December	Control – Tetramin	0.000	-2.989	0.002*
	Control – Larval	10.500	-1.429	0.240
	Tetramin – Larval	0.000	-2.903	0.002*
08 December	Control – Tetramin	0.000	-2.887	0.002*
	Control – Larval	0.000	-2.887	0.002*
	Tetramin – Larval	14.000	-0.641	0.589
09 December	Control – Tetramin	12.000	-0.962	0.394
	Control – Larval	6.000	-1.939	0.065
	Tetramin – Larval	15.500	-.0402	0.699
10 December	Control – Tetramin	6.000	-1.932	0.065
	Control – Larval	14.00	-0.643	0.589
	Tetramin – Larval	12.000	-0.980	0.394
11 December	Control – Tetramin	7.000	-1.780	0.093
	Control – Larval	6.500	-1.845	0.065
	Tetramin – Larval	5.000	-2.085	0.041*
12 December	Control – Tetramin	7.000	-1.777	0.093
	Control – Larval	16.500	-0.241	0.818
	Tetramin – Larval	6.000	-1.939	0.065
13 December	Control – Tetramin	7.500	-1.718	0.093
	Control – Larval	14.500	-0.567	0.589
	Tetramin – Larval	8.500	-1.551	0.132
14 December	Control – Tetramin	0.000	-2.945	0.002*
	Control – Larval	12.000	-0.969	0.394
	Tetramin – Larval	0.000	-2.934	0.002*
15 December	Control – Tetramin	6.000	-1.992	0.065
	Control – Larval	8.000	-1.607	0.132
	Tetramin – Larval	4.000	-2.325	0.026*
18 December	Control – Tetramin	5.000	-2.163	0.041*
	Control – Larval	12.000	-0.974	0.394
	Tetramin – Larval	8.500	-1.627	0.132
19 December	Control – Tetramin	6.000	-2.292	0.065
	Control – Larval	17.500	-0.082	0.937
	Tetramin – Larval	6.000	-2.286	0.065
20 December	Control – Tetramin	6.000	-2.292	0.065
	Control – Larval	14.500	-0.580	0.589
	Tetramin – Larval	3.000	-2.690	0.015*

* indicates significant differences observed at the $\alpha < 0.05$ level.

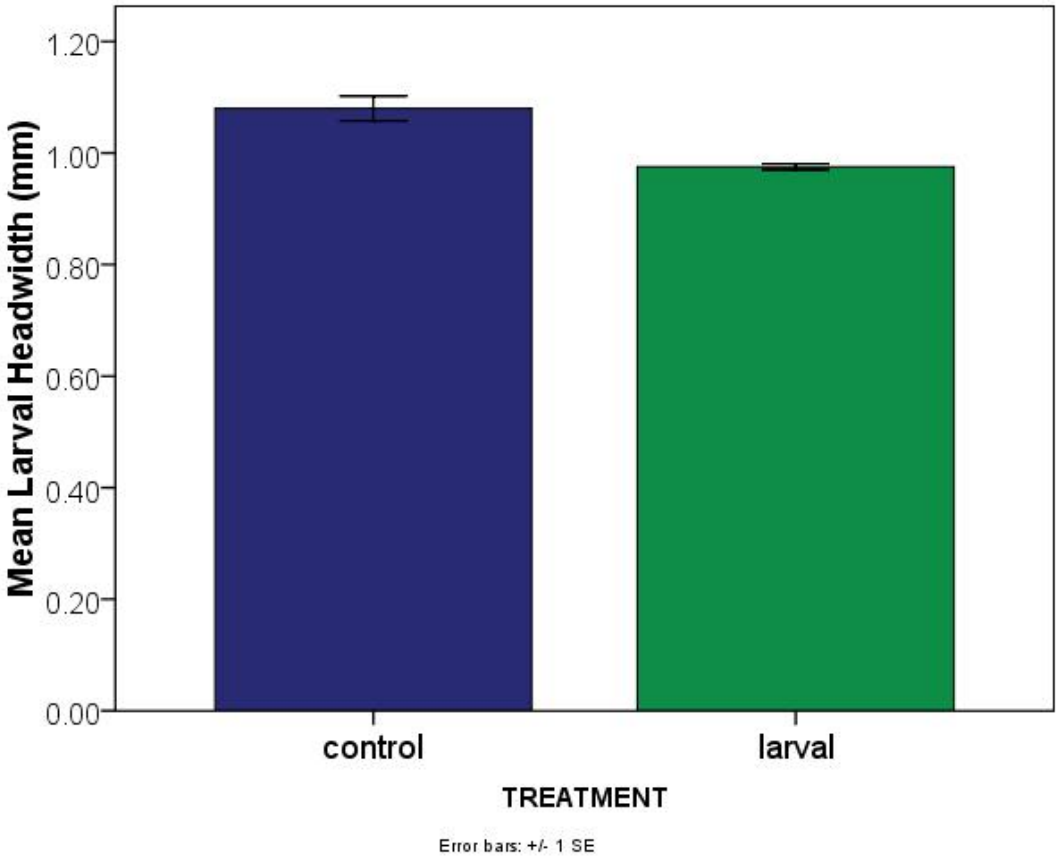

Figure 5.4. Mean (+/- SE) headwidths (mm) for *Culex quinquefasciatus* larvae that remained in the control (blue) and larval (green) rearing treatments at day 20 post-hatch. The control treatment consisted of mosquito larvae reared without mosquitofish. The larval treatment consisted of mosquito larvae reared in separated pans with mosquitofish fed a diet of 20-30 conspecific 3rd and 4th instar larvae per day.

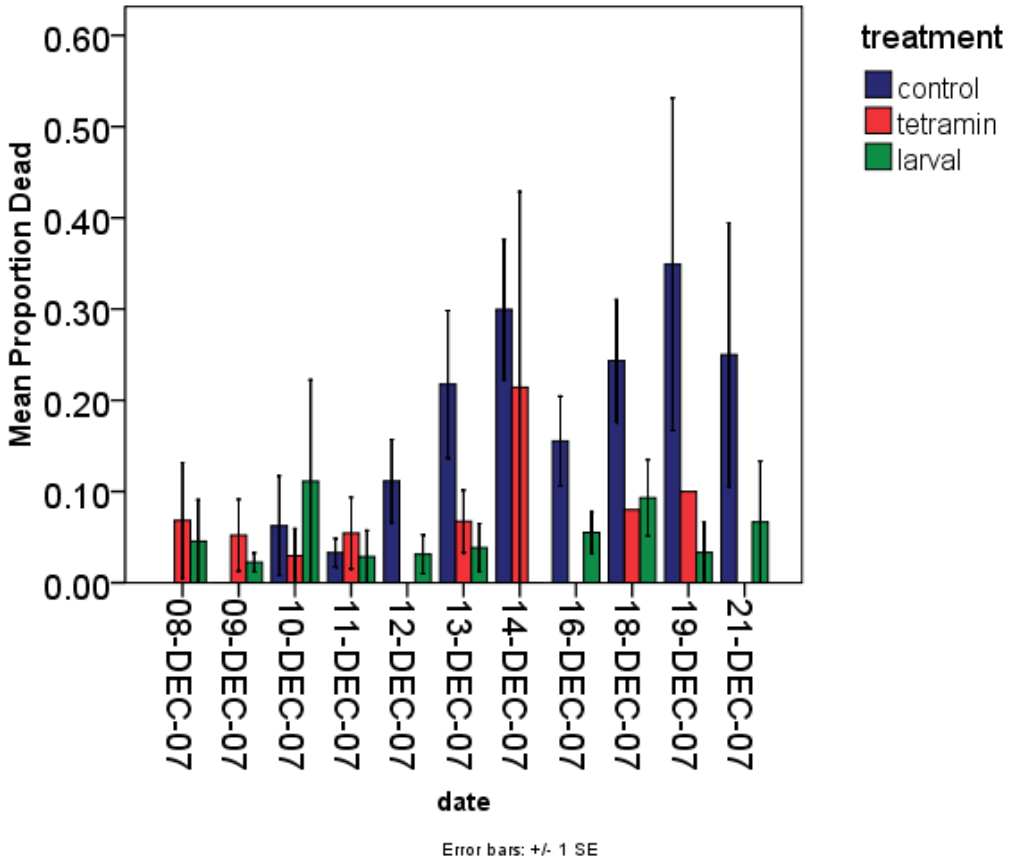

Figure 5.5. Mean (+/- SE) proportion of dead *Culex quinquefasciatus* pupae per collection date from the three rearing treatments. The control treatment (blue) consisted of mosquito larvae reared without mosquitofish. The tetramin treatment (red) consisted of mosquito larvae reared in a separated pan with mosquitofish fed Tetramin flakes on the other side. The larval treatment (green) consisted of mosquito larvae reared in separated pans with mosquitofish fed a diet of 20-30 conspecific 3rd and 4th instar larvae per day.

Pupal Exuvia

Figure 5.6 displays the mean *Cx. quinquefasciatus* pupal cephalothorax length for each treatment over the dates of collection. From this figure, it is clear that pupal sizes were larger at the beginning of pupation from the tetramin rearing treatment, while in the larval treatment, the size of individuals remained fairly constant. The size of individuals from the control rearing followed the general pattern expected for mosquitoes, such that smaller individuals, which were assumed to be males, emerge first followed by the larger females. Variation in the pupal exuvia cephalothorax length per day was so skewed that transformation and specialized techniques (e.g. weighted least squares regression) did not alleviate it sufficiently to use a parametric analysis technique. A two-way KW-ANOVA was employed to analyze the date the exuvia was collected, rearing treatment, and the interaction between date and rearing treatment. Significance was observed for all factors (date: $\chi^2 = 198.191$, d.f. = 11, $P < 0.001$; treatment: $\chi^2 = 103.823$, d.f. = 2, $P < 0.001$) and the interaction between date and treatment ($\chi^2 = 119.068$, d.f. = 19, $P < 0.001$). The post hoc analysis then consisted of 36 total Mann-Whitney U tests for each individual treatment combination on each date (Table 5.3).

Date of exuvia collection was important in that it was expected that males would emerge before females in *Cx. quinquefasciatus* as they do in many mosquito species (Holzapel and Bradshaw 2002). Using this assumption and the fact that male mosquitoes are in general smaller than females, a K-Means Cluster analysis was used within each treatment based on date (Table 5.4). This artificially created two groups that were significantly different from each other based on mean exuvia cephalothorax length. If it

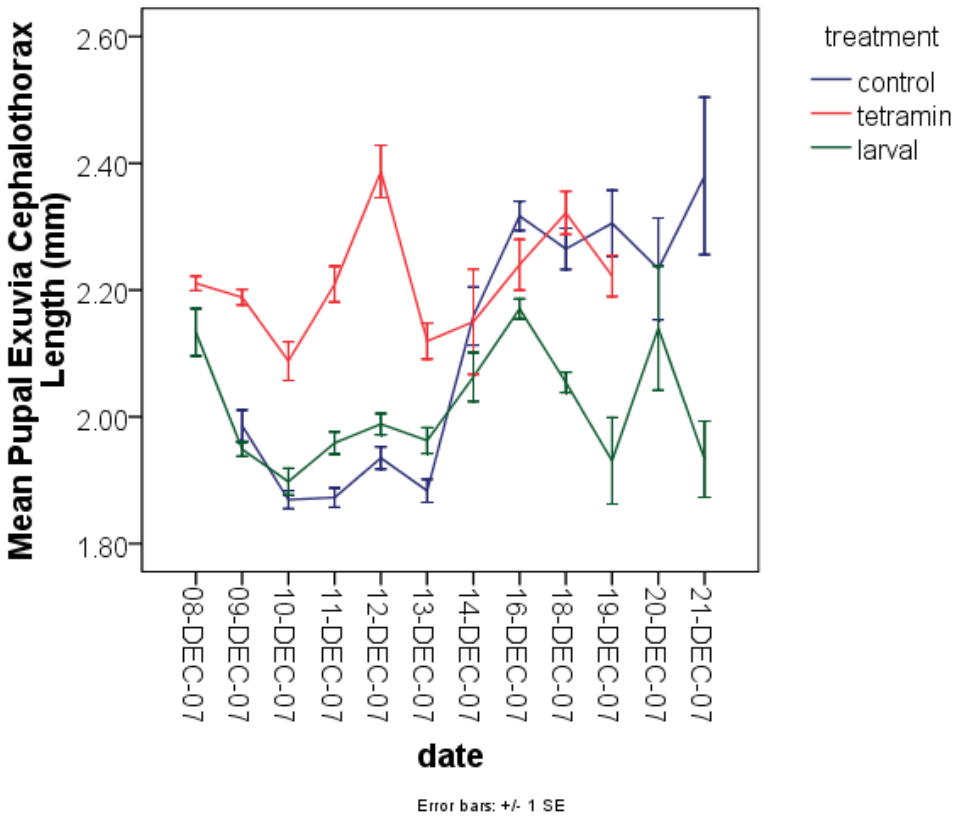

Figure 5.6. Mean (+/- SE) pupal exuvia cephalothorax lengths per collection date for each of the three rearing treatments. The control treatment (blue) consisted of mosquito larvae reared without mosquitofish. The tetramin treatment (red) consisted of mosquito larvae reared in a separated pan with mosquitofish fed Tetramin flakes on the other side. The larval treatment (green) consisted of mosquito larvae reared in separated pans with mosquitofish fed a diet of 20-30 conspecific 3rd and 4th instar larvae per day.

Table 5.3: Individual Mann-Whitney U test results for daily *Cx. quinquefasciatus* pupal exuvia cephalothorax length for each combination of the three rearing treatments. The overall test of effects using a two-way Kruskal-Wallis test indicated that the interaction between date and treatment was significant. The reported P values represent the Exact Significance results. Sample sizes were equivalent across treatments within dates (n = 6 per treatment).

		Mann-Whitney		
Date	**Treatments**	**U**	**Z score**	***P* value**
08-December	Control – Tetramin	n/a	n/a	n/a
	Control – Larval	n/a	n/a	n/a
	Tetramin – Larval	1618.500	-1.730	0.084
09 December	Control – Tetramin	2403.000	-5.954	<0.001*
	Control – Larval	4027.000	-1.446	0.148
	Tetramin – Larval	9568.500	-12.372	<0.001*
10 December	Control – Tetramin	907.000	-5.867	<0.001*
	Control – Larval	1482.00	-0.955	0.339
	Tetramin – Larval	635.000	-4.424	<0.001*
11 December	Control – Tetramin	227.500	-7.922	<0.001*
	Control – Larval	1737.000	-3.578	<0.001*
	Tetramin – Larval	450.500	-6.215	<0.001*
12 December	Control – Tetramin	90.500	-6.996	<0.001*
	Control – Larval	1981.000	-2.084	0.037*
	Tetramin – Larval	231.000	-6.926	<0.001*
13 December	Control – Tetramin	268.000	-5.933	<0.001*
	Control – Larval	1811.500	-2.796	0.005*
	Tetramin – Larval	620.000	-3.820	<0.001*
14 December	Control – Tetramin	149.500	-1.246	0.213
	Control – Larval	3098.000	-5.577	<0.001*
	Tetramin – Larval	228.000	-1.038	0.299
16 December	Control – Tetramin	121.000	-0.402	0.709
	Control – Larval	226.500	-1.438	0.150
	Tetramin – Larval	151.000	-0.341	0.753
18 December	Control – Tetramin	383.000	-1.114	0.265
	Control – Larval	907.500	-5.249	<0.001*
	Tetramin – Larval	313.500	-5.576	<0.001*
19 December	Control – Tetramin	58.000	-1.372	0.188
	Control – Larval	34.500	-3.433	<0.001*
	Tetramin – Larval	14.500	-2.970	0.002*
20 December	Control – Tetramin	n/a	n/a	n/a
	Control – Larval	12.500	-0.463	0.662
	Tetramin – Larval	n/a	n/a	n/a
21 December	Control – Tetramin	n/a	n/a	n/a
	Control – Larval	3.500	-2.588	0.007*
	Tetramin – Larval	n/a	n/a	n/a

* indicates significant differences observed at the $\alpha < 0.05$ level.

Table 5.4: Results of the K-Means Cluster analysis of pupal exuvia cephalothorax lengths from three rearing treatments. Clustering was based on dates within each treatment group and the assumption that males emerge before females and are smaller than females (number of clusters = 2). The smaller cluster was then assumed to consist primarily of males and the larger cluster to be primarily females.

Treatment	"Male" Cluster Centroid	# cases	"Female" Cluster Centroid	# cases
Control	1.90	351	2.38	148
Tetramin	1.70	410	2.90	256
Larval	1.60	614	2.70	180

was assumed that the smaller group consists of males and the larger group females, further analyses could be run on the sex, size and rearing treatment of the individuals. Figure 5.7 displays the mean pupal exuvia cephalothorax lengths for each treatment and cluster generated group. The Brown-Forsythe F-test indicated that there was an overall significant difference (Brown-Forsythe = 1006.050, d.f. = 5, 1300.765, $P < 0.001$). Significant differences among groups as indicated by Tamhane's T2 test are indicated in Figure 5.7.

Initially, a regression equation was generated in an attempt to relate the pupal cephalothorax length with pupal exuvia cephalothorax length and adult wing length, similar to Koendraadt (2008). The laboratory colony was used to generate this equation, which also allowed for the identification of the individual mosquito's sex that proved to be a strong predictor in the equation. Without this information in the experimental setup, the use of a regression equation to determine mosquito adult size fell apart. Despite this the data for the regression is still presented, as it is the first to relate pupal characteristics

with adult size in the species. Figure 5.8 panel A displays the scatterplot of adult wing length as a function of pupal cephalothorax size. The significant regression equation generated from these data was: adult wing length = 1.240 * (pupal cephalothorax length) + 0.266 * (sex of the mosquito) + 0.832 ($R^2 = 0.924$). The second panel (Fig. 5.8B) is a scatterplot of adult wing lengths as a function of pupal exuvia cephalothorax length. The regression was also significant for this relationship with the resulting equation: adult wing length = 0.638 * (pupal exuvia cephalothorax length) + 0374 * (sex of the mosquito) + 1.943 ($R^2 = 0.905$).

Oviposition

A total of 238 egg rafts were laid in all the oviposition substrates. No egg rafts were laid in any of the deionized water substrate cups (Fig. 5.9). The deionized water substrate was dropped from the analysis prior to the ln(x+1) transformation and ANOVA (Table 5.5). There was no significant difference in oviposition substrate choice among the rearing treatments where the mosquitoes had the option of their own natal habitat and those with and without mosquitofish associated cues. However, the mosquitoes appeared to prefer certain oviposition substrates, as this was a significant factor in the analysis. Post hoc analysis with Tukey's HSD revealed that mosquitoes preferred to lay their eggs in water that had previously contained mosquitofish which had been fed conspecific larvae regardless of rearing and hence, larval experience (Fig. 5.9).

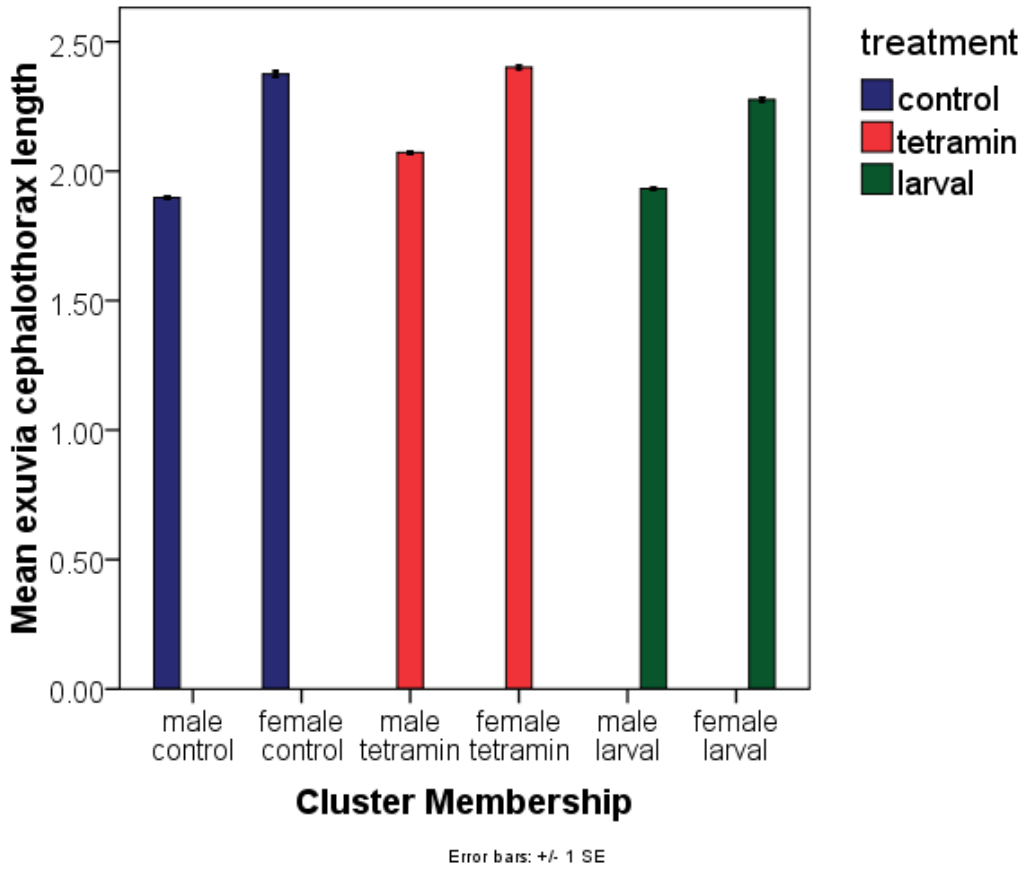

Figure 5.7. Mean (+/- SE) *Culex quinquefasciatus* pupal exuvia cephalothorax lengths based on groups created within each rearing treatment with a K-Means Cluster analysis to separate groups that may be considered males and females based on timing of emergence and size. Different letters indicate significant differences observed with Tamhane's T2 test with significance observed at the $\alpha = 0.05$ level.

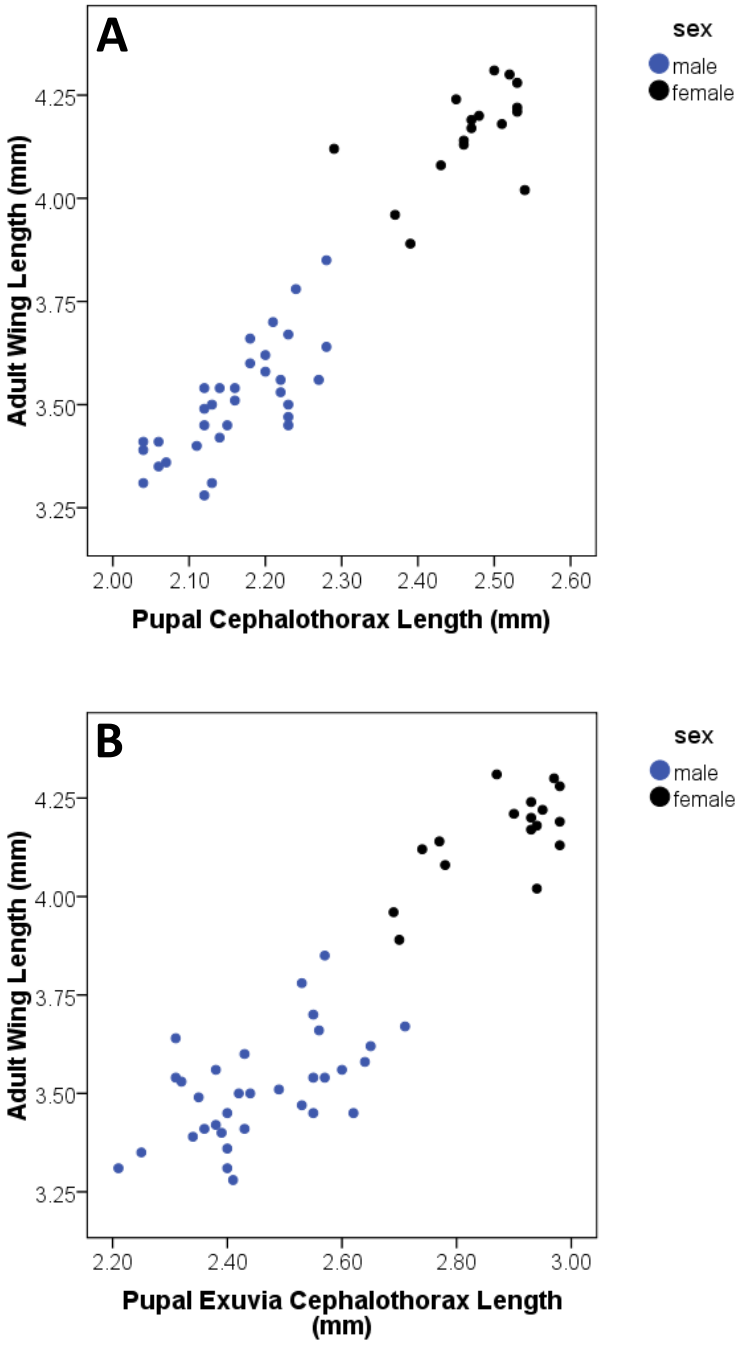

Figure 5.8. Scatterplots of *Culex quinquefasciatus* pupal cephalothorax length by adult wing length (A) and of pupal exuvia cephalothorax length by adult wing length (B) of the same individuals. Regression equations were generated for these data and are presented in the text.

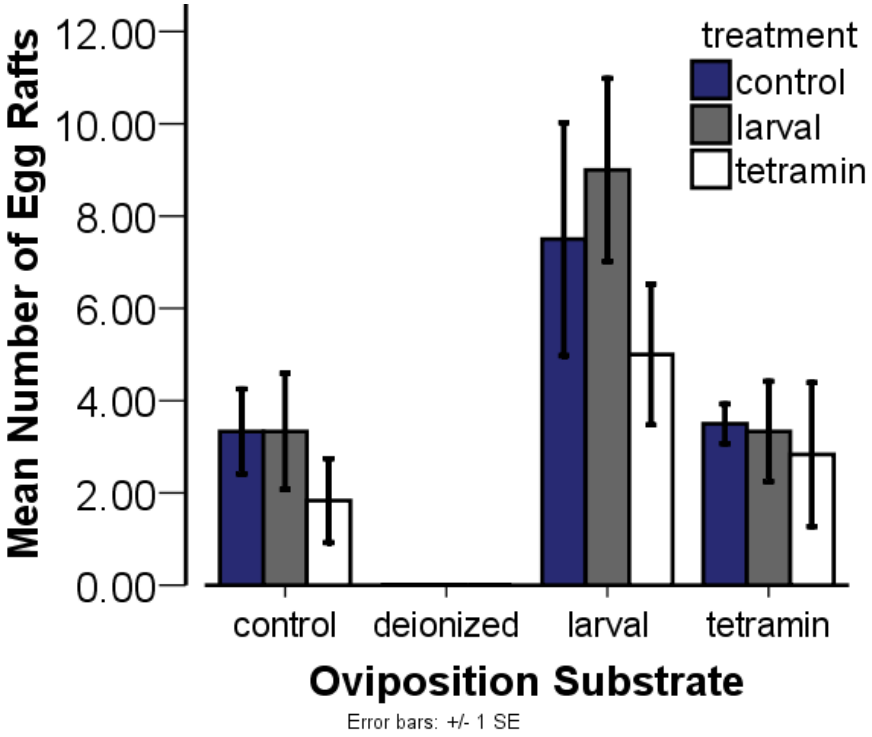

Figure 5.9. Mean (+/- SE) number of *Culex quinquefasciatus* egg rafts laid in each of four possible oviposition substrates by each rearing treatment. The oviposition substrates consisted of deionized water, water from the control treatment without fish, water from the tetramin rearing treatment in which mosquitofish were fed Tetramin® flakes and water from the larval rearing treatment in which the mosquitofish were fed 20-30 conspecific 3rd and 4th instar larvae per day. Letters indicate significant differences as determined by Tukey's HSD test ($\alpha = 0.05$).

Table 5.5: Analysis of variance results for mean number of *Culex quinquefasciatus* egg rafts laid in the four different oviposition substrates by mosquitoes reared in three different rearing treatments with and without mosquitofish.

Factor	**d.f.**	**F statistic**	***P* value**
Treatment	2	1.951	0.154
Oviposition Substrate	2	5.199	<0.001*
Treatment x Oviposition Substrate	4	0.284	0.887
Error	45		

* indicates significant differences observed at the $\alpha < 0.05$ level.

Discussion

Non-consumptive effects can take many different manifestations in prey species. In this experiment, the impact of mosquitofish presence and diet on larval *Cx. quinquefasciatus* development, pupation rate, size at metamorphosis, emergence success, and the choice of females at oviposition were examined. The data suggest that mosquitofish presence impacts all of these factors, depending on the diet the mosquitofish received. The data on oviposition choice also showed that females did not select a habitat most like their natal habitat at oviposition and those reared without mosquitofish did not select substrates without mosquitofish cues.

Larval Development

Larvae reared with mosquitofish fed Tetramin® flakes developed larger, faster than those reared in the larval mosquitofish diet treatment or the control rearing

treatment. By the second collection date, at the second instar, the variation in the headwidths of larvae reared with mosquitofish fed Tetramin was already greater than observed in the other treatments. On the third date, the larvae from the Tetramin rearing treatment appeared to be progressing into the next instar before the other treatments (Fig. 5.2). This is supported by the fact that pupae were observed in the Tetramin rearing treatment one day before the other treatments (Fig. 5.3). The obvious conclusion that could be suggested for this accelerated development is that the mosquitofish did not consume all of the Tetramin and so there was an increase in available food for the developing mosquito larvae. This is entirely possible but was not evaluated in this study.

Non-consumptive effects (NCE) and NCE intensity can be mediated by resource availability through interactions known as trait-mediated indirect effects (Beketov and Leiss 2007, Peckarsky et al. 2008, Preisser et al. 2009). These effects are often associated with studies of larger three-level food chains (Abrams 2008) rather than the two-level predator-prey interactions often studied in examinations of NCE. There really is more to the foodweb in the rearing pan than just the mosquito and the mosquitofish. The microorganism community may be an important component of the foodweb and the experiment that was not examined in this study. Mosquitoes are known to consume not only organic particles but bacteria, algae and fungi, (Merritt et al. 1992), the composition of which can have strong effects on development and adult size (e.g., Kaufman et al. 1999, Peck and Walton 2005, 2006). *Culex quinquefasciatus* larvae reared in highly-eutrophic water from a dairy waste pond took half as long to develop from egg to adult as those reared in wetland water; and this was suggested to be due to a difference in both

bacterial density and nutrient availability (Peck and Walton 2005). Low bacterial density was shown to reduce overall growth rate for both *Cx. quinquefasciatus* and *Cx. tarsalis* reared on bacteria selected for specific C:N:P composition (Peck and Walton 2006). So, it is possible that mosquitofish and the additional resource availability (i.e. extra Tetramin) in this study had an impact on the microorganism community, which in turn affected mosquito larval development. Nevertheless this could be viewed more simply as an increase in available food resources and thus, a straightforward developmental acceleration that is not due to NCE. In future studies, it would be beneficial to examine more of the trophic interactions in the rearing pan by examining microorganism response to mosquitofish, mosquito larvae and resource quantity and quality.

Pupation Rate

The apparent differences in pupation rate between the Tetramin-rearing treatment and the remaining treatments may be similarly attributable to increased food resources as in the observations of larval development. The pupation rate never peaked again after the first burst of pupation at the start of the study, and the larger mean pupal exuvia cephalothorax measurements observed in the Tetramin treatment suggest that females were emerging at about the same time as males. Generally males are observed to emerge before females and are typically of smaller size (Brust 1967), resulting in a bimodal pupal abundance distribution. This expected distribution was only observed in the larval rearing treatment (Fig. 5.3).

In the larval rearing treatment a bimodal pupal abundance distribution was observed; however, the other data sources in this study do not necessarily support that

these two peaks in mean abundance correspond to males and females, respectively. Data from measurements of the pupal exuvia cephalothorax length indicated that the size of individuals over time did not have a discernable pattern and remained fairly constant throughout the collection period (Fig. 5.6). Also, the number of larvae present in the larval rearing treatments after 20 d post-hatch and their small size (Fig. 5.4) suggest that development to pupation was protracted for some individuals in the population. The manifestation of NCE as delays in development is well-known (Lima 1998). Prey reduce foraging behavior under the risk of predation, which, in turn, leads to reduced growth rates and the potential for reduced future reproduction (Lima and Dill 1990, Lima and Steury 2005). However, the data presented here do not suggest a straightforward developmental delay. Pupation rate peaked at the same time as in the Tetramin rearing at a lower level, but significantly higher than in the control treatment (Fig. 5.3). Thus, there was production of pupae throughout and it can be assumed that these consisted of both males and females, despite the abundance of larvae at the conclusion of the rearing portion of the experiment, because egg rafts were produced across all treatments and replicates. This coupled with the smaller pupal exuvia cephalothorax sizes suggests smaller individuals overall, which will be discussed in a later section in greater detail.

One other aspect of the larval rearing conditions that may have some bearing on the response observed by developing mosquito larvae was the diet the mosquitofish were fed. The mosquitofish were maintained on a diet consisting exclusively of 20-30 3rd and 4th instar *Cx quinquefasciatus* larvae per day. This was selected to maximize exposure to cues thought to be important to prey perception of predation risk and to serve as a

control for the Tetramin diet offered to the mosquitofish in the other rearing treatment. However, *G. affinis* cannot subsist on a diet consisting of only mosquito larvae (Reddy and Pandian 1972). In the wild they will feed on zooplankton as well. So, it is possible that there was an added effect of the nutritional stress of the mosquitofish on the development of the mosquito larvae in the larval rearing treatments although, this was not evaluated in this study.

In the control rearing treatments, mean pupal abundance remained fairly constant throughout with no obvious discernable peaks contrary to what was expected. There was no obvious bimodal distribution in abundance data, but the data on pupal exuvia cephalothorax length show a bimodal distribution of sizes over time suggesting that smaller males emerged first followed by the larger females (Fig. 5.6).

Overall pupation rate appeared to be accelerated in the tetramin treatment, altered in the larval treatment, and constant for the control treatment. This suggests that mosquitofish may have affected development in the mosquitofish-containing treatments by accelerating development when food resources were presumably in excess (Tetramin treatment) and prolonging them under resource conditions similar to the control rearing treatment. Acceleration of development would presumably reduce exposure to the predator by reducing contact time and when food is abundant this strategy might be optimal. When food is not abundant a better strategy might be to produce adults over an extended period of time such that there are always adults that can contribute to the next generation. This illustrates the importance of measuring resource dynamics when

conducting studies of NCE. Resource abundance and quality both have the potential to drastically alter the observed effect of predator cues on the development of prey species.

Emergence success

Mosquitoes have mobile pupae which readily dive when confronted with shadows and water movement which in turn, have the potential to be cues of predators or other threats. If pupae spend large amounts of time diving and moving away from perceived threats, it is possible that the energy stores reserved for metamorphosis and the adult stage could be consumed rather than devoted to adult resource needs. It is not known whether pupal mosquitoes respond behaviorally to mosquitofish associated cues as much of the work has been on observing the larval behavior for its obvious contribution to overall mosquito survival and fitness (e.g., Juliano and Gravel 2002). If one assumes that pupae can perceive predation risk as in the other life stages, it might then be assumed that NCE would be evident in the successful emergence of mosquitoes. Thus, mosquitoes from predator-containing treatments in this study would have used more resources in responding to potential predator cues and may have a higher mortality associated with the pupal stage prior to emergence. This was not, however, the result observed in this study.

In this study, it was found that, toward the end of the rearing portion of the experiment, the control rearing treatments had a significantly higher proportion of dead pupae than the other treatments (Fig. 5.5). The reason for this is unknown. The pupae from this treatment were handled as in all other treatments with no observed reason for a significant reduction in pupal mortality prior to emergence. It is difficult to determine

whether a reduction in mortality is a manifestation of NCE in the mosquitofish-containing treatments, or whether some un-measured factor (e.g. a pathogen or parasite) contaminated the control treatment and caused significant mortality; or if this un-measured factor was present, if it was present in all treatments but only manifested in the control treatment. If such a pathogen or parasite were present in the experiment, it leads one to consider the complexity of the simplified laboratory foodweb and what impact such a factor might have on the expression of NCE.

Pupal Exuvia

Once the pupal stage has been reached, no further feeding occurs and pupal size can be used as an indicator of the final size of the adult. The collection of data on pupal exuvia cephalothorax length proved to be one of the more interesting aspects of the study. The pupal cephalothorax length and pupal exuvia cephalothorax length were able to be related with the adult wing length with a high level of variation explained by the equation; however, it required knowledge of the sex of the mosquito to be a functional model. Thus, these data were not able to be used further in the experiment. The results are similar to those of Koendraadt (2008) in *Ae*. *aegypti* in that pupal characters can be used to estimate adult size plus it was shown here that pupal exuvia can be used in the same manner. The caveat is that, for the equations generated herein to function well there is a need to know the sex of the pupa as well. Other characters might prove reliable in determining sex, but the spine on the anal paddle which was also measured (preliminary data not shown) did not correlate well with the sex of the mosquitoes.

It would be expected that exuvia size would correspond to sex, due to males being generally smaller and emerging earlier than females under optimal conditions. For the first six days of emergence in the current study the exuvia from the Tetramin-rearing treatment were significantly larger than those from the other treatments; and as was mentioned previously, this is when the peak in pupal abundance also occurred. This suggests that males and females emerged at approximately the same time, and this may have been due to increased resource availability based on the extra food available from the mosquitofish diet. Exuvia from the larval rearing remained fairly constant throughout the experiment, which did not correspond with the bimodal pupal abundance recorded over the course of the experiment. If one takes into consideration that egg rafts were collected from cages from the larval rearing treatment in amounts consistent with the other treatments, it might suggest that smaller females were emerging from the larval rearing treatment. If one compares this pattern to that of the control, it becomes even more plausible. The size of exuvia in the control treatment follows a distinct bimodal distribution over time, suggesting that smaller males did emerge early followed by larger females.

Two clusters were artificially created within each treatment based on dates on the assumption that males are smaller and emerge before females (Brust 1967, Holzapel and Bradshaw 2002). This allowed for an analysis of the size of males and females in the treatments based on these cluster assignments (Fig. 5.7). Interestingly, this analysis suggested that females of both the Tetramin and control treatments were not significantly different from each other, but were significantly larger than those from the

larval rearing. This supports the suggestion that NCE caused accelerated development in the Tetramin treatment and that smaller females were emerging from the larval rearing treatment. This analysis also suggested that males significantly differed in size among all three treatments with males from the Tetramin treatment being the largest followed by those from the control and finally the smallest from the larval rearing treatment. This supports the suggestion that those individuals in the tetramin treatment may have had access to more resources and that individuals from the larval treatment were emerging at a smaller size than in the other two treatments. Although artificial, the clustering and subsequent analysis does bring together several aspects of the study which suggest NCE operating in different ways during the study.

Oviposition

This aspect of the study was perhaps the most surprising in terms of an un-anticipated result. Females did not choose oviposition substrates from their natal habitat nor did naïve mosquitoes avoid habitats with mosquitofish cues, but rather preferred substrate where mosquitofish had been held and fed conspecific larvae. We expected that mosquitoes would preferentially lay in their natal habitat and that substrates containing mosquitofish cues would receive only eggs from mosquitoes that had been reared in that particular treatment. This was not the response observed in this study.

The effect of natal habitat preference induction has only been discretely documented in two instances for mosquitoes (i.e., *Cx. quinquefasciatus*: McCall and Eaton 2001; *Ae. aegypti*: Kaur et al. 2003) and in both instances, it has been to either a novel chemical (Kaur et al. 2003) or a component of a known attractant blend at an

abnormally high concentration (McCall and Eaton 2001). This had the effect of making the natal habitat more unique, at least from the human perspective. It has also been suggested that these studies reflect habituation to the odors rather than a positive association (Alonso and Schuck-Paim 2006). In the current study the aim was to determine if the mosquitoes remembered their natal habitat based on a generalized rearing habitat associated chemicals and with cues from mosquitofish fed specific diets. All the treatments had similarities and what was presumed to be distinct differences. It is possible that the cues associated with the habitats were not unique enough to be remembered by mosquitoes; however, if that is the case, it suggests that the descriptions of this ability in the literature bear little relevance to actual field situations. Another hypothesis for the observed oviposition behavior is that there is a hierarchical decision-making process by which a mosquito makes an oviposition decision and that certain cues have higher relevance than others in that process. This kind of evaluation/decision-making process has been suggested in the behavioral decisions made by prey species under threat of predation (Lima and Dill 1990). For example, under the conditions of starvation, an animal is more likely to take risks under the threat of predation than when it has eaten, which suggests prioritizing and hierarchical decision-making (Lima and Steury 2005). If a female mosquito is fully gravid and under the perceived threat of predation but lacking resources to seek another habitat, there may be a decision made based on current physiological state and environmental conditions at the time that leads to reduced predator avoidance.

No egg rafts were laid in the deionized water control substrate (Fig. 5.9). This is the standard laboratory oviposition substrate and the substrate that was used to collect the egg rafts used in this study. It has been demonstrated that mosquitoes may drink water immediately prior to or during oviposition and this has been suggested as either a sampling of the water to determine suitability or as a means to help expel the eggs from the abdomen (Hudson 1956, Weber and Tipping 1990, 1993). This may be an important factor for an experimental design such as the one used in the current study where there is the potential for odor saturation within the confines of the cage which may preclude odor-guided flight to a substrate (Kramer and Mulla 1979). With this in mind, it is also interesting to note that about the same number of egg rafts were laid in the control and Tetramin rearing treatments which, as has been suggested, may have differed in the amount of available resources. If the Tetramin treatment had significantly higher resource availability, it seems logical to suggest that females should have laid eggs in this substrate over others, as it would provide for the developing larvae, but that was not the case in this study.

Mosquitoes preferentially laid eggs in a substrate that had contained mosquitofish potentially stressed from consuming a diet consisting only of 20-30 3rd and 4th instar *Cx. quinquefasciatus* larvae per day. It is known that substances from conspecific larvae and pupae can be attractive substances to ovipositing females and that these factors often interact with other factors to determine where a female lays her eggs (Bentley and Day 1989). It is possible that the cues associated with conspecific larvae may indicate to an ovipositing mosquito that the site is suitable for oviposition and that

these cues confer more relevance than those associated with the mosquitofish. This seems counterintuitive based on the results of the immature development that suggested that cues associated with the larvae consumed by the mosquitofish conferred NCE. It is possible that any immediate cues from larval consumption by mosquitofish that could be classified as "alarm cues" are very short-lived or that these cues are not perceived by adults. If no predation risk is perceived, then no NCE can be manifested (Lima and Steury 2005). Perhaps feeding the mosquitofish a diet of egg rafts or of adult mosquitoes would have provided an appropriate cue that the ovipositing females could recognize as a threat.

Conclusions

Integrating multiple types of data in this experiment allowed for the determination that the mosquitofish, *G. affinis* does appear to confer NCE during development on *Cx. quinquefasciatus* despite a lack of long-term evolutionary history. This lack of long-term evolutionary history has been suggested as a reason for reduced response to fish exudates in mosquitofish oviposition avoidance studies (Van Dam and Walton 2008, Walton 2009). However, adult females do not seem to relate generalized habitat cues with future oviposition and naïve females do not appear to avoid mosquitofish associated cues but were attracted to them in this study. The observation of developmental NCE but not adult associated NCE suggests that perhaps my design did not address all of the complexity of the laboratory foodweb. Microorganisms are important to not only developing mosquito larvae but to ovipositing females as well (e.g., Hazard et al. 1967, Beehler et al. 1994, Isoe and Millar 1995). The microorganisms

represent not only another level to the foodweb in terms of food resources, but also represent a factor that mediates oviposition in *Culex* spp., a factor perhaps even stronger than the threat of predation. Future studies should attempt to address the role that the microorganisms may play in the trophic interactions of mosquito larvae and their predators as well as the potential for NCE among microorganism groups.

CHAPTER VI

MEMORY LENGTH IN THE SOUTHERN HOUSE MOSQUITO, *Culex quinquefasciatus* SAY (DIPTERA: CULICIDAE)

Introduction

Associating odors with resources has been explored across the three major life history domains of culicids; i.e., sugar-feeding, host-seeking, and oviposition. Associating novel odors with a sugar-meal was demonstrated for *Culex quinquefasciatus* Say by Tomberlin et al. (2006) and has been extensively discussed in previous chapters of this dissertation (Chapters II and VII). Several examples exist of mosquitoes returning to sites associated with hosts (e.g., Charlwood et al. 1988, Renshaw et al. 1994, McCall et al. 2001)) and to specific host types on which they had previously fed (Hii et al. 1991, Mwandawiro et al. 2000). Conditioning of odors associated with larval habitats and oviposition preference has been demonstrated with both *Aedes aegypti* L. (Kaur et al. 2003) and *Cx. quinquefasciatus* (McCall and Eaton 2001). Despite this, relatively little is known about the specifics of learning in mosquitoes, especially when compared to model organisms like *Drosophila melanogaster* Meigen.

Memory length is one aspect of mosquito learning that is not well known. In contrast, extensive associative learning studies on *Drosophila* have been conducted. Through the use of different mutant phenotypes in *Drosophila,* four different memory phases have been proposed, with each being dependent upon each other during memory

consolidation (DeZazzo and Tully 1995). The different phases of memory each have a defined duration and each was determined by use of specific mutant phenotypes that lack the specific memory type (Tully et al. 1994, DeZazzo and Tully 1995, Meller and Davis 1996, McGuire et al. 2005). Short-term memory (STM) was discovered using the *dunce* and *rutabaga* mutants and is defined as memory that lasts from right after the end of training until about two hours after training (Tully et al. 1994). The second memory phase, medium-term memory (MTM), was discovered with the use of *amnesiac* mutant fruit flies and is defined as memory that is greatest at about one hour post-training and at a minimal level at seven hours post-training and is susceptible to disruption with anesthetic (Tully et al. 1994). The remaining two types of memory are dependent upon the type of training received by the flies. For long-term memory (LTM) formation to occur, training trials need to be spaced with time intervals between stimulus presentations which leads to protein formation and memory that can last from one day to one week (Tully et al. 1994). If the training trials are not spaced but repeated one after the other (massed), the resulting memory type is anesthesia-resistant memory (ARM; discovered with the *radish* mutant) which lasts from two hours to four days after training and no LTM will be observed (Tully et al. 1994). In addition to the fruit fly, *Drosophila,* these same phases have been described in the honey bee, *Apis mellifera* L., though in much less detail with respect to mechanisms (Meller and Davis 1996).

Memory length in the context of mosquito life history could have impacts in all three of the major domains. In terms of sugar-feeding, memory that spans at least a 24 hour period would have the potential to shorten search times for sugar resources, as

males often sugar-seek before or after daily swarming events (Foster 1995). For a female mosquito to be a successful vector it must take more than one blood-meal. If a mosquito forms a memory about a particular host type and preferentially returns to that host it could have implications for localized disease transmission (McCall and Kelly 2002); and thus, it would be particularly useful to know how long a mosquito might remember an odor-resource association. Based on mosquito life history it would be expected that LTM would most likely be involved for host-seeking as oviposition and host-seeking are often separated by as much as a week (Reeves et al. 1990). Long-term memory would also be suggested for odor associations created between larval habitats and oviposition sites, as the length of the gonotrophic cycle may span seven to ten days (Reeves et al. 1990).

Another aspect of learning that has relevance to mosquito life history is the age of the mosquito at the time of learning. Older *Drosophila* (> 20 d old) have significantly reduced LTM formation (Tamura et al. 2003, Mery 2007). Similarly, older cockroaches (50 wk old) took longer to complete a maze and did not improve after repeated trainings over time as occurred in the 10 wk old cockroaches (Brown and Strausfeld 2009). Honeybees exposed to odors, either through a traditional proboscis extension reflex training or with general presentation of scented sugar solutions at an early age (but older than 1-4 d), improves learning of odors by older bees (Arenas and Farina 2008, Arenas et al. 2009).

There are no data on the impact of age on learning for mosquitoes. Based on mosquito life history, it is suggested that, because mosquitoes are relatively short-lived, experiences learned early in life may have a greater impact and/or be more likely to be

remembered. This scenario may be thought of as similar to the concept of imprinting known from the vertebrate literature (e.g., Hess 1958).

Drosophila research on learning has opened up another interesting area in examining selection for learning and memory parameters based on ecological context (Mery and Kawecki 2002, Papaj and Snell-Rood 2007). Mery and Kawecki (2002) found that in using conditioning for oviposition avoidance, they could select for faster learning and better memory in fruit flies within 15 generations. This supports selection for learning and memory under ecologically-relevant scenarios (Papaj and Snell-Rood 2007). If flies can be selected for their enhanced ability to learn, it might be that, in the absence of selection pressure while in the field, this could result in the loss of some of this ability when confined to laboratory cages. Mosquitoes can be selected for specific behavioral traits such as stenogamy (e.g., Somboon and Suwonkerd 1997) or an ability to feed on artificial diets (Griffith and Turner 1996). Thus, it seems reasonable to suggest that, if there is some cost associated with maintaining learning and memory structure and behavior under laboratory colony induced selection, where all resources are available within the confines of the cage, this might be lost or decline over time.

In this study, the question was asked, how long does a mosquito remember, using the current method of conditioning mosquitoes based on Tomberlin et al. (2006) and the model mosquito, *Cx. quinquefasciatus*. The expectation was that the mosquitoes would exhibit memory length comparable to *Drosophila,* as they are both dipterans with similar life expectancies. This question was taken a step further to ask, does the age of the mosquito impact memory length as has been suggested by information in *Drosophila*

(Tamura et al. 2003, Mery 2007) and other insects (Arenas and Farina 2008, Arenas et al. 2009, Brown and Strausfeld 2009)? It was predicted that younger mosquitoes might display longer memory associations. Given that ecological factors may drive selection of learning associated genes (Mery and Kawecki 2002, Papaj and Snell-Rood 2007), the question was asked, does the population from which the mosquitoes were derived impact memory length. In this study the prediction was that laboratory colony mosquitoes are less likely to experience selection for learning as their resources are in such close proximity at all times; thus, they would have shorter memory length, unlike field populations. Finally, because there is evidence to suggest that males and females may respond to conditioning differently (Tomberlin et al. 2006; Chapters II and VII), does memory length differ between males and females? Based on the previous work in this area it was predicted that females might exhibit lower levels of conditioning at the longest memory test intervals.

Materials and Methods

Mosquitoes

Two sources, or populations, of *Culex quinquefasciatus* Say were used in this study; one representing laboratory colony and the other a field-collected population. The laboratory colony consisted of mosquitoes originally derived from material collected in Gainesville, FL, USA in 1992 but obtained from the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), USDA-ARS, Gainesville, FL, USA in January 2009 for colony establishment at Texas A&M University, College Station, TX, USA. Thus, the original colony had been in culture for seventeen years before the initiation of

this experiment. Larvae were reared using a standard method of two egg rafts per liter of deionized water in white enamel pans on a diet consisting of a ground Tetramin® Tropical Flakes (Tetra Holding (US) Inc., Blacksburg, VA, USA) slurry consisting of 3 parts ground Tetramin® to 1 part deionized water.

The field collected populations were obtained by collecting egg rafts at a single field site location over the course of the experiment (April – September 2009). Egg rafts were collected using a modified Reiter media (Reiter 1986) consisting of 75 gm Bermuda grass (Kaytee Natural Bermuda Grass, KAYTEE Products, Inc., Chilton, WI, USA) and 4.6 gm dried active baker's yeast (MP Biomedicals, Inc., Solon, OH, USA) in 18.92 liter of tap water fermented in sealed 5-gallon buckets for approximately seven days. In the evening oviposition media was deployed in a 11.4 liter white dish pan (Sterilite Corporation, Townsend, MA, USA) filled approximately half-full (~5.7 liter) and egg rafts were collected the following morning. Egg rafts were collected for each memory length tested; so, all material was F_0 generation. Larvae were reared at a density of one egg raft per liter of deionized water to ensure that each egg raft was *Cx. quinquefasciatus,* and samples of 3rd and 4th instar larvae were pulled from developing cohorts so identification could be confirmed. In addition adult samples were preserved from every field collected cohort and will be deposited in the Texas A&M University Insect Collection for future reference.

For both the laboratory colony and the field collected mosquitoes, pupae were collected and mixed before placing approximately 100 individual pupae on the second day of pupation (to ensure collection of male and female pupae) into each of two cages

(small Plexiglass cages: 19.5 x 19.5 x 19.5cm). One cage was held without sugar but with access to water for tests involving 1-2 d old mosquitoes and the other cage was given access to a 10% sucrose solution on a soaked cotton wick up to 24 h before testing of the 3-5 d old mosquito age group. All mosquitoes were maintained in a Rheem Environmental walk-in growth chamber (Ashville, NC, USA) at 25-27°C and approximately 50-70% relative humidity with a 14:10 L:D cycle.

Sugar-feeding Time

The amount of time that male and female mosquitoes sugar-feed was evaluated to ensure that by the end of conditioning the mosquito had not exceeded the time required for a full sugar-meal, which would render them without motivation to continue sugar-seeking. This was accomplished by feeding individual mosquitoes a 10% (w/v) technical grade sucrose (Sigma-Aldrich, Co., St. Louis, MO, USA) solution dyed with red food coloring (to enhance visualization of the filling mosquito gut; Strawberry Red Color, Royallee Brand, Bangkok, Thailand; concentration = 12 drops:30 ml solution) from a new 200 µl calibrated micropipette (Drummond Scientific Company, Broomall, PA, USA). The amount of time from the beginning of feeding until the mosquito pulled the proboscis out of the pipette (this was assumed to represent satiation) was recorded for males and females from the laboratory colony and field collections at the 1-2 d and 3-5 d old ages.

Conditioning

Mosquitoes were first allowed to acclimate to the laboratory conditions for 30 minutes before conditioning by moving the cage from incubator into the main

laboratory. All conditioning and testing was conducted under a biological safety cabinet (Logic, Purifier© Class II Biological Safety Cabinet, Labconco Corporation, Kansas City, MO, USA) to promote the movement of air and reduce the potential for habituation and odor contamination. Mean airflow speed was 0.47 m/s where the mosquitoes were located in the middle of the cabinet and 3.48 m/s on average at the front of the cabinet as measured with an anemometer (Testo 435-1, Testo, Inc., Sparta, NJ, USA). Prior to conditioning, the mosquitoes were each isolated into individual clean glass shell vials (70 mm tall x 20.5 mm diameter, 4 dram : 14.787 ml volume) placed on a small square of clean office paper (~4 x 4 cm; Discovery® Premium Select, Soporcel North America, Inc., Norwalk, CT, USA). Overall, the conditioning procedure was similar to that used by Tomberlin et al. (2006) and the same as that used in Chapter II. This method involves conditioning individual mosquitoes by offering them a 200 µl calibrated micropipette (Drummond Scientific Company, Broomall, PA, USA) with the first 1-2 cm filled with a 10% (w/v) technical grade sucrose (Sigma-Aldrich, Co., St. Louis, MO, USA) solution. The outside, distal, ~1 cm of the pipette was coated with the target odor of jasmine flavor extract (Winners Brand, Bangkok, Thailand). The mosquitoes are offered the odor coated pipette for 15 s by encouraging the mosquito to rest at the bottom of the vial and then lifting the vial approximately 30° and placing the pipette directly onto the mosquito's proboscis. This was repeated for a total of three times, with a 30 s resting period between trials. The mosquitoes were then labeled; and if testing was to be conducted at a later time, a small plug of clean cotton was placed in the top of each vial until testing. The mosquitoes were left in the safety cabinet if the interval between

conditioning and testing was 5 h or less; otherwise, they were returned to the walk-in growth chamber until testing. A minimum of 15 males and females from each age and population were conditioned to for each test interval (N = 720). Following the completion of the entire time series, an additional 30 males and females for each time period were conditioned to confirm the observed trends, with a larger within-time sample size being used in the case of the laboratory colony derived population.

Testing

For each testing category (age 1-2 d and 3-5 d old by laboratory colony and field collected), mosquitoes were tested at each of six different times from conditioning, consisting of less than 5 minutes (as in Tomberlin et al. 2006), 1 h, 2.5 h, 5 h, 10 h, and 24 h. If the conditioning to testing interval was 5 h or longer, the mosquitoes were kept in the walk-in growth chamber until testing. They were then allowed to acclimate to the laboratory conditions as previously described for approximately 30 minutes before testing.

The testing protocol and evaluation of response was the same as that used in Tomberlin et al. (2006). The mosquito is offered an empty pipette in the same way as during testing and the small sheet of paper used allows for the maintenance of the same relationship between the pipette, the mosquito and the observer throughout the experiment. The pipette is empty and either swabbed with the target odor (jasmine flavor extract) or, the non-target odor (geraniol) that the mosquito has never had experience with prior; or, the pipette is a blank pipette and is not swabbed with any odor. The pipette so treated is presented to the mosquito for 15 s just under the shell vial to allow

for a response that requires the mosquito to move. A positive response to the assay is recorded when the mosquito moves the proboscis up and down and walks toward the pipette in what appears to be a searching behavior. Negative responses are recorded when the mosquito moves away from the pipette or does not make a directed movement toward the pipette. If the mosquito does not make any detectable movement or response this is also recorded as a negative response.

Innate Odor Preference

For this experiment, the single target odor of jasmine flavor extract was selected for conditioning (Winners Brand, Bangkok, Thailand). Jasmine flavor extract was chosen, as it was used successfully in preliminary work with both *Anopheles minimus* Theobald and *Cx quinquefasciatus* in Thailand. It is also an odor the mosquitoes do not appear to be repelled by and thus, is easier to work with when conditioning mosquitoes (personal observation). The non-target odor selected was geraniol (98%, Sigma-Aldrich, Co. St. Louis, MO, USA), as it has been used in work with *Anopheles cracens* Sallum & Peyton (Chapter VII), and it is an odor that is known to be detected by the *Cx. quinquefasciatus* antennae but not considered an attractant (Bowen 1992). Single target odor experimental designs have successfully been used to demonstrate learning in *Microplitis croceipes* Cresson (Hymenoptera: Braconidae) on multiple occasions (Lewis and Tumlinson 1988, Wackers et al. 2002, Tertuliano et al. 2004, Wackers et al. 2006).

The innate response of the mosquitoes to the odors was observed by presenting individual males and females to the pure odor swabbed on the outer ~1 cm of the distal end of an empty 200 µL micropipette for 15 s as in the conditioning procedure. The

mosquitoes' responses were recorded as positive or negative as in the conditioning procedure.

Statistical Analysis

For the data on sugar-feeding time, data points more than 210 s (3.5 min) were dropped since they were identified as being greater than two standard deviations from the mean. The data were also $\log_{10}$ transformed for normalization and use in a full factorial analysis of variance (ANOVA) using the general linear model procedure in SPSS 16.0 (SPSS 2007). The factors in the model were the population of the mosquitoes, the age of the mosquitoes, the sex of the mosquitoes and the interactions among these terms on the time of sugar feeding. The data consisting of the percentages of mosquitoes responding positively to the odors used in the experiment were calculated, but no further data manipulations or statistical analyses were performed.

For the analysis of factors associated with conditioning, a full factorial logistic regression model was constructed using syntax commands for SPSS 16.0 (SPSS 2007). Backward stepwise variable selection based on the maximum -2log-likelihood was used. However, it never resulted in a significant model, as determined by Hosemer-Lemeshow statistics. So, interaction terms remaining in the model through the last iteration of variable selection but that did not contribute significantly to the model were removed manually, and the model selection re-run until a significant model was observed. Predicted probabilities generated by the model were plotted and subjected to non-parametric statistical analysis with either Kruskal-Wallis analysis of variance or Mann-Whitney U tests as appropriate.

All statistical analyses were conducted in SPSS 16.0 (SPSS 2007), with some data manipulations and charts created in Microsoft Excel 2007 (Microsoft, Corp. Redmond, WA, USA). Significance for all statistical tests was observed at the $\alpha = 0.05$ level.

Results

Sugar-feeding Time

Sugar-feeding time for females was generally shorter than that for males (Fig. 6.1), with a mean of 100.3 s for females while males took 151.4 s on average. The final model for the ANOVA of sugar-feeding time is presented in Table 6.1. Significant factors included the population of the mosquito, with colony mosquitoes taking significantly longer sugar-meals (Table 6.1; Fig. 6.2); the sex of the mosquito, where males took significantly longer to take a sugar-meal (Table 6.1; Fig. 6.1), and there was a significant interaction between population and age of the mosquito, with 3-5 d old mosquitoes taking a longer time to sugar-feed than 1-2 d old mosquitoes from the field collected population (Table 6.1; Fig. 6.3). Although significant differences were detected, the mean time minus the standard deviation for all categories did not approach the total feeding time of 30 s to which the mosquitoes were subjected; so, no adjustment of the conditioning protocol was made.

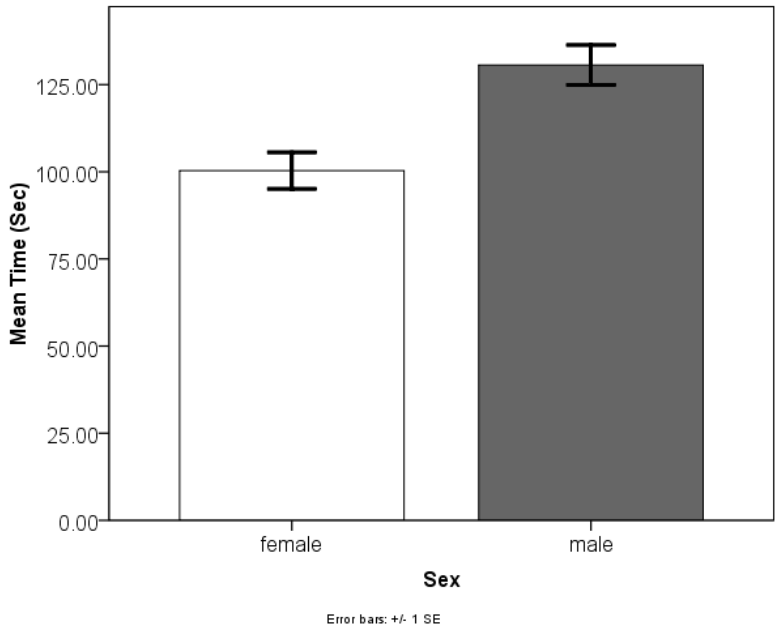

Figure 6.1. Mean (+/- SE) sugar-feeding time (s) for female (white) and male (gray) *Culex quinquefasciatus* adults.

Table 6.1: Full factorial analysis of variance for sugar-feeding time based on the factors of mosquito population (laboratory or field collected), age of the mosquito (1-2 d old or 3-5 d old), and sex of the mosquito (male or female).

Factor	d.f.	F-statistic	*P*-value
Population	1	5.744	0.019*
Age	1	2.331	0.130
Sex	1	19.545	<0.001*
Population x Age	1	5.353	0.023*
Population x Sex	1	0.919	0.340
Age x Sex	1	0.834	0.364
Population x Age x Sex	1	0.006	0.938
Error	88		

*Indicates significance observed at the $\alpha = 0.05$ level.

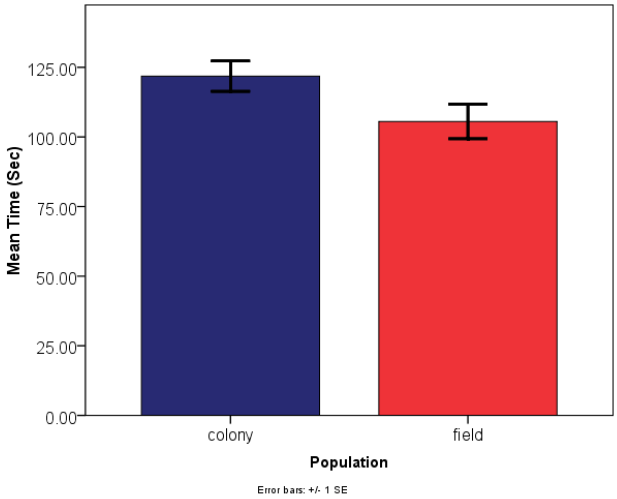

Figure 6.2. Mean (+/- SE) sugar-feeding time (s) for laboratory colony (blue) and field collected *Culex quinquefasciatus* mosquitoes (red).

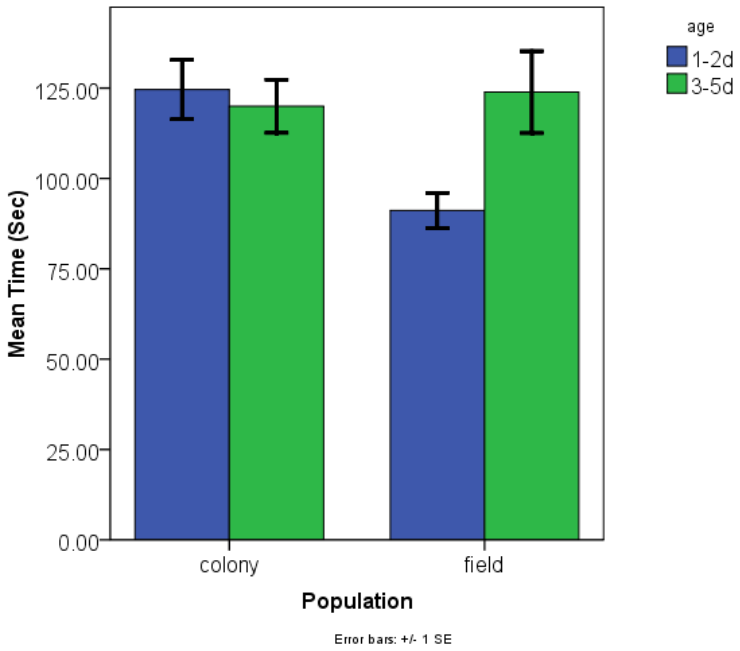

Figure 6.3. Mean (+/- SE) sugar-feeding time (s) for laboratory colony and field collected *Culex quinquefasciatus* mosquitoes by age (1-2 d old: blue; 3-5 d old: green).

Conditioning

The percent positive response of mosquitoes to the target odor following conditioning for each population, age and sex by time period between conditioning and testing is plotted in Figure 6.4. Overall the median positive response to the target odor following conditioning was 40%. In general, the trends present in positive responses to the target odor are not obvious, with the exception of 1-2 d old female laboratory-derived mosquitoes, where there was a steady reduction in percent positive response to the target odor over time. In contrast, the only positive responses to the target odor for 3-5 d old mosquitoes were at the shortest time period of less than 5 minutes (Fig. 6.4). There was also a lack of responses for 1-2 d old male mosquitoes from the field collected population at 24 h, which is due to the fact that approximately 95% of them died before testing could be conducted.

Table 6.2 displays the final model after both manual removal of non-significant interaction terms and backward stepwise variable selection using maximum -2log-likelihood. Model selection ended at step 13 had a -2 log-likelihood of 397.488 and Hosemer-Lemeshow statistics indicated the model was significant ($\chi^2 = 14.290$, d.f. = 7, $P = 0.046$). Logistic regression results differ from least squares regression in that the model compares the binary outcomes between categories ;and for groups with more than two categories, a dummy variable is created to compare within each group. This provides log odds (or odds ratios) and generates predicted probabilities for the categories. The odds ratios and Wald statistics for each factor in the model are presented in Table 6.2 as well.

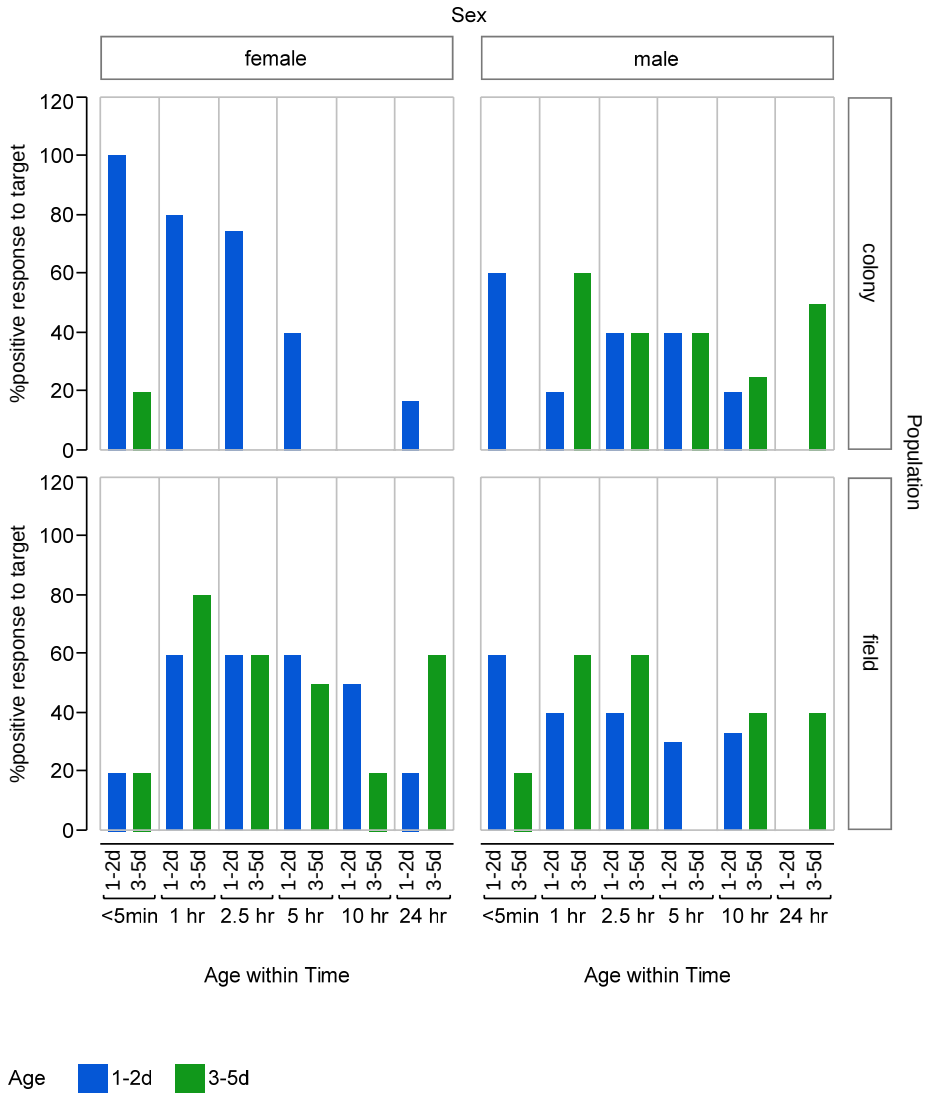

Figure 6.4. Percent positive response to the target odor following conditioning by female and male *Culex quinquefasciatus* mosquitoes from laboratory colony and field populations for six different time intervals between conditioning and testing by age (1-2 d old: blue; 3-5 d old: green).

Table 6.2. Final model for the full factorial logistic regression analysis of response to conditioning based on the factors of age of the mosquito (1-2 d old or 3-5 d old), the test the mosquito was administered (target, non-target, or blank odor pipette), the interaction between population the mosquito was derived from (laboratory or field collected) by the sex of the mosquito, the interaction between population, age of the mosquito and sex of the mosquito and the interaction between the population, age, sex and time interval between conditioning and testing.

Factor	Log Odds (β)	Wald Statistic	d.f.	*P*-value	Odds Ratio
Age (1-2d)	0.487	3.011	1	0.083	1.627
Test		88.898	2	<0.001*	
Test (blank)	-2.528	53.138	1	<0.001*	0.080
Test (non-target)	-3.322	48.121	1	<0.001*	0.036
Population (lab) x Sex (female)	-2.912	7.931	1	0.005*	0.54
Population (lab) x Age (1-2d) x Sex (female)	3.533	9.513	1	0.002*	34.252
Population (lab) x Age (1-2d) x Time x Sex (female)	0.000	4.445	1	0.035*	1.000

*Indicates significance observed at the $\alpha = 0.05$ level.

The significance of the test factor in the model is important, as it indicated that there was a significant difference in odds of responding to the target odor when compared to the non-target or blank (Table 6.2). This result is confirmed by examining Figure 6.5 which displays the mean predicted probabilities for positive response to the blank, non-target, and target odors. There were significantly higher odds of positive response to the target odor regardless of any of the other factors. A Kruskal-Wallis analysis of variance followed by individual Mann-Whitney U tests confirms that the mean predicted probabilities, are in fact, significantly different (K-W $\chi^2 = 400.019$, d.f. =

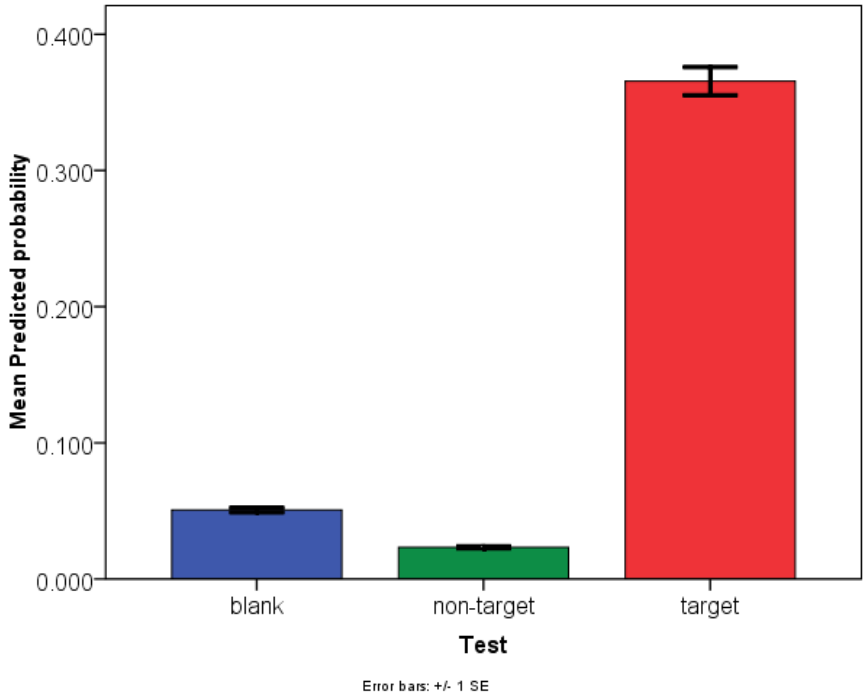

Figure 6.5. Mean (+/- SE) predicted probabilities of positive response generated by the logistic regression model for *Culex quinquefasciatus* mosquitoes offered an un-scented blank pipette (blue), the non-target odor (geraniol: green) or the target odor (jasmine: red).

2, P <0.001; M-W U (blank vs. non-target) = 7197.000, P <0.001; M-W U (blank vs. target) = 5782.00, P <0.001; M-W U (non-target vs. target) = 2730.000, P < 0001). From this result it can be inferred that conditioning was successful.

The only factor that was not significant on its own in the model was the age of the mosquitoes. However, the odds ratio suggests that there were 1.627 higher odds that a mosquito from the 1-2 d old age group would respond positively (Table 6.2).

The interaction between the population from which the mosquitoes were derived and the sex of the mosquitoes was a significant factor in the overall model (Table 6.2). There were -2.912 odds that a female mosquito from the laboratory colony would respond positively, which is supported by the mean predicted probabilities plotted in Figure 6.6. There was a significantly lower mean predicted probability that females from the laboratory colony population would respond positively (Fig. 6.6; M-W U = 9859.000, P <0.001).

A significant interaction between the population from which the mosquitoes were derived, the age of the mosquitoes, and their sex was also determined (Table 6.2). The logistic regression model indicates there were 3.533 higher odds that a female mosquito from the laboratory colony, aged 1-2 d old would respond positively. A plot of the mean predicted probabilities illustrates this relationship (Fig. 6.7). This is likely attributable to a surprising lack of positive responses from laboratory colony females aged 3-5 d old (Fig. 6.4). The mean predicted probabilities of 1-2 d old mosquitoes of all test categories are significantly higher than those of 3-5 d old mosquitoes (M-W U (females, laboratory

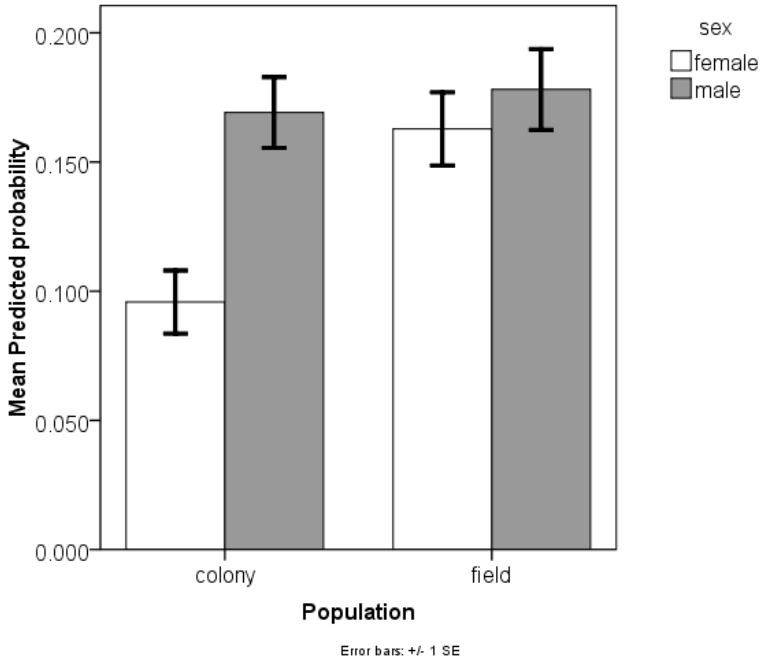

Figure 6.6. Mean (+/- SE) predicted probabilities of positive response generated by the logistic regression model for female (white) and male (gray) *Culex quinquefasciatus* mosquitoes derived from either the laboratory colony or field collected populations.

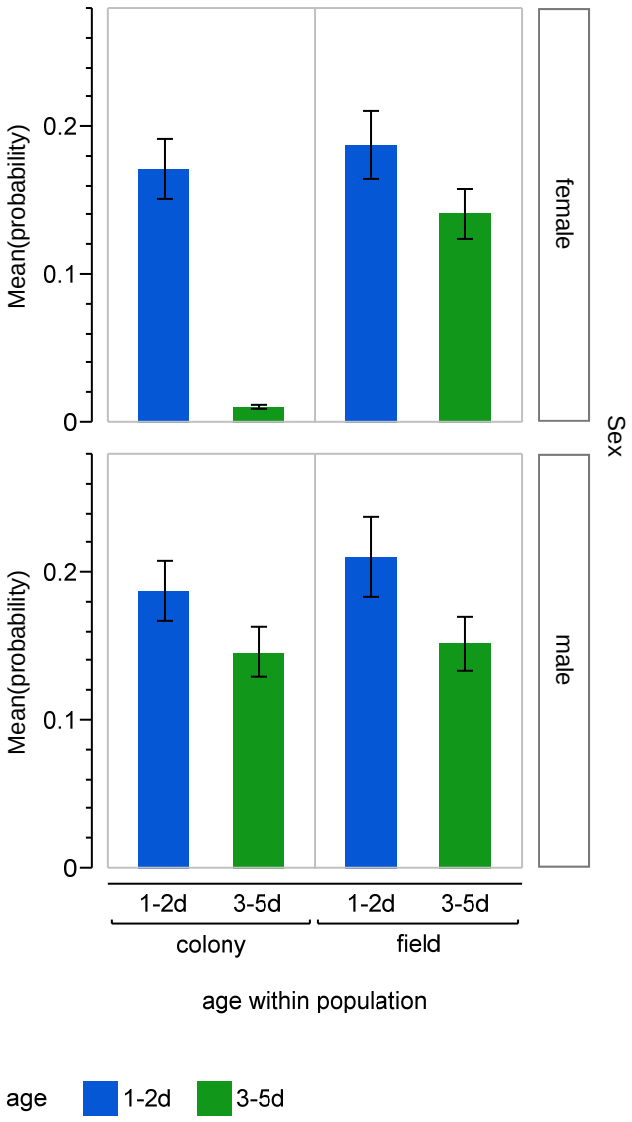

Figure 6.7. Mean (+/- SE) predicted probabilities of positive response generated by the logistic regression model for male and female *Culex quinquefasciatus* mosquitoes from laboratory colony and field collected populations by age (1-2 d old: blue; 3-5 d old: green).

colony) = 600.000, P <0.001;M-W U (females, field collected) = 2204.000, P < 0.001; M-W U (males, laboratory colony) = 2724.000, P < 0.001; M-W U (males, field collected) = 1602.000, P = 0.001). It thus can be inferred that mosquitoes aged 1-2 d old, regardless of population, had a higher probability of positive response following conditioning.

The only significant factor in the model relating to time between conditioning and testing, or the length of mosquito memory, was the interaction between the population from which the mosquito was derived, the age of the mosquito, the sex of the mosquito and the time between conditioning and testing (Table 6.2). The model indicates that there were significantly higher odds that a female mosquito from the laboratory colony, aged 1-2 d old would respond positively based on the time between conditioning and testing which is presented in the plot of mean predicted probabilities (Fig. 6.8) which, in turn, represents the trend observed in the actual data (Fig. 6.4). The mean predicted probabilities plot does not show significant differences among any of the other groups, but does display an unusually high predicted probability for 1-2 d old males from the field-collected mosquitoes at the 10 h time interval (Fig. 6.8). This high value does not match the observed data (Fig. 6.4), but perhaps, is an artifact of the missing 24 h male data for this population and age. Although not a significant comparison, there does appear to be a trend of higher mean predicted probabilities for the longest time period among mosquitoes in the 3-5 d old age group, with the only exception being the females from the laboratory colony population (Fig. 6.8). The

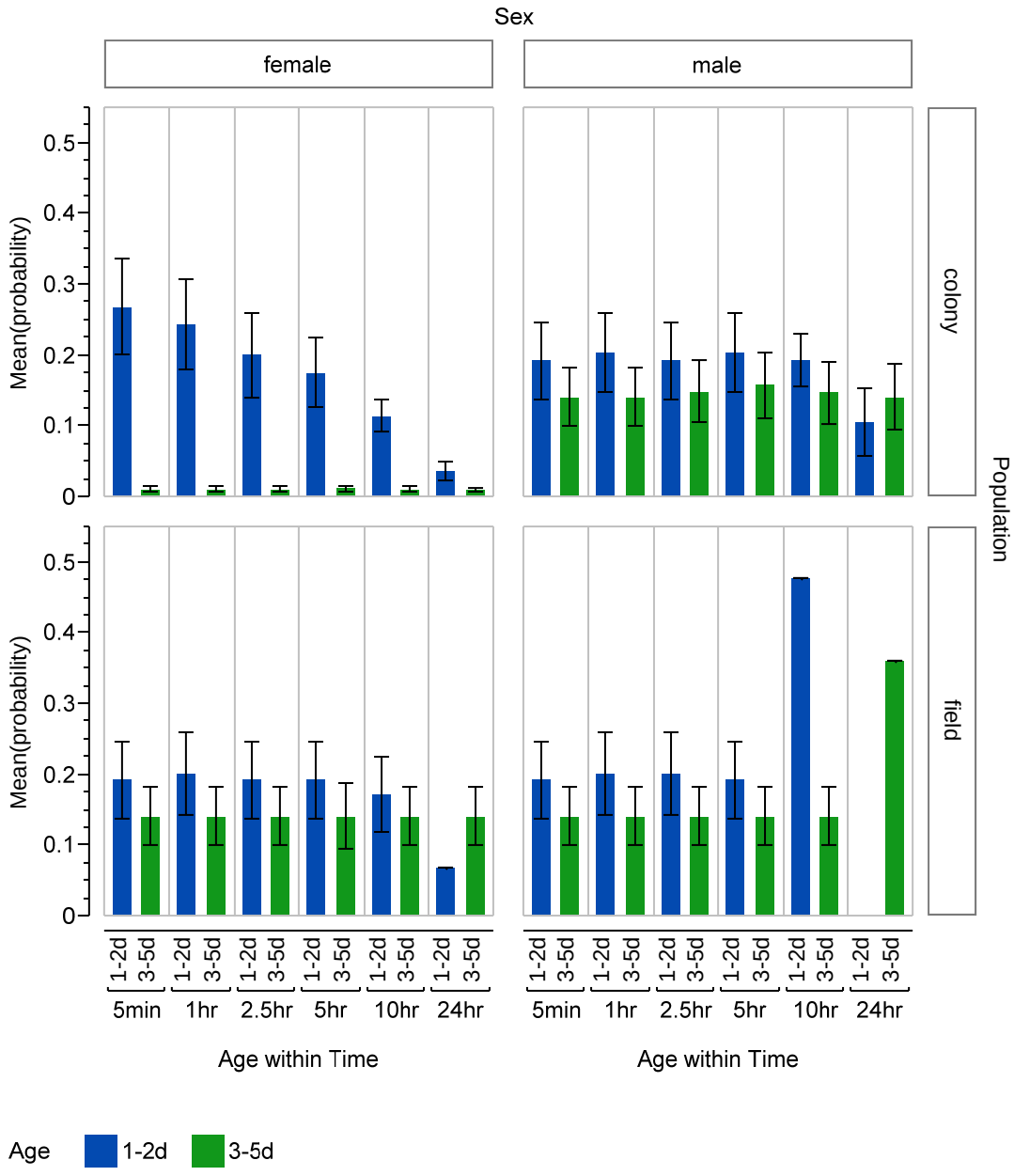

Figure 6.8. Mean (+/- SE) predicted probabilities of positive response generated by the logistic regression model for female and male *Culex quinquefasciatus* mosquitoes from laboratory colony and field populations (wild) for six different time intervals between conditioning and testing by age (1-2 d old: blue; 3-5 d old: green).

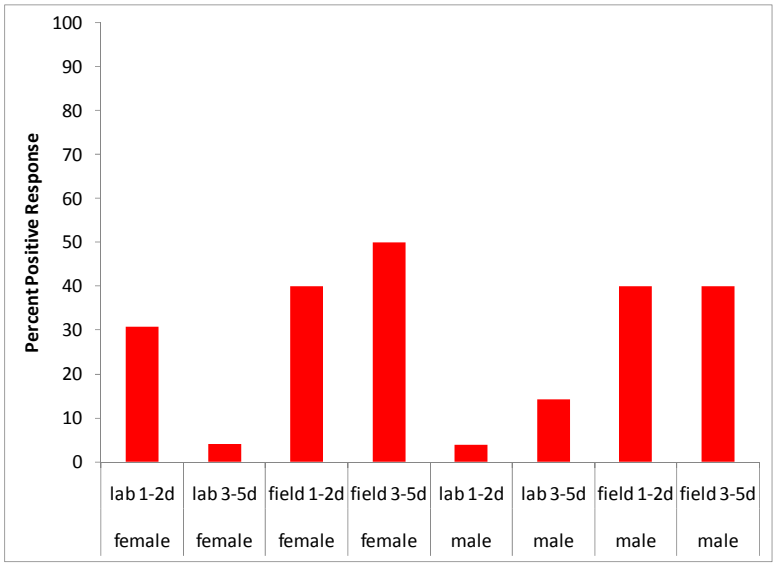

Figure 6.9. Percent positive response to the odor of jasmine flavor extract by naïve male and female *Culex quinquefasciatus* mosquitoes aged 1-2 d and 3-5 d old from laboratory colony and field derived populations.

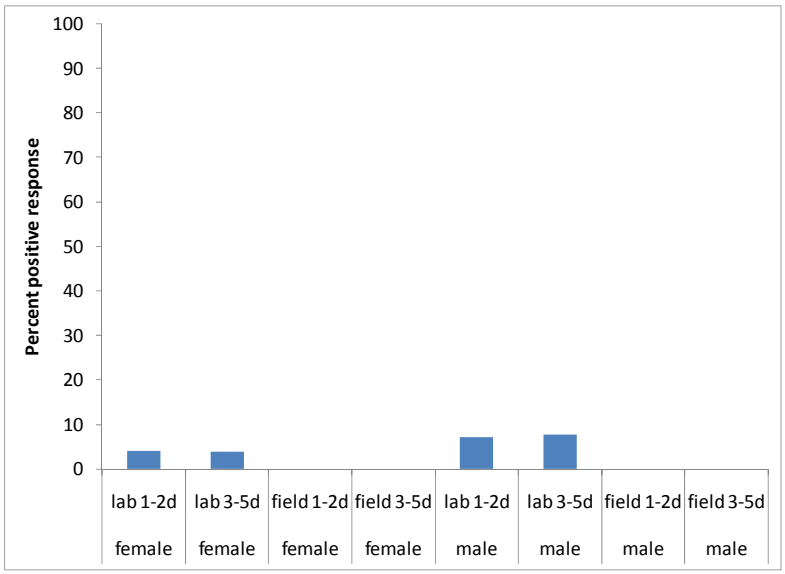

Figure 6.10. Percent positive response to the odor of geraniol by naïve male and female *Culex quinquefasciatus* mosquitoes aged 1-2 d and 3-5 d old from laboratory colony and field derived populations.

overall trend can also be observed that mean predicted probabilities are higher on average for all other time periods with available data, except for the longest time period (Fig. 6.8); however, this is not a significant trend in the model-generated predicted probabilities.

Innate Odor Response

Figures 6.9 and 6.10 display the percent positive response to the odors of jasmine flavor extract and geraniol, respectively. Overall innate responses to the odors were higher for jasmine than for geraniol. Field-collected mosquitoes had a higher innate response to jasmine than laboratory colony derived mosquitoes (Fig. 6.9). Interestingly the opposite trend was observed in the innate responses to geraniol, as only the laboratory colony mosquitoes responded positively to geraniol (Fig. 6.10).

Discussion

In this study the seemingly simple question was asked; i.e., how long does a mosquito remember, and came to discover that this not a simple question at all. Once all the relevant factors were accounted for, it appears that memory length in mosquitoes is dependent upon the interactions of the factors of the population from which the mosquito was derived, the age of the mosquito and the sex of the mosquito. Overall it was found that mosquito age is important. Younger mosquitoes (1-2 d old) that received conditioning as their first experience with sugar-feeding had higher probability of displaying a positive response. It was also found that probability of response was higher in young mosquitoes at all time intervals except for the longest time interval (24 h), although this was not significant.

Sugar-feeding time data were collected as a means to fine tune the assay; however, they yielded interesting results. Males take a significantly longer time to complete a sugar-meal than females. The significant difference between sugar-feeding times for males and females has been observed previously in *An. cracens* (Chapter VII) and for other species and populations of *Cx. quinquefasciatus* (Appendix B). Sugar-feeding times are not regularly published in the literature, so this represents an interesting contribution. This also represents an interesting finding in that male mosquitoes take a significantly longer time to feed. One possible explanation is that males are not under the same level of pressure to feed fast that females are when blood-feeding (Gibson and Torr 1999). Another possible explanation is that the females are filling the crop with the sucrose solution rather than the midgut (Friend et al. 1989) and this could be a shorter process. However, by the appearance of the mosquitoes as they were feeding, it appeared they were taking full meals and expanding the entire abdomen (personal observation). It is also interesting to note that younger mosquitoes taking their first sugar-meal from the field-collected population took shorter sugar-meals. A potential hypothesis for this observation may be that by 3-5 d of age, the older mosquitoes had experience with 10% sucrose in the cage and they had been conditioned to anticipate access to sugar and thus, took a longer time to feed to satiation. Another possible explanation might be that the volume of the meal differs; but, that was not evaluated by the current experimental design and there are numerous other factors that may account for the significant difference.

There is evidence to suggest that population level differences may occur in mosquitoes for associative learning. In this experiment it was determined that there was a significant interaction between the sex of the mosquito and the population from which they were derived. Female mosquitoes from the laboratory colony did not respond well to conditioning, with positive responses observed only for the shortest time interval (less than 5 min).There are two possible hypotheses that might help to explain these observed results. First, this difference may be due to the natural shift from carbohydrate-seeking behavior to blood-seeking, which is complicated by the fact that the observer for the experiments is a potential host. It has been suggested that working on associative learning of host-associated odors can lead to complications due to the observer/experimenter being a potential contaminating source of host odor (Alonso and Schuck-Paim 2006) and is one reason why this experiment examined associating sugar-meals and odors. However, by 3 d of age, female *Cx. tarsalis* can start to take blood-meals and develop eggs under optimal field conditions (Reisen and Reeves 1990); and at 5 d of age, the colony at Texas A&M is offered a blood-meal when it is under frequent use in experiments. A trend in female behavior is further suggested by the significant differences between male and female conditioned responses observed by Tomberlin et al. (2006) and in the previous chapter on sucrose concentration and associative learning (Chapter II) as well as in *An. cracens* (Chapter VII). This trend was not observed in the females derived from field-collected populations used in this study, which may or may not suggest long-term culture may select for females to be ready to blood-feed at 3-5 d of age.

The second hypothesis is that, the conditioning procedure used in this study does not lead to LTM formation. Tully et al. (1994) determined that less than ten trials of spaced conditioning did not induce LTM formation in *Drosophila*. Spacing of conditioning trials is required to initiate the protein synthesis required for LTM formation and is known as the spacing effect (Pagani et al. 2009). In the current study, three spaced trials were used, and there has been no other evaluation of trial number or length of time between trials to determine how that affects memory. There has yet to be any examination of how trial number or inter-trial-interval affects mosquito conditioning. It can be hypothesized that the number of trials used in this study was not enough to induce even medium-term memory in 3-5 d old females, because the length of memory that was able to be measured was 5 minutes or less and would fall within the short-term memory category as established with *Drosophila* (Tully et al. 1994). The fact that this observation was not made in field-collected female mosquitoes supports the suggestion that population is an important factor in interpreting learning data.

Mery and Kawecki (2002) suggested that selection for traits associated with learning are broader than just a simple associative task. They showed that learning itself is under selection and the selection pressure is in the context of ecologically-relevant information. In the current study it was observed that female mosquitoes 3-5 d of age did not respond well to conditioning and another possible explanation for this observation is that, if there is a cost associated with maintaining learning ability (Papaj and Snell-Rood 2007), colony females are not under selection pressure to maintain it. All the resources a mosquito needs are within the confines of the colony cage. However, if this is the case,

then it might be expected that males would also lose this ability, and this was not observed in the current study.

Another aspect of memory length that has been examined, particularly within the context of aging research, is age of the individual. Studies that examined age in *Drosophila* focused on the impact of advanced age on memory formation and retrieval, demonstrating that advanced age leads to reduced ability for LTM (Tamura et al. 2003, Mery 2007). The same can be said for cockroaches where advanced age appears to impair both acquisition and retrieval in cockroaches (Brown and Strausfeld 2009). Honey bees having early experiences with odor have been examined for their effect on learning later in life and not as memory length per se (Arenas and Farina 2008, Arenas et al. 2009). In the current study, two different age groups were used; i.e., representing newly-emerged mosquitoes with no prior access to sugar or the associated odors (1-2 d old) and those that had experience with sugar but not with the conditioning odors (3-5 d old). Mosquitoes at 3-5 d of age are not considered old, but this represents an important transitional stage for the completion of the female life cycle, since she shifts from sugar-seeking to host-seeking. It was found that mosquitoes that had never sugar-fed previously at 1-2 d of age had significantly higher odds and predicted probability of positive response following conditioning. This result was independent of memory length.

When considering both memory length and age of mosquito, the data indicate a trend that was not found to be significant, but nonetheless remains interesting. The mean predicted probabilities generated from the logistic regression model show, with the

exception of 3-5 d old laboratory colony females, that, regardless of population or sex, higher probabilities of positive response by 1-2 d old mosquitoes for all memory length intervals, except 24 h where the trend flips. At the longest memory interval of 24 h, where LTM is often considered (Tully et al. 1994), mosquitoes age 3-5 d old had higher mean predicted probabilities. This suggests that perhaps the conditioning protocol does not result in LTM for younger mosquitoes or that LTM formation is not fully developed in younger mosquitoes. If LTM is not developed in younger mosquitoes, it casts doubt on the hypothesis that long-term imprinting occurs at the beginning of adult life. The statistical results do not provide evidence that there is a significant trend in the data, and it is also possible that the lack of responses from 1-2 d old males from the field-collected material has created this artifact.

The first step to any behavioral analysis is the establishment of a good behavioral assay; and though many different permutations can and should be evaluated for this conditioning protocol, it appears to be effective in inducing memory formation. However, from this experiment, it has become evident that, in order to adequately compare mosquito memory to the best studied organism available on insect memory, *Drosophila*, more data need to be collected. The genome data for *Cx. quinquefasciatus* are not currently at the level of depth that would be required to effectively compare genetic mutants as has been done to determine the memory phases for *Drosophila;* thus, there is a need to to get more creative about the way experiments are designed. This might include experimental designs that include longer memory time intervals to examine the decay of anesthesia resistant memory and LTM. Unlike the genetic

experiments in *Drosophila*, data on conditioning and LTM memory formation using pharmacologic techniques to block protein formation could be conducted in mosquitoes. Understanding more about mosquito memory is not only important as it relates to mosquito life histories and their ability to transmit pathogens; it also opens doors for modifying and suppressing it. If memory is an important factor in driving disease transmission and the depth of knowledge achieved, as in *Drosophila* in terms of the available genetics and the ability to manipulate, then mutant mosquitoes incapable of different memory types could be produced.

Memory length in mosquitoes appears to be dependent on many factors and future work should take into account such factors as the genetic background of the mosquitoes under study. Learning ability and presumably, memory retrieval may be under as much selection pressure as other ecologically-relevant factors such as host preference. Plasticity in learning ability in mosquitoes may be closely tied to genes responsible for foraging activity as in *Drosophila* (Mery et al. 2007, Papaj and Snell-Rood 2007); and if that is the case, it opens up a whole new area of research taking into consideration the ecology of the mosquito when asking questions about learning and memory.

CHAPTER VII

EVIDENCE FOR OLFACTORY-BASED ASSOCIATIVE LEARNING IN *Anopheles cracens* SALLUM & PEYTON (DIPTERA: CULICIDAE)

Introduction

While associative learning has been studied in a diverse number of insect groups (e.g., Carew and Sahley 1986, Papaj and Prokopy 1989, Turlings et al. 1993, Hammer and Menzel 1995), the application of insect-learning principles and techniques to mosquito (Diptera: Culicidae) vectors has not been widely explored. Examples of learning in mosquitoes have thus far largely consisted of field observations on site fidelity where anophelines were found to come back to a previous feeding or resting site after removal (Charlwood et al. 1988, Hii et al. 1991, Renshaw et al. 1994, McCall et al. 2001). Olfactory-based appetitive learning in which an insect associates an odor with a food resource could have a significant impact on vector-host interactions and hence, disease transmission and has the potential to more accurately model malaria dynamics (McCall and Kelly 2002). There are some examples in the literature using mark-release-recapture techniques to observe the propensity of female mosquitoes to return to a host that had provided them with a successful initial blood-meal, presumably based on the odors associated with the host (Hii et al. 1991, Mwandawiro et al. 2000). In more controlled laboratory settings female *Culex quinquefasciatus* Say were found to be capable of associating a novel odor with a blood-meal or a sugar-meal (Tomberlin et al.

2006). Repeated exposure of *Cx. pipiens pipiens* biotype *molestus* Forskal to flower-associated odors in conjunction with sugar-feeding increased the innate attraction of the mosquitoes to these odors in a wind tunnel (Jhumur et al. 2006). In contrast, conditioning ability seemed to be lacking in *Aedes aegypti* L. (Alonso et al. 2003).

Sugar-feeding is an important part of mosquito life history requirements and is important for both males and females (Foster 1995). In *Anopheles gambiae* Giles, increased sugar-feeding can lead to decreased blood-feeding and hence, has the potential to reduce disease transmission (Gary and Foster 2001). A recent study showed that the application of insecticide to sugar host plants could provide at least localized control of adult *Culex* spp. (Muller and Schlein 2006, Schlein and Muller 2008). Thus the importance of sugar-feeding is not directed only toward individual mosquito energy needs but can be viewed in the more encompassing issues of vector control and disease transmission.

In light of the interest in sugar-feeding in anophelines and their importance as malaria vectors, the ability of *Anopheles cracens* Sallum & Peyton 2005, a member of the *An. dirus* complex and malaria vector in Thailand, to associate a novel odor with a food resource, a sugar-meal was tested, using a controlled laboratory approach. As far as is known, this represents the first application of olfactory-based appetitive techniques to determine the associative learning ability of an anopheline to a food resource. In utilizing learning in association with a sugar-meal, attempts were made to overcome the complication of a human observer in testing the behavior of an anthropophilic species during learning experiments (Alonso and Schuck-Paim 2006), and it allowed for the

investigation of the behavior of males. Based on published accounts of *Anopheles* spp. site fidelity and work with other species, it was expected that evidence of associative learning in this species would be found.

Materials and Methods

Mosquitoes

The colony of *An. cracens*, formerly *An. dirus* B which was formerly *An. balabacensis* Perlis form, is stenogamous and has been in culture in the Department of Parasitology at Chiang Mai University, Chiang Mai, Thailand since the 1980's when it was originally obtained from the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand (Sucharit and Choochote 1983). Immatures were reared at a density of approximately 80 larvae per plastic pan containing 2 liter of filtered tap water and were maintained on finely ground TetraBits® (Tetra GmbH, Melle, Germany) sprinkled on the surface of the water *ad libidum*. Pupae were transferred for emergence to 30 x 30 x 30 cm screen cages with a damp terry cloth towel placed over the top to maintain humidity. Adults had access to 10% sucrose (Wang Kanai, Bangkok, Thailand) and 5% multivitamin syrup (SevenSeas, OLIC (Thailand) Limited, Ayudhaya, Thailand) in distilled water via a soaked cotton wick in each cage. Blood meals for colony propagation were provided by use of a restrained white laboratory rat (*Rattus norvegicus* Berkenhout). Maintenance of all life stages took place in the Chiang Mai University, Department of Parasitology Insectary at 24 to 26ºC and relative humidity of 70 to 80%, with a 14:10 L:D cycle consisting of natural and artificial light (Somboon and Suwonkerd 1997).

For all portions of the experiment, 3 to 5 d-old male and female *An. cracens* were separated from the main colony and placed in a small screen-covered cup (approximately 200 ml in volume located within the main cage) and were deprived of the sucrose/multivitamin solution, but had access to distilled water soaked cotton for 24 h to ensure readiness to sugar-feed. Observations and conditioning took place during the later portion of the mosquito scotophase (approximately 1400 to 1800 h).

Sugar-feeding Time

In order to achieve the best possible response of each mosquito to the conditioning procedure, the mosquito must still be "hungry", hence retaining the drive to search for a sugar-meal after conditioning to adequately assess learning. For this reason, the amount of time that male and female *An. cracens* fed on sugar was observed to ensure that the mosquitoes would still have the potential to be responsive after conditioning. The mosquitoes were handled as in all experiments by depriving them of access to sucrose for 24 h prior to observation. Mosquitoes were moved from the insectary to a laboratory room (room temperature of approximately 24 to 26º C) with an operating fumehood and allowed to acclimate for at least 30 min (the procedure for all portions of the experiment). Observation of sugar-meal feeding was accomplished by use of red food coloring (Strawberry Red Color, Royallee Brand, Bangkok, Thailand) at a concentration of 20 drops in 50 ml of 10% sucrose in distilled water solution. Food coloring has been successfully used without behavioral interference (Lindh et al. 2006). Males and females were allowed to feed from a 200 µl calibrated glass micropipette (Drummond Scientific Company, Broomall, PA, USA) until they pulled the proboscis

out of the pipette. It was assumed that the distention of the abdomen in conjunction with removal of the proboscis from the pipette indicated satiation. These data were used to define the amount of time that the mosquitoes should be allowed to feed during the conditioning portion of the experiment by taking the mean feeding time minus the standard deviation (Fig. 7.1) and dividing by three. Food coloring was not used for the conditioning portion of the experiment to maintain consistency with a previously published study (Tomberlin et al. 2006).

Innate Odor Response

Another important component of the conditioning experiment with potential to impact the interpretation of the responses of individual mosquitoes is the innate response to the odors presented during conditioning. Conditioning to odors to which the mosquitoes have a strong innate attraction leads to complicated interpretation of learning; however, the use of odors that have little to no innate attractive properties has the potential to facilitate learning interpretation. For this study the chemicals myrcene (technical grade, Sigma-Aldrich, Co., St. Louis, MO, USA) and geraniol (98%, Sigma-Aldrich, Co. St. Louis, MO, USA) were selected based on their use in observing associative learning in other insects (e.g., myrcene: Rains et al. 2006) and the lack of attraction seen in other mosquito species (e.g., geraniol: Bowen 1992). Myrcene was chosen as the target odor to which the mosquitoes would be conditioned and geraniol was selected as the non-target odor with which the mosquitoes would not have any experience or exposure prior to the testing portion of the experiment. In other studies involving learning in other insect groups a single odor has been successfully used to

demonstrate learning and learning attributes (e.g. *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae); Lewis and Tumlinson 1988, Wackers et al. 2002, Tertuliano et al. 2004, Wackers et al. 2006; *Cotesia* spp. (Hymenoptera: Braconidae); Bleeker et al. 2006; *Anastrepha obliqua* (Macquart) (Diptera: Tephritidae); Cresoni-Periera and Zucoloto 2006). For this reason and the short time frame of the experiment myrcene was selected as the only training odor.

Observations on innate response were made by exposing male and female *An. cracens* for 15 s to empty 200 µl calibrated glass micropipettes which had the distal 1 to 1.5 cm coated with either 100% myrcene or 100% geraniol. A positive response (presumed to indicate attraction) was recorded if the mosquito moved toward the pipette opening and probed the surface or the paper on which they were standing, and a negative response (presumed to indicate lack of attraction) was recorded if the mosquito moved away from the pipette or did not make any observable movement (Tomberlin et al. 2006). These data allowed for the observation of the innate attraction of *An. cracens* to these odor chemicals. A high level (> 50%) of innate attraction was not observed to either chemical by either sex (Fig. 7.2). Handling of the mosquitoes prior to observations was conducted as in all other portions of the experiment.

Conditioning

Conditioning of the mosquitoes to the odor of myrcene was conducted as in Tomberlin et al. (2006), but with a few modifications. As in the previous study, individual mosquitoes were isolated into separate inverted clean glass shell vials (60 mm in height x 16.5 mm in diameter) placed on a clean sheet of disposable white office

paper (Double A, Chachoengsao, Thailand) under an operating fumehood (as described in the methods for sugar-feeding time). The mosquitoes were exposed to the odor in conjunction with a sugar-meal by offering the mosquito a myrcene-coated pipette (as in the procedure for innate odor response) filled with a 10% sucrose solution. As defined by observations on feeding time, males were allowed to feed for three intervals of 10 s separated by a 30 s inter-trial interval and females were allowed to feed for three intervals of 8 s separated by the same inter-trial interval.

The major difference in this study, as compared to those previously conducted, was the handling of the mosquitoes after conditioning. Each mosquito was labeled and kept in its shell vial by placing a piece of clean cotton (Ambulance, Ayudhaya, Thailand) in the open end, adding 2 to 3 drops of distilled water to the cotton, and placing it back into the insectary for 22 to 24 h before testing. This procedure was done in an attempt to overcome the potential confounding effects of odor habituation by moving the mosquito away from the conditioning area and evacuating the odors from the laboratory and fumehood where conditioning took place before testing was conducted.

Testing

Twenty-two to 24 h after conditioning, the mosquitoes were brought back into the laboratory and placed under the fumehood as previously described and allowed to again acclimate for approximately 30 min. As in previous studies, each mosquito was presented with an empty pipette which was coated with either the target odor (myrcene) to which they were conditioned, the non-target odor (geraniol) to which they were naïve, or a control pipette which had no odor associated with it and was not modified

(Tomberlin et al. 2006). Each mosquito was exposed for 15 s and the response was recorded as positive or negative as previously described.

Statistical Analysis

Data for sugar-feeding time were collected as the number of seconds that male and female mosquitoes fed; means and standard deviations were generated for these data (Fig. 7.1). A one-way Analysis of Variance (ANOVA) was used to compare the mean time to satiation for male and female *An. cracens*. The evaluation of innate response was recorded and presented as percent positive response (Fig. 7.2) and no further analysis was conducted on these data. The data for the conditioning portion of the experiment were collected as binary data points which were analyzed using binary logistic regression (Hardy and Field 1998, Hartz et al. 2001).

The binary logistic model was evaluated using the backward stepwise variable selection method based on the change in likelihood ratio (entry at $P = 0.05$ and exit at $P = 0.10$). In this study, the factors of interest were the generation from which the mosquito was derived, the sex of the mosquito, the odor used to test the mosquito (target, non-target, or control), and the amount of time between conditioning and testing. Binary logistic regression provides an overall measure of model fit, log-odds and predicted probabilities. When a significant factor was found in the model, an ANOVA coupled with Tukey's HSD post hoc analysis was run on the generated predicted probabilities to determine which groups differed from each other. All statistics were computed with the SPSS 14.0 software package (SPSS 2005) and statistical significance

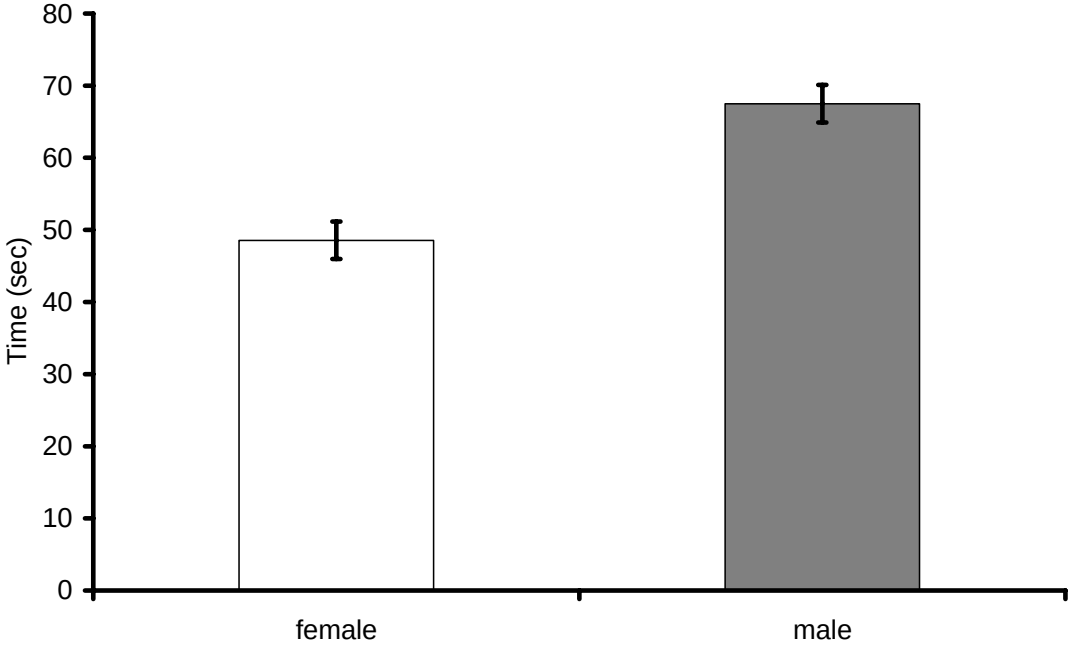

Figure 7.1. Mean sugar-feeding time (±SE) in seconds to satiation (as defined in the methods) of male and female *Anopheles cracens*. A significant difference was observed between mean male and female feeding times (ANOVA: $F = 23.88$; d.f. = 1, 59; $P < 0.001$).

was observed at $\alpha = 0.05$. Plots were generated either within SPSS 14.0 or with Microsoft Excel 2003 (Microsoft, Corp. Redmond, WA, USA).

Results and Discussion

Sugar-feeding Time

Results of the sugar-feeding time observations indicated that males of *An. cracens* took a significantly longer time than females to feed to satiation (Fig. 7.1; $F = 23.88$; d.f. = 1, 59; $P < 0.001$). Males had a mean feeding time of 67.50 s (± SE 2.85; n = 26) while females were satiated after a mean of 48.56 s (± SE 2.60; n = 34). The amount of time that mosquitoes of different species sugar-feed is not widely reported, and these data show a significant difference in the amount of time that male and female mosquitoes need to extract a sugar-meal until satiated from a glass pipette. Females took a shorter time to imbibe a sugar-meal than males, which is potentially related to a greater overall need to take a liquid meal quickly as in taking blood from a moving host during blood-feeding.

Innate Odor Response

Observations of innate responses of male (n = 8) and female (n = 10) *An. cracens* indicated that males had no observable innate attraction to either myrcene or geraniol. However, 30% of the females presented with myrcene responded positively and 18.2% of the females exposed to geraniol responded positively (Fig. 7.2). Different procedural modifications could be made to determine whether the differences in innate response and conditioned response are real. One method that could potentially sort out the ability to be conditioned as well as the ability to discriminate odors after conditioning would be to

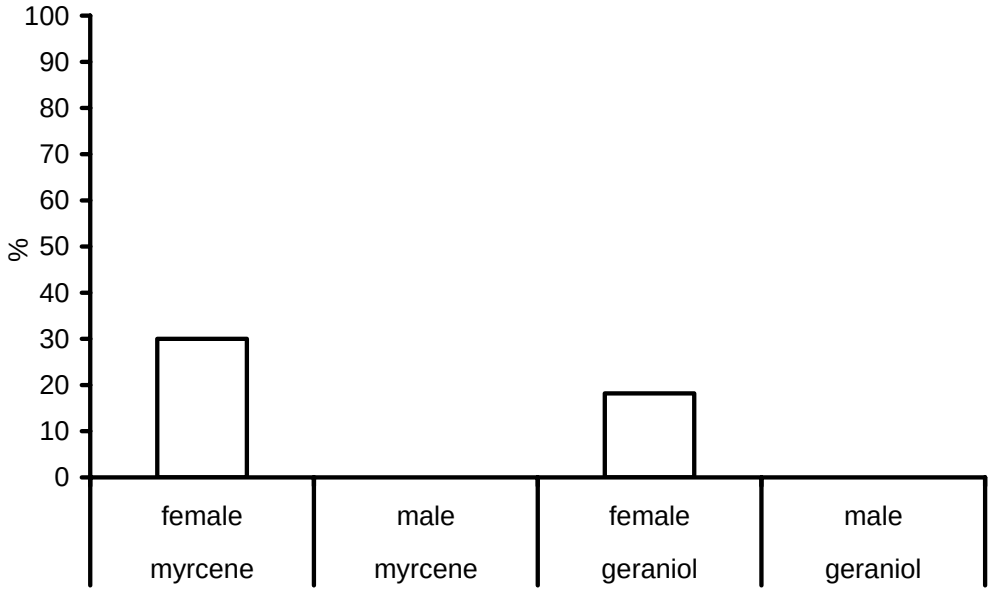

Figure 7.2. Percent positive response of naïve 3 to 5 d old male and female *Anopheles cracens* to the odors of myrcene and geraniol after a 15 s exposure.

Table 7.1: Final binary logistic regression model for female *Anopheles cracens* conditioned to the odor of myrcene (Hosmer-Lemeshow χ^2: 3.709, d.f. = 8, *P*= 0.882). The odor of geraniol was used as the non-target odor and the blank test consisted of an un-altered blank pipette. The model is defined for the response variable of positive outcome. * indicates a significant difference at the $\alpha = 0.05$ level.

Variable	Log Odds (β)	d.f.	Wald	p	Odds Ratio (e^{β})
Generation		3	1.87	0.599	
Generation (1)	19.831	1	4.38×10^{-6}	0.998	2.6×10^{8}
Generation (2)	18.443	1	3.97×10^{-6}	0.998	1.0×10^{8}
Generation (3)	20.320	1	4.82×10^{-6}	0.998	6.7×10^{8}
Test		2	3.15	0.207	
Test: Blank	-2.223	1	3.15	0.076	0.108
Test: Non-target	-20.437	1	6.04×10^{-6}	0.998	0.000
Constant	-19.978	1	4.66×10^{-6}	0.998	0.000

use a three port olfactometer where the mosquito then has simultaneous choice of the blank, the non-target, or the target odors.

Appetitive Olfactory-based Associative Learning

An overall model of male (n = 50) and female (n = 58) mosquitoes indicated that the sex of the mosquito, generation of the mosquito and the test odor to which the mosquito was exposed were all significant factors in the model (Hosmer-Lemeshow χ^2 = 2.892; d.f. = 7, P = 0.895). However, plots of the predicted probabilities suggested that a large portion of the observed significance was within the data for male mosquitoes; thus, the data were analyzed separately for each sex.

Female Mosquitoes. Model selection for the regression of female *An. cracens* ended at step two, with the only factor dropping out being the time between conditioning and testing and none of the factors were significant in modeling the data as tested by the Wald statistic (Collett 2003); Table 7.1). A plot of the percent positive response by females to the tested odors (Fig. 7.3) reveals that only 21.1% of females conditioned to myrcene responded positively to myrcene during the testing portion of the experiment. However, no females responded positively to the non-target odor of geraniol and 5.3% of females responded to the blank control pipette.

It is interesting to note that females responded positively at a lower level during testing than during observations on innate response to the odor chemicals (Fig. 7.2). There is an 8.9% difference between the percentage of mosquitoes that responded positively to myrcene without prior experience and those conditioned to myrcene. Not

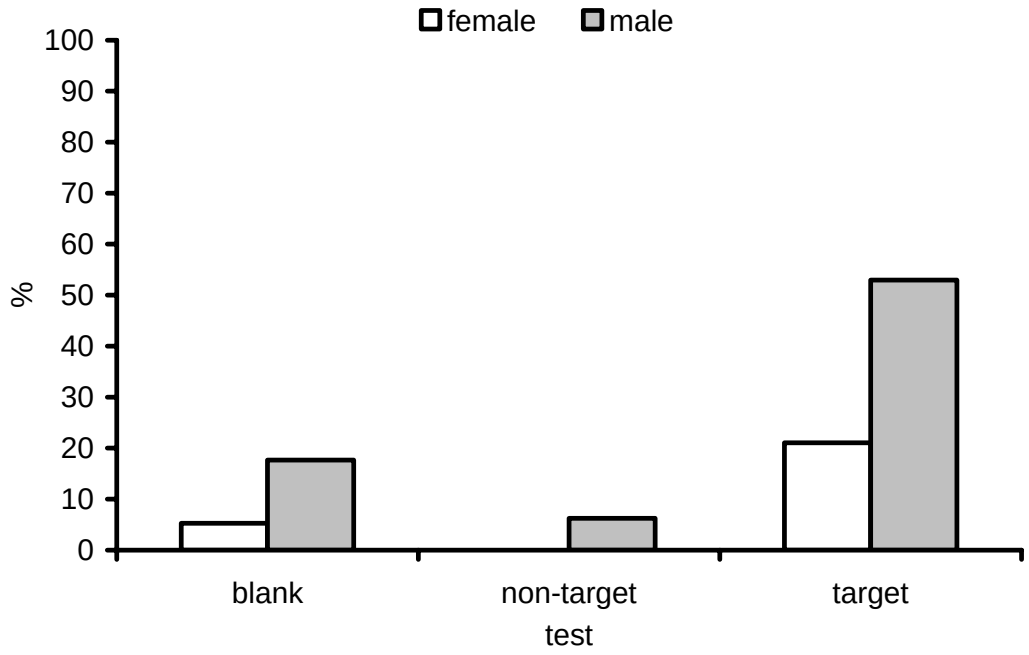

Figure 7.3. Percent positive response of conditioned 3 to 5 d old male and female *Anopheles cracens* to a single test of either a blank control pipette, a geraniol coated non-target odor pipette, or a myrcene coated target odor pipette for 15 s.

only were fewer females responding to myrcene after conditioning but 12.9% fewer were responding to geraniol after conditioning with myrcene. These data suggest that females are recognizing and are perhaps able to discriminate between the odors, but they are not responding by displaying the behavioral response used to evaluate learning. It is possible that females at the age of three to five days are switching to a host seeking/blood-feeding behavioral pattern, as in anautogenous *Culex tarsalis* Coquillet which are ready to host-seek at about three days of age (Reisen and Reeves 1990), such that they will opportunistically take a partial sugar-meal, but do not have the behavioral impulse to sugar-feed again at this age. For this species, it is not known whether females sugar-feed daily or whether blood can be used as a direct energy source. Assuming that sugar-seeking and host/blood-seeking are two separate behavioral repertoires, if females can obtain energy directly from blood, as in other anophelines (e.g. *An. gambiae* Gary and Foster 2001), and hormonal control of host seeking and blood feeding are beginning to dominate the female's behavioral patterns at three to five days old, the partial sugar-meal taken during conditioning may fulfill the energy deficit caused by starvation and no further sugar-feeding will be required within the 22 to 24 h post-conditioning period. A procedural modification that could aid in sorting out the confounding effects of female mosquito age would be to condition females younger than three to five days old, perhaps as their first sugar-meal experience.

There are simpler explanations for the lack of demonstrable learning ability in females. It is possible that the small sample sizes used in this study for demonstration of innate odor response overestimate the actual innate responses; in which case, there is

little reason to suspect that female *An. cracens* have classical conditioning abilities to a sugar-meal. Due to time limitations on the study, a larger sample size was not achieved. It is also possible that the odor chemicals used or the time of day that the mosquitoes were being conditioned and tested was not adequate for conditioning of female *An. cracens.*

Table 7.2: Final binary logistic regression model for male *Anopheles cracens* conditioned to the odor of myrcene (Hosmer-Lemeshow χ^2: 3.424, d.f. = 8, P= 0.905). The odor of geraniol was used as the non-target odor and the blank test consisted of an un-altered blank pipette. The model is defined for the response variable of positive outcome.

Variable	Log Odds (β)	d.f.	Wald	*P*	Odds Ratio (e^{β})
Generation		3	3.04	0.386	
Generation (1)	2.085	1	2.46	0.117	8.046
Generation (2)	2.123	1	2.69	0.101	8.358
Generation (3)	1.543	1	1.32	0.250	4.680
Test		2	8.61	0.016*	
Test: Blank	-1.835	1	4.63	0.031*	0.160
Test: Non-target	-3.008	1	6.50	0.011*	0.049
Constant	-1.389	1	1.57	0.211	0.249

* indicates a significant difference at the α = 0.05 level.

Male Mosquitoes. The model generated from the male-conditioning data indicated that the variable test (which refers to the test administered to a given mosquito) was a significant factor in the model (Table 7.2). Variable selection ended at step three; however, the model generated at step two was selected for use in generating predicted probabilities, as the third model reduced overall variation in the generated predicted

probabilities to only three values. The Hosmer-Lemeshow χ^2 value for this model was 3.424 (d.f. = 8) and was not significant ($P = 0.905$), indicating an acceptable model (Collett 2003), with the only factor removed from the model being the time between conditioning and testing of the mosquito. The model indicates that the odds of a mosquito responding positive to the blank control pipette were only 0.160 and the odds of responding to the non-target odor pipette were only 0.049 when compared to responding to the target odor.

A plot of the actual percent positive male responses (Fig. 7.3) shows a trend similar to that observed in females, but at a much higher level; and the responses otherwise match the overall trends observed in the model (Fig. 7.4). Males tested with a myrcene-coated pipette responded positively at 51.9% after conditioning to myrcene. Responses to the blank control were higher than those to the geraniol non-target odor at 17.6% and 6.3% positive responses respectively. The predicted probabilities generated from this model were normalized with the Log_{10} transformation and subjected to an ANOVA coupled with Tukey's HSD post hoc analysis. These results revealed that there was significant difference among the probabilities for the tests administered ($F = 38.420$; d.f. = 2, 47; $P < 0.001$) and that the probability of responding positively to each of the administered tests were significantly different from each other (Fig. 7.4). The data indicate that males display classical conditioning ability.

The importance of learning in males has been less thoroughly examined in mosquitoes probably due to their lack of direct importance in disease transmission. In

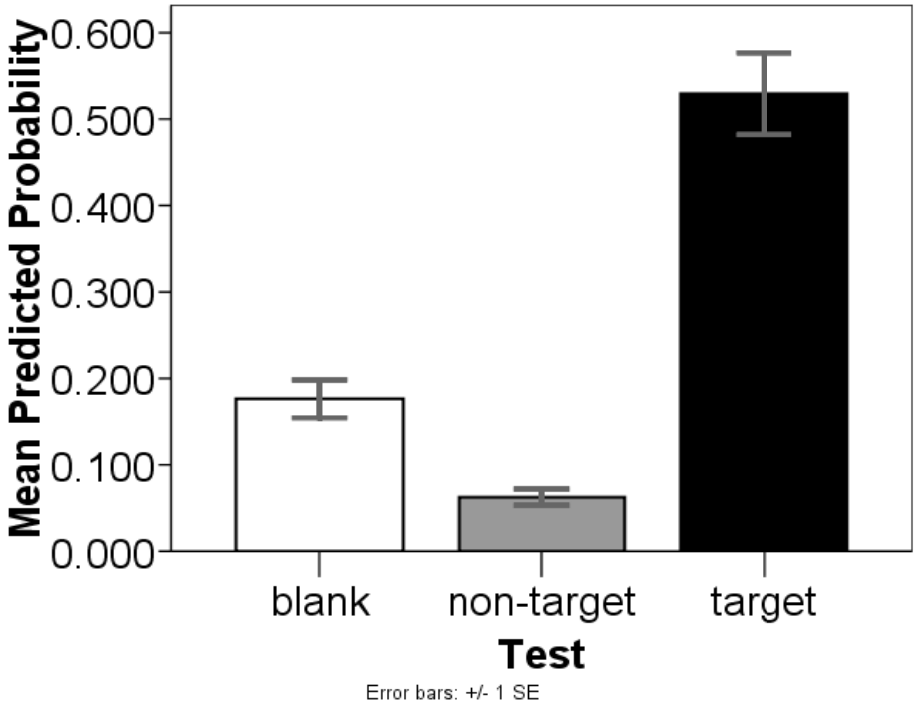

Figure 7.4. Final binary logistic regression model generated mean predicted probabilities (± SE) of a positive response by male *Anopheles cracens* to either a blank control pipette, a geraniol coated non-target pipette or a myrcene coated target odor pipette after conditioning to the odor of myrcene. A Log_{10} transformation for normality followed by ANOVA and Tukey's HSD revealed that the probability of a positive response to each test differed significantly.

other mosquito species, sugar-feeding events by males may occur about every 24 h before or after swarming (Reisen et al. 1986); thus, it seems that memory across a 24 h period might be advantageous for male mosquitoes that must sugar-seek at this interval. However, the swarming and sugar-feeding behavior of natural populations of *An. cracens* is not currently known.

There is a great deal of information yet to be gathered on associative learning in mosquitoes before these data can be incorporated into predictive disease models. However, studies such as the one just described emphasize that examining only one species within the Culicidae does not define the learning abilities of all mosquitoes. This study adds to our current understanding of the associative learning capabilities of the Culicidae through investigation of mosquitoes in the genus *Anopheles,* which had not been previously studied in a controlled laboratory setting. Furthermore the examination of sugar-feeding allowed for the examination of the learning abilities of male mosquitoes, which often get overlooked when working with host-seeking and blood-feeding by females. This study documents an interesting observation that either females of this mosquito species do not learn under the same conditions as males, or they do not learn odors in association with a sugar meal. Future research should take advantage of different procedural techniques, such as an olfactometer or windtunnel and shorter time intervals between conditioning and testing, to try and determine the learning abilities of females in this species.

CHAPTER VIII

GENERAL CONCLUSION

For a long time, mosquito research has focused on the problem solving aspects of medical entomology and defining the epidemiologic triad in different locations, with different pathogens and different vectors. While there is no doubt that this is important, because it directly impacts human and animal health, there is so much more to consider when one incorporates the complexity that mosquito learning behavior brings to the equation. Associative learning of odors with resources can be beneficial to an animal that lives in a habitat where resources change rapidly, giving them the flexibility to adapt to changing conditions within their lifespan. Mosquitoes exemplify this type of life history where they must contend with the shift from aquatic to terrestrial habitat, search for carbohydrates, search for hosts from which to take blood, and finally return to the aquatic habitat to deposit eggs. This dissertation covers a broad range of topics where associative learning and memory may interact with the three major life history domains of mosquitoes.

This research refined the methods used to condition mosquitoes in an individually-measured assay. It applied the statistical analysis method of binary logistic regression to the conditioned responses which allowed for simultaneous testing of the factors potentially involved in associative learning. Conditioning of mosquitoes to odors associated with sugar allowed for the determination of female and male mosquito behavior while reducing the confounding effects of host associated odors and the

observer. Using different sucrose concentrations, it was suggested that mosquitoes may respond differently to not only conditioning, but to the sucrose concentration itself. The data generated from this research open up new questions about mosquito sugar foraging in nature and how associative learning may play a role.

The conditioning assay was then taken on the road, to Thailand, to examine olfactory-based associative learning in another genus of mosquitoes with a completely different life history. The ability of *Anopheles cracens* Sallum & Peyton to associate a sugar-meal with myrcene was evaluated at a 24 hour time interval. Evidence for learning was found in males, but not in females. This generates many questions about the inherent differences between males and females and their reliance on olfactory cues in general. It also leads to the question of whether the assay conditioned mosquitoes for memory with enough persistence to last 24 hours in females.

Memory length has been studied in *Drosophila* with the use of genetic knockout mutants and dissected into multiple components that differ by onset and duration (Tully et al. 1994). Mosquito memory length was evaluated in an experiment that incorporated the factors of mosquito population, mosquito, age and mosquito sex. The data showed that younger mosquitoes had higher levels of positive response after conditioning and suggested that field-collected mosquitoes may have longer memory capabilities than laboratory colony-derived mosquitoes. It also suggested a new hypothesis for differences observed in male and female responses to conditioning. These represent the first dataset on mosquito memory and generate many questions related to the conditioning assay. The length of a mosquito's memory has implications for both host-seeking and oviposition.

A female mosquito's second blood-meal is the most important in terms of pathogen transmission. Several field studies have suggested that different mosquito species may be able to remember olfactory information about their first host and return to that host type or location at the second meal (Charlwood et al. 1988, Hii et al. 1991, Mwandawiro et al. 2000, McCall et al. 2001). Following blood-feeding on a chicken or a human in this study, mosquitoes were offered a choice at the second blood-meal of odors associated with either human or the chicken. No significant effect was observed; and while it may be possible that there is no effect and mosquitoes were not conditioned by the first experience, it is also possible that there was a flaw in the experimental set-up that prevented adequate observation of the behavior. This is definitely an area that should be pursued further, because it has the most direct effect on pathogen transmission and can easily bridge into the study of plasticity associated with host preference in some species.

Although demonstrated in only two published studies (McCall and Eaton 2001, Kaur et al. 2003), there is growing consensus that mosquitoes learn about their larval habitat and preferentially lay eggs in habitats with similar chemical cues. The work described here does not support this hypothesis, based on generalized habitat cues or the presence of predators. However, the data do support the observation that *Culex quinquefasciatus* Say does not avoid habitats with predatory fish cues (Van Dam and Walton 2008, Walton 2009). Perhaps more interesting is the fact that predatory fish induced non-consumptive effects in the developing larvae and pupae in the separated habitats. These data do not close the door on the idea that mosquitoes can learn about

their larval habitats nor does it disprove that mosquitoes do not detect fish cues at oviposition; they merely suggest that not all species respond the same way about habitat cues and predatory fish.

The value of this work really lies in questioning the assumptions we often make regarding mosquitoes, questions such as those involving the assumption that studying a single species equates to knowing about all mosquitoes (Klowden 2007) or the assumption that males are unimportant to research dealing with pathogen transmission and blood-feeding females. It also touches on the assumptions made about pathogen transmission and how mosquitoes may select hosts. Long held assumptions about host preference are becoming less and less established doctrine and revealing themselves to be more flexible than was ever known. All of this can only lead to better understanding of the link between host preference and pathogen transmission and thus, better interventions in the future.

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APPENDIX A

PRELIMINARY DATA ON OLFACTORY-BASED ASSOCIATIVE LEARNING IN *Anopheles minimus* THEOBALD (DIPTERA: CULICIDAE)

Description of data

These data resulted from preliminary studies of associative learning ability of *Anopheles minimus* species A Theobald that were collected concurrently with the data on *An. cracens* (Chapter VII) and utilized the same methods. The source of the *An. minimus* A colony used for this study is described in Somboon & Suwonkerd (1997). Data were collected on male and female sugar-feeding time (Figure A1), innate odor response to jasmine flavor extract (Winners Brand, Bangkok, Thailand) (Figure A2), and conditioned response to jasmine flavor extract (Figure A3). These data were not included with the dataset for *An. cracens* due to small sample sizes yet they represent the first data collected on this species for sugar-feeding and olfactory conditioning.

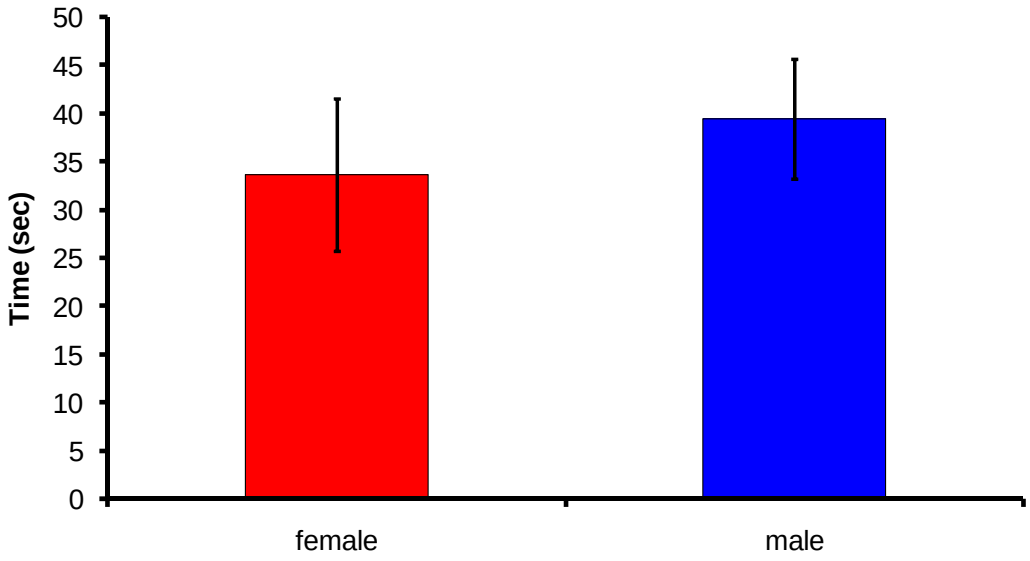

Figure A1. Mean (+/- SE) sugar-feeding time of male (n = 11) and female (n = 23) *Anopheles minimus s. s.* for 10% sucrose solution dyed with food red food coloring (for method see Chapter V).

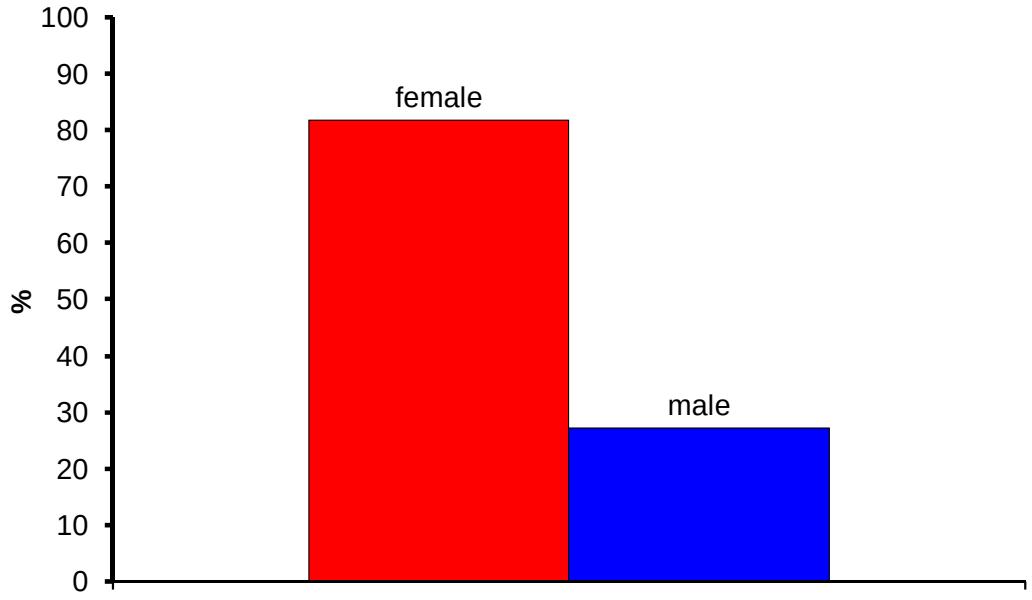

Figure A2. Percent positive response to jasmine flavor extract by naïve male and female *An. minimus* using methods described in Chapter VII for *An. cracens*. Sample size for males = 11 individuals and for females = 11 individuals.

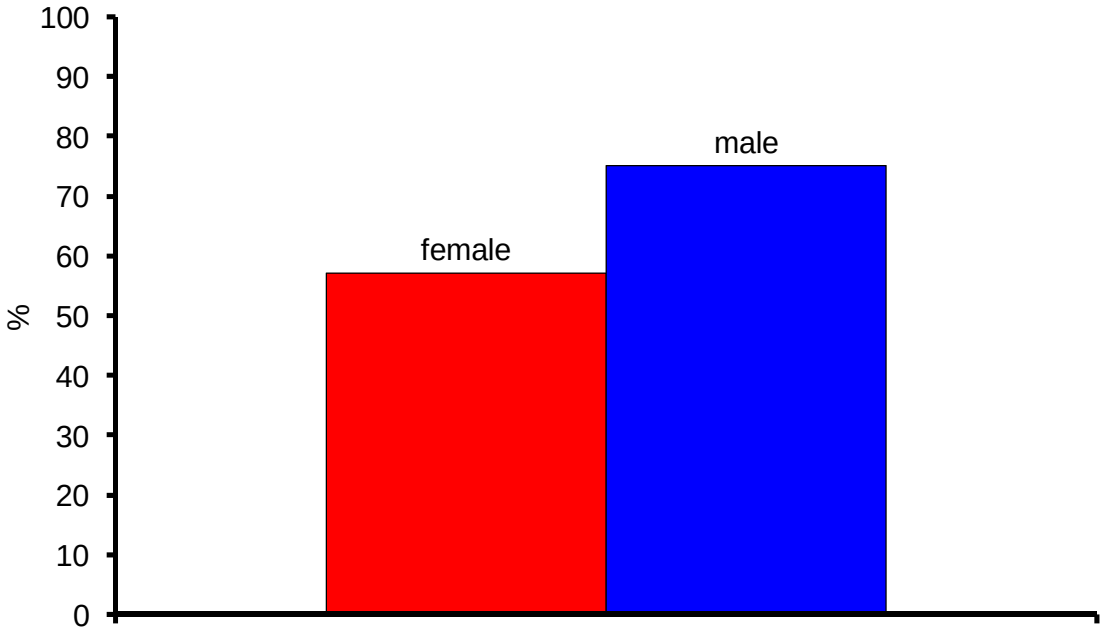

Figure A3. Percent positive response to jasmine flavor extract following conditioning by male and female *An. minimus* following the methods described in Chapter VII. Sample size for males = 8 and for females = 7 individuals. The percent positive response after conditioning is lower than the naïve response of individuals (Fig. A2).

APPENDIX B

SUGAR-FEEDING TIMES OF DIFFERENT MOSQUITO SPECIES AND POPULATIONS

Description of data

These data were collected for the purpose of fine tuning the conditioning assay to ensure that mosquitoes receive some sugar but presumable maintain the motivation to continue sugar-seeking after conditioning. Data were collected using 10% (w/v) technical grade sucrose (Sigma-Aldrich, Co., St. Louis, MO, USA) solution dyed with red food coloring (Strawberry Red Color, Royallee Brand, Bangkok, Thailand) at a concentration of 12 drops dye to 30 ml of sucrose solution. This mixture was offered to each mosquito using a new 200 µl calibrated micropipette (Drummond Scientific Company, Broomall, PA, USA). Figure B1 displays the mean sugar-feeding times for male and female *Anopheles cracens* Sallum & Peyton, *Anopheles minimus* Theobald and *Culex quinquefasciatus* Say collected in Chiang Mai, Thailand, a laboratory colony derived from Gainesville, FL, USA (in continuous culture for 19+ yr) and field collected material from College Station, TX USA all aged 3-5 days and starved for 24 h before testing.

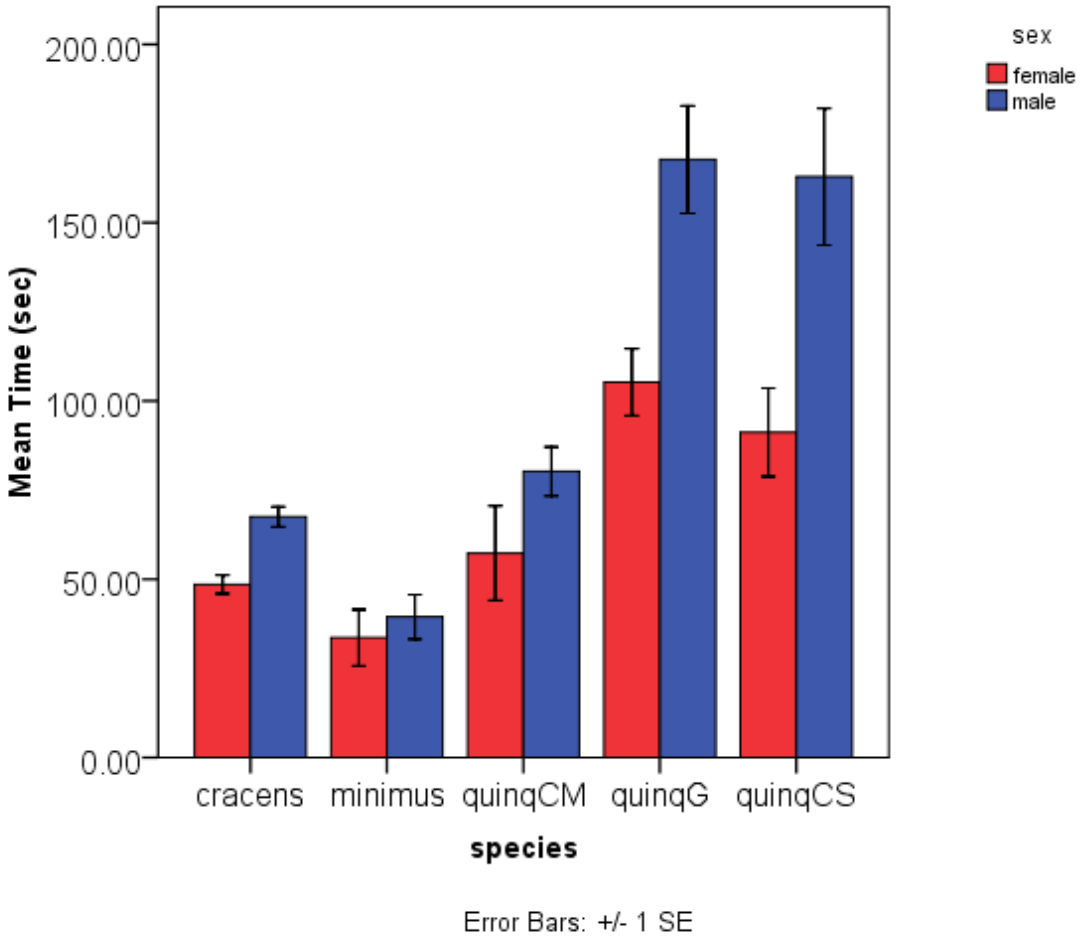

Figure B1. Mean (+/- SE) sugar-feeding times for male (blue) and female (red) *Anopheles cracens* (cracens), *An. minimus* (minimus), *Culex quinquefasciatus* from Chiang Mai, Thailand (quinqCM), *Cx quinquefasciatus* from a colony derived from Gainesville, FL, USA (quinqG) and *Cx. quinquefasciatus* from College Station, TX, USA (quinqCS).

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