8-2011

ZOOS AS EXPERIMENT ENVIRONMENTS: BIOLOGY OF LARVAL AND ADULT MOSQUITOES (DIPTERA: CULICIDAE)

Holly Tuten
Clemson University, htuten@gmail.com

ZOOS AS EXPERIMENT ENVIRONMENTS: BIOLOGY OF LARVAL AND ADULT MOSQUITOES (DIPTERA: CULICIDAE)

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Entomology

---

by
Holly Coleen Tuten
August 2011

Accepted by:
Peter H. Adler, Committee Chair
William C. Bridges, First Reader
Kimberly S. Paul, Second Reader
William Wills, Third Reader

ABSTRACT

Zoos are a unique environment where humans and animals are in close daily contact, potential mosquito habitats exist, exotic plants and animals are introduced regularly, and wild animals roam. Studies of mosquito behaviors in zoos will lead to a better understanding, both within and outside zoos, of disease transmission routes and mosquito biology. To investigate whether the unique assemblage of habitats in zoos affects mosquito behavior, I sampled larvae and adults in the Greenville Zoo and the Riverbanks Zoo, South Carolina, USA, from March 2008 to January 2011. The objectives of my study were to investigate mosquito oviposition behavior, blood-host usage, and transmission of the causative agent of dog heartworm (*Dirofilaria immitis*); document the structure of the mosquito pyloric armature; and provide zoos with suggestions for mosquito control. My results underscore the medical and veterinary importance of studying mosquito blood feeding ecology in zoos, and the experimental utility of zoos for studying mosquito behavior.

A total of 1,630 larvae and 4,349 adults representing 16 species was collected and identified. The most common species were *Aedes albopictus*, *Ae. triseriatus*, *Culex erraticus*, *Cx. restuans*, and *Cx. pipiens* complex. Principal components and multiple logistic regression analyses showed that across both zoos the overall larval mosquito presence (regardless of species) was predicted by ambient and site temperature, precipitation, dissolved oxygen, presence of natural habitats, and absence of aquatic vegetation. Pairwise species associations indicated significant habitat-based relationships

between larvae of *Ae. albopictus* and *Ae. triseriatus*, and *Cx. pipiens* complex and *Cx. restuans*. Recommendations to zoo personnel, regarding larval mosquito habitat management, were to reduce or eliminate artificial containers and shade sources greater than or equal to 2 m above standing water, use mosquito larvicides when source reduction is not possible, and receive training in recognizing and mitigating larval mosquito habitats. Mosquitoes fed on captive animals, humans, and wild animals, and took mixed bloodmeals. Blood hosts included 1 amphibian species, 16 bird species, 10 mammal species (including humans), and 2 reptile species. Minimum flight distances (dispersal) from host locations ranged from 15.5 m to 327.0 m, with a mean of 94.1 m ± 13.4 m. No mosquitoes tested (n = 45) were positive for *D. immitis*. The pyloric spines of *Ae. albopictus*, *Ae. j. japonicus*, *Ae. triseriatus*, *An. punctipennis*, *Cx. pipiens* complex, *Cx. restuans*, *Or. signifera*, and *Tx. rutilus* were photographed and measured. Differences exist in qualitative and quantitative spine structure, with *Aedes* spp. forming one general group, *Culex* spp. another, and *An. punctipennis* and *Or. signifera* a third. The one specimen of *Toxorhynchites rutilus* examined was most like *Culex* spp. mosquitoes.

Larval mosquito-habitat, adult mosquito-host associations, and pyloric armature and spine structures generally conformed to previously published accounts, indicating that mosquito biology inside zoos represents mosquito biology outside zoos. Therefore, zoos can be used for experiments not feasible in the field. However, novel variation (e.g., new, exotic host records) recorded in mosquito species warrants further investigation in zoos.

My study demonstrates that zoos can be used as experiment environments to study mosquito behaviors (e.g., oviposition cues, innate versus learned host preferences, mosquito dispersal, and home range memory), and that findings can be extrapolated to non-zoo areas, while also providing medical and veterinary benefits to zoo animals, visitors, and the public.

DEDICATION

To my friends and family and the “World’s Greatest Medical Entomologist”

&

To Chris M. Stone, this work would not be what it is without your enthusiastic support and our rambling conversations. I can’t wait to see where our adventures take us next.

ACKNOWLEDGMENTS

I will forever be gladly indebted to W. Wills for guiding me into this profession. Thank you for finding me in the forest.

I gratefully acknowledge the guidance of P.H. Adler, a graceful leader and outstanding person. “The best of leaders when the job is done, when the task is accomplished, the people will say we have done it ourselves.” – Lao Tzu.

I would like to thank the staff of both zoos including K. Benson DVM, H. Miller DVM, J. Bullock, B. Foster, K. Gilchrist, J. Lineberger, A. Norris, and S. Reno. Additionally, my thanks go to C. Beard, T. Brewer, C. Climer (“Exo-AP” protocol), C. Evans (Richland Co. DHEC collections), K. Korneva, J. McCall, J. McCreadie, M. Nelder, D. Swanson (identifications of Ceratopogonidae and Corethrellidae), and P. Vigueira. And, I am grateful to the lab of Dr. W. Foster at The Ohio State University for serving as my surrogate lab. I would also like to thank the W.C. Nettles Endowed Research Grant for partial research funding, the Nettles Travel Grant and Clemson GSG Professional Enrichment Grant for funding most of my professional presentations, the Terminix and Cochran Fellowships, the Filaria Research Reagent Repository in Athens, GA for materials and training (thank you E. Burkman), the SC Department of Health and Environmental Control for equipment, and the National Science Foundation Graduate Research Fellowship Program for financial support.

TABLE OF CONTENTS

Page

Table of Contents (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

Great strides have been made in the past century to reduce the incidence of arthropod-borne diseases, and the key to most of this success has been the identification and control of arthropod vectors (Service 1978, Geong 2001). However, although we have achieved a significant decline in infection rates, we still coexist with many arthropods that have the potential to transmit agents of illness between humans and the animals that serve as zoonotic reservoirs. I am primarily interested in areas where different genomes and pathogens can converge (e.g., airports, factory farms, rest areas, shipping ports, and zoos). Associated with the epidemiological threat of these areas are the vectors that can spread disease agents out of them.

Zoos are a unique environment where mosquitoes, humans, and exotic and native animals and plants interact. They are places where humans and animals are in close daily contact, potential mosquito oviposition and larval development habitats exist, exotic plants and animals are introduced regularly, and wild animals are present. The only requirement for a potential outbreak (e.g., West Nile virus or avian blood pathogens) would be the introduction of the pathogen (for instance, by migrating birds) into a competent population (such as mosquitoes breeding on zoo grounds).

Nineteen articles have been published that are related to aspects of mosquito ecology in zoos including surveys of larval mosquito habitats and environmental characteristics related to oviposition (Beier and Trpis 1981b, Derraik 2004, Derraik and Slaney 2005, Derraik et al. 2008, Tuten 2011), incrimination of mosquito vectors (Beier and Stoskopf 1980, Beier and Trpis 1981a, McConkey et al. 1996, Huijben et al. 2003, Grim et al. 2004, Ejiri et al. 2009), identification of blood hosts (Nelder 2007, Ejiri et al. 2011), recommendations for mosquito control in zoos (Derraik 2005), management of mosquitoes in zoos (Griner 1974, Shimonsky 2009, Shimonsky 2010), and a review of mosquito-associated illnesses in zoo animals (Adler et al. 2011). These studies have identified mosquitoes as the arthropods of greatest medical and veterinary concern in zoos, and documented larval mosquitoes on zoo grounds, epinortics in captive, endangered birds, mosquito bloodmeals from multiple hosts in succession, and mosquito biting of humans.

Awareness is increasing about the need for an understanding of mosquito ecology at the intermediate and microhabitat scales (Rey et al. 2006, Gu et al. 2008, Chaves et al. 2010, Ferguson et al. 2010). This understanding will be a synthesis of the interaction between mosquito host choice, larval performance, ovipositional preference, and vector potential within the context of environment. Human-created and modified environments, such as zoos, present unique arenas in which to study the habitats, distributions, and successions of multiple mosquito species. Analyses of mosquito oviposition and blood feeding behaviors in zoos will lead to a better understanding of mosquito involvement in disease

transmission routes. These data will contribute to the larger knowledge on larval mosquito habitats, mosquito-host relationships, and host fidelity patterns of mosquitoes.

If potential vector species can be identified, then zoos can implement cost-effective source reduction of larval habitats associated with the vector species. Most zoos perform continuous surveillance for zoonoses and keep a close watch on "sentinel" animals, which serve as an early warning system for zoo-associated pathogens (McNamara 2007). Animals are tested and vaccinated at the first signs of disease within and beyond the confines of zoos, and disease detection and notification networks exist among veterinarians, physicians, and public health agencies (Adler et al. 2011). Despite this vigilance, a critical component is missing in the monitoring of zoonoses in zoological parks—the ecology of the vectors themselves.

My study focuses on larval habitats, blood hosts, dog heartworm (*Dirofiliaria immitis*) transmission, and pyloric armature of mosquitoes in South Carolina zoos. The results of my study will identify potential zoo-associated threats and the conditions that foster their survival, allowing for a more rapid response to a disease outbreak and more efficient detection methods aimed at preventing one. The unifying idea for my investigations is that the unique environment of zoological parks is a testing ground for experiments undertaken to gain a better understanding of mosquito ecology as a basis for control measures in zoos and by extrapolation, beyond zoos. I intend to provide a better understanding of the potential health risks posed by mosquitoes interfacing with animals

and humans in a zoo setting. Additionally, I aim to use the zoos as natural experiments for the investigation of mosquito biology. My research will benefit zoos by providing an empirical basis for mosquito control recommendations.

The specific objectives of my research on mosquito ecology in South Carolina zoos are

1. To document mosquito species present as larvae and adults.
2. To determine environmental variables associated with larval mosquito distributions.
3. To document mosquito blood hosts.
4. To determine potential vectors of *Dirofilaria immitis*.
5. To interpret structure of the pyloric armature of zoo-associated mosquitoes.

The specific hypotheses of my research on mosquito ecology in South Carolina zoos are

1. Mosquito species present as larvae and adults will not differ from environments outside the zoos.
2. Larval mosquito distributions will be predictable on the basis of environmental variables.
3. Blood feeding of mosquitoes will conform to previous host usage patterns in non-zoo areas.
4. Mosquito vectors of *Dirofilaria immitis* will be present and conform to previous vector reports.
5. The pyloric armature of zoo-associated species will vary among species.

CHAPTER TWO

LITERATURE REVIEW

Around 3,200 species of mosquitoes (Diptera: Culicidae) are recognized worldwide, and females of most of these species will consume vertebrate blood at some point during their adult life (Foster and Walker 2009). Female and male mosquitoes obtain nutrients from plant nectar and insect honeydew as adults, and from decomposed leaf matter, suspended particles, microinvertebrates, and small macroinvertebrates in aquatic habitats as larvae (Merritt et al. 1992, Foster and Walker 2009). Most adult female mosquitoes also supplement their carbohydrate-rich diet with vertebrate blood rich in amino acids. Bloodmeal supplements are used for energy required in flight, foraging, and egg development (Hocking 1971, Foster and Walker 2009). Additionally, blood feeding females will usually obtain blood from more than one vertebrate host during their lifetime, with each bloodmeal typically driving a gonotrophic cycle of egg fertilization, maturation, and deposition (Hocking 1971, Washino and Tempelis 1983). This tendency to feed on multiple hosts, with meals punctuated by non-feeding egg-laying periods, is one of the primary reasons female mosquitoes are efficient intermediate hosts and vectors of animal pathogens (Foster and Walker 2009).

Mosquitoes in zoos

A zoo is an enclosed space in which artificial habitats host natural phenomena (e.g., migrating birds stopping over on zoo grounds, breeding and birth of exotic animals) to

create ecologies that exist in few other places. These habitats usually have well-defined parameters in which many inputs are controlled by necessity of operation. Additionally, zoos are environments where native and non-native mosquitoes and native and non-native animal hosts interact. They are islands of diversity; areas where animals and plants not normally found in association with each other will co-occur. The ecologies of zoos could be different than surrounding areas because of human-mediated introduction of non-native flora and fauna. Although gene flow might occur across zoo boundaries between some subpopulations (e.g., mosquitoes), it will not occur between other, primarily, non-native organisms limited in distribution (e.g., plants at the zoo such as bromeliads or foreign animals such as penguins).Therefore, different selection pressures might operate on mosquitoes feeding and breeding within zoos compared to those outside of zoos. If these pressures are strong enough, they could outweigh the effects of gene flow (thereby facilitating new behaviors adapted to zoo environments). These behaviors could then facilitate species dispersal into habitats similar to zoos (e.g., city parks). Alternatively, few or no differences between mosquitoes within and outside zoos might exist. Altogether, zoos represent unique environments in which to study mosquito ecology in controlled yet heterogeneous conditions and to monitor mosquito-borne diseases.

Research on mosquitoes in zoos is scarce but it supports further investigation. Studies have not only confirmed mosquito presence in zoos (Pombi et al. 2003), but have documented mosquitoes breeding on zoo property (Beier and Trpis 1981b). Twelve

species of adult and six species of larval mosquitoes were found during a previous study in the Greenville Zoo, South Carolina (Nelder 2007). The study author also documented locations of adult captures and the locations of larvae (Table 2.1). Some adults were captured for which no larval specimens were found. During the same study, mosquitoes at the Riverbanks Zoo, Columbia, SC, were found taking blood meals from hosts as diverse as birds to hippopotamus (Nelder 2007).

Table 2.1. Species of mosquitoes captured as adults and their associated larval habitats at the Greenville, SC, USA zoo 2004-2006 (Nelder 2007).

Species of mosquito captured as adults	Habitat of same species found as larvae
Aedes aegypti	No larvae found
Aedes albopictus	Bamboo shoots, white buckets, concrete depressions, red planters, rain gutters, storm drain covers, tree holes, black, blue, and white tarpaulins
Aedes japonicus japonicus	No larvae found
Aedes triseriatus	Red planters, rain gutters, tree holes
Aedes vexans	No larvae found
Anopheles crucians	No larvae found
Anopheles punctipennis	Blue tarpaulins
Coquilletiddia perturbans	No larvae found
Culex erraticus	No larvae found
Culex quinquefasciatus/pipiens complex	Rain gutters and blue and white tarpaulins
Culex restuans	White tarpaulins
Orthopodomyia signifera	Blue tarpaulins

Further research is timely and necessary for a contemporary understanding of vector dynamics in zoos. Two recent studies in New Zealand zoos documented that densities of adult mosquitoes were higher in zoos than in nearby native forest and suggested that zoos can serve as foci of enzootic outbreaks (Derraik 2004, Derraik et al. 2008). Various forms of avian *Plasmodium* (the causative agent of avian malaria) were detected molecularly in *Culex* mosquitoes at the Baltimore Zoo and related directly to the death of a penguin in the zoo (McConkey et al. 1996). And, at the Baltimore Zoo, mosquitoes on zoo grounds were documented carrying avian malarial parasites (Grim et al. 2004). An established

documentation of mosquito blood-meals in South Carolina zoos would provide a map of potential transmission routes if *Plasmodium* or any other mosquito-transmitted pathogens became problems on zoo grounds. Currently, the head veterinarian at the Riverbanks Zoo is only aware of *Yersinia* spp.(not transmitted by mosquitoes) in zoo birds and primates (K. Benson, personal communication). Additionally, every bird death at the Riverbanks Zoo is subject to full necropsy and one dead bird was previously found positive for West Nile virus (WNv); this information is unknown for the Greenville Zoo. Although there is a national consortium (funded by the CDC) of zoos and public health officials, the National Zoological West Nile Virus Surveillance Group, it does not include routine mosquito monitoring in zoos by entomologists (McNamara 2007).

Environmental characters of larval mosquito habitats

To gain an understanding of the distribution of larval habitats requires a holistic view of not only larval requirements for survival and growth but also distribution of adults and cues that female adult mosquitoes use to locate and assess oviposition sites. Any predictive model of larval distribution will incorporate all of these factors to produce the most accurate picture possible by considering both the proximate and ultimate mechanisms affecting individual success. Female mosquitoes use many different cues for choice of oviposition site including visual, tactile, and olfactory cues (Bentley and Day 1989). Female mosquitoes should have strong ovipositional preferences, as larvae are not able to change the environment the adult female commits them to. Therefore, decisions

affecting success and failure will be strongly reinforced through the survival of subsequent generations (Spencer et al. 2002).

Although ovipositional choice seems to be dependent on a complex suite of interacting cues, some specific cues can be volatilized chemicals derived from both decaying and living animal and vegetable matter at the breeding site, turbidity or color of the water, reflectance of the surface, wetness of the habitat, texture of the habitat surrounding the water body, temperature of the water, and pH. Ovipositional choices can have pronounced effects on mosquito populations in terms of distribution and abundance of both larvae and adults (Reiskind and Wilson 2004) (Spencer et al. 2002). However, although many laboratory studies have been conducted on female ovipositional choices, particularly those related to volatile chemicals, oviposition behavior in field conditions is still in need of study (Reiskind and Wilson 2004). We know that some species prefer tree hole habitats while other species prefer human-made containers and these preferences could be due to selection for different life-history strategies such as increased ability to compete in species rich environments or ability to develop faster in warmer environments (Sota et al. 1992).

The act of oviposition is the result of a complex suite of behaviors on the part of the female that range from large-scale cues affecting flight movement to small-scale cues such as assessing water temperature in a microhabitat. Females can assess a site for previous and contemporary conspecifics and detrimental predators and thereby oviposit

in sites that have indicators of potential larval success or avoid sites that would be harmful to larvae (Kitron 1989, Torres-Estrada et al. 2001, Sunahara 2002, Arav and Blaustein 2006). However, there is evidence that different females use the same cues in different ways. For example, *Aedes triseriatus* has been recorded ovipositing in the presence of conspecific eggs in one study but avoiding ovipositing when conspecifics are present in another (Kitron 1989, Beehler 1991). Nonspecific eggs can deter oviposition in *Culex* spp. or alternately not affect decision making (Dhileepan 1997, Reiskind and Wilson 2004). However, in a large-scale study conducted in the Florida Keys, species from the genera *Aedes*, *Culex*, and *Ochlerotatus* multiply infested the same artificial containers (Hribar et al. 2001). Altogether, these data hint that the co-occurrence of mosquito species may be dependent on the environmental context. During a longitudinal field study colonization patterns, and any changes in composition, of species in different breeding habitats would help to elucidate these conflicting results.

Gravid female mosquitoes have an arsenal of abiotic cues with which they can assess a potential larval habitat. For species that lay eggs in natural and artificial containers either as rafts or as eggs deposited above the water line (which will hatch when the containers flood), aspects related to pool size such as permanence and risk of desiccation can be assessed in a variety of ways. They could appear to prefer an optimal surface area to depth ratio (thereby providing a large surface for matter exchange with the surrounding environment in a location ephemeral enough to allow for larval development in the absence of established predators). Depth of container can provide larval protection when

water is abundant, as pupae gain protection from aerial predation with increasing depth of water (Rodriguez-Prieto et al. 2006). But a study conducted in a resource-limited environment designed to mimic tree holes found that increasing depth decreased larval survival and increasing horizontal surface area increased larval survival (Wynn and Paradise 2001). However, depth might not be assessed directly whereas surface area is (Lester and Pike 2003, Arav and Blaustein 2006). And choice of surface area could be independent of presence of predators (Lester and Pike 2003). Alternately, oviposition preference can be positively correlated with depth of the water body while holding surface area constant, but this preference could be species specific (Dhileepan 1997). Empirical observation of native Japanese species indicates that choice of pool size can vary by species (Sunahara 2002). We do not know whether turbidity of the water body is used as an ovipositional cue but it has been correlated with larval abundance of several species in an Iowa wetland (Mercer et al. 2005).

Multiple aspects related to plants in, on, and around the water body could have direct effects on oviposition site choice for species that lay eggs singly in still, weedy water. Plant volatiles acquired from living plants and tested in a laboratory setting induced oviposition in an *Anopheles* species at low concentrations but had a repellent effect at high concentrations (Torres-Estrada et al. 2005). This same effect was observed in the same species and another *Anopheles* species when using plant volatiles from dried plants and cyanobacteria mats acquired from breeding habitats (Rejmankova et al. 2005). The amount of vegetation correlated with larval presence in natural habitats has also been

shown to vary by species (Gimnig et al. 2001). For females of some species which tend to choose smaller containers dependent on external inputs for environmental enrichment there is a preference for containers with high detritus amounts (such as leaves fallen from nearby trees) in choice tests for containers enriched with organic nutrients (Beehler and Mulla 1995, Reiskind and Wilson 2004, Yee and Yee 2007). Additionally, oviposition choice could be specific for certain types of trees, as indicated by the predominant leaf substrate or chemicals obtained from certain leaves (Novak and Peloquin 1981, Lampman and Novak 1996, Trexler et al. 1998). When given a choice between a substrate in artificial tree holes and no substrate, and both treatments lacked plant volatiles, females still chose dark substrates and this could be due to camouflage for larvae provided by darker substrates (Huang et al. 2007).

These nutrients could be indirect indicators of other habitat aspects that might not be evident during the normal hours of oviposition at crepuscular times of day. One factor that might be assessed indirectly is that of shade, and first-instar larvae have been associated with shaded habitats, indicating an ovipositional preference (Foley et al. 2002). However, although some species are known as "shade-loving", this seems to be related to anecdotal evidence rather than explicitly investigated mechanisms (Foley et al. 2002). Some mosquitoes also preferentially oviposit in plants that contain the water body, such as bromeliads and trees, whereas others have become specialists in human-affected landscapes and oviposit in water-bearing artificial containers such as buckets and gutters (Haramis 1984, Beehler 1991, Sota et al. 1992, Hribar et al. 2001, Gottfried et al. 2002).

Females might also be using specific aspects of the water itself to assess a site. Artificially darkened water elicits more of a response than lighter choices (Beehler 1991, Dhileepan 1997). Scant empirical evidence exists on the role of water temperature as an ovipositional cue for mosquitoes it does have an effect on larval development and would therefore be a logical habitat aspect to record (Derraik and Slaney 2005). Additionally, temperature affects larval development times, emergence rates, and population densities in laboratory experiments (Alto and Juliano 2001a). When combining the effect of water temperature and desiccation, significant differences have been found in emergence rates and adult population numbers with varying combinations of water level, evaporation, and temperature (Alto and Juliano 2001b). These are all aspects intrinsically related to shade and a female might use a parameter such as water temperature to assess a site's shade cover and resistance to desiccation as well as its susceptibility to flooding.

The relationship between oviposition choice, water temperature, and larval development underscores the need to understand larval habitat requirements in order to better understand female ovipositional choice and larval habitat distribution. Many aspects other than water temperature can affect larval presence, survivorship, distribution, species succession, and abundance. Although an understanding of larval habitat requirement is essential to the creation of any predictive model, the process of defining the most important habitat predictors of larval presence will also inform our perception of adult population dynamics. Various factors related to larval development can have marked effects on the emergent adult population in terms of individual size, distribution and,

hence, dispersal (Schneider et al. 2004). If larval biological and ecological factors have an influence on subsequent adult stages, then to control adult populations the associated larval stages must be characterized. Previous studies related to larval distribution, survival, and abundance provide good starting parameters for this characterization.

Soluble nitrogen content can have a limiting factor on larval growth in tree hole assemblages dependent on stemflow for nutrient input (Kaufman and Walker 2006). However, larval abundance can also be unrelated to total nitrogen content (Costanzo et al. 2005). Adversely, the number of immature mosquitoes has been significantly correlated with dissolved nitrate (Mercer et al. 2005). Other dissolved nutrients and ionic content derived from local stemflow could affect larval abundance (Paradise and Dunson 1997). Larval abundance can be correlated with conductivity (a measure of dissolved ionic compounds) but also can be independent of it, depending on species (Costanzo et al. 2005). In addition to nitrogenous compounds and ionic concentrations, pH has been determined experimentally to have effects on abundance of larvae through indirect effects on the trophic structure of tree-hole environments (Paradise 2000).

Just as water-body associations with plants can affect female oviposition choice they can also have an effect on larval development. In Puerto Rico, larval and pupal abundance were enhanced in artificial containers with leaf litter or algae that were near trees (Barrera et al. 2006). A study in Thailand found location of larvae in shade environments to be species dependent, and one species predominated in temporary habitats in artificial

containers near animals (Vanwambeke et al. 2007). But abundances of two common American species are shade independent (Costanzo et al. 2005). Plants can also influence larval mosquito abundance by influencing the richness of species assemblages associated with leaf litter. When given a choice between animal and plant detritus, larvae of two species fed preferentially on animal detritus (Kesavaraju et al. 2007). Presence of macroinvertebrate detritus benefits container-dwelling mosquitoes by enhancing growth rate, survival, and adult mass (Yee et al. 2007).

The type of larval mosquito habitats, whether it's natural, artificial, temporary, permanent, covered, or uncovered, can have an effect on species distributions. A study in Vero Beach, FL, found eggs of two species significantly positively associated with urban settings and negatively associated with rural or open settings. However, no distinct rural vs. urban patterns emerged for the two other species assessed (Rey et al. 2006). Flooding of a habitat could lead to alternations of species present in the same container, as some raft-laying species will exploit a habitat during dry times while eggs of floodwater mosquitoes accumulate. When the containers flood, those eggs will hatch and there will be a switch in the dominant species. A succession of species has been noted in the same environments, depending on seasonal rainfall in three *Anopheles* species in Kenya (Gimnig et al. 2001).

Blood hosts of mosquitoes

Since the incrimination of mosquitoes as vectors of pathogens in 1878, numerous studies have been conducted on mosquito-host associations (Foster and Walker 2009). Just as numerous are the methods that have been used to capture blood-fed mosquitoes and determine host identity. Host-seeking and blood-engorged mosquitoes have been captured with nets, and vacuum aspiration from hosts and vegetation where mosquitoes rest after blood feeding (including traps designed to be attractive resting sites), fan collections of host-seeking mosquitoes in flight (e.g., light traps that attract host-seeking mosquitoes), and fan collections of mosquitoes in habitats where they lay eggs (i.e., "gravid traps") (Silver 2008). To determine bloodmeal identity early studies relied on eyewitness accounts of mosquitoes feeding on animals. With advances in technology, methods have become increasingly sophisticated and allow for elucidation of host identity based on biochemical characterizations of mosquito bloodmeals.

Briefly, the primary methods used to determine mosquito-host associations include recording mosquito visits to hosts through direct observation and bait traps (e.g., choice experiments between caged animals), identifying hosts using serological methods including precipitin tests, fluorescent antibody technique, passive hemagglutination inhibition technique, enzyme-linked immunosorbent assay, and most recently DNA-based methods that amplify host DNA with the polymerase chain reaction (PCR) followed by variants on any or all of the following steps: restriction enzyme digestion, gel separation, heteroduplex analysis, reverse line-blot hybridization, excision and

purification of isolated host-derived DNA, sequencing of the DNA, and identification of the sequence using a GenBank BLAST (Washino and Tempelis 1983, Mukabana et al. 2002, Kent 2009). One of the most common PCR primers in current use was designed by Kocher et al. (1989) to universally (e.g., all vertebrates) amplify a portion of the vertebrate mitochondrial cytochrome *b* gene. To date this primer has been used in at least eleven studies on mosquito bloodmeal identity (Kent 2009).

From these data, generated over a century of study, general patterns in mosquito-host associations have been characterized, yet many specific associations (e.g., host fidelity) are still debated. We know that many factors contribute to the likelihood of an individual mosquito feeding on an individual host. These factors can be environmental (e.g., ambient temperature), behavioral (e.g., host avoidance of a mosquito), temporal (e.g., time of day), physiological (e.g., parasitism of the mosquito or host), and genetic (e.g., *Culex pipiens* complex subpopulations with preferences for either birds or mammals) (Hocking 1971, Washino and Tempelis 1983, Rossignol et al. 1985, Bentley and Day 1989, Fonseca et al. 2004). Most mosquito species have either "fixed" (i.e., specific host preferences regardless of host diversity) or "opportunistic" (i.e., no host preferences, so diversity of bloodmeals reflects local host diversity) feeding patterns (Hess et al. 1968, Edman et al. 1972, Washino and Tempelis 1983). Within those species that have "fixed" patterns they are further characterized as "anthropophagic" (i.e., human-feeding), "zoophagic" (i.e., feed on vertebrates other than humans), "mammalophagic" (i.e., mammal-feeding), and "ornithophagic" (i.e., avian-feeding) (Reisen 2009).

The basis of mosquito host biases likely has coevolutionary (e.g., optimized digestive enzymes) and environmental (e.g., feeding on most abundant hosts) components. Mosquitoes employ a wide arsenal of tools to locate hosts including olfactory and visual cues (Gibson and Torr 1999), and sound (Borkent and Belton 2006). Some of these cues, such as volatiles emitted by human skin microbiota, could even determine mosquito host choice within a species (Braks et al. 1999). Some mosquitoes might have physiological and behavioral mechanisms allowing them to exploit a particular host at the expense of being able to efficiently use blood from a wide range of host types, such as the apparent evolved anthropophily of *Aedes aegypti* (Harrington et al. 2001). But retaining plasticity in host usage can be advantageous. For instance, feeding on new hosts could help some invasive mosquito species adapt to new environments and subsequently become vectors of introduced and native pathogens (Juliano and Lounibos 2005, Bataille et al. 2009). Mosquito host usage might be influenced by location of the host in relation to the larval habitat and previous environmental stimuli conditioning the mosquito (Hocking 1971, Smith et al. 2004, Foster and Walker 2009). Depending on the species, a mosquito might stay very close to the larval habitat from which it emerged (e.g., ≤30 meters) or disperse over distances exceeding 100 kilometers; however, two kilometers is the typical upper lifetime flight distance of a mosquito and most mosquitoes average 50 meters or less (Foster and Walker 2009, Silver 2009).

Studies of mosquito hosts and populations have been integral to sorting out the epidemiology of many diseases. Mosquitoes can act as both vectors of pathogens (e.g.,

transmission of WNv from birds to humans) and intermediate hosts necessary to the life cycle of pathogens (e.g., several filarial nematodes and *Plasmodium* spp. have obligate developmental stages in mosquito hosts). A single mosquito species or genus can be the critical vector serving as the most fundamental component of an epidemiological outbreak (Hamer et al. 2009). In such scenarios, identification of the main vector and its hosts can lead to mosquito management efforts that interrupt pathogen transmission. However, an understanding of the ecology of the vector is necessary to control efforts (Juliano and Lounibos 2005) and if the ecology is not well understood then efforts to control pathogen transmission might fail (Ferguson et al. 2010). Monitoring and censuses of mosquito populations can lead to anticipation of potential vectors, and early interventions in the event of outbreaks (Britch et al. 2008).

Mosquito vectors of *Dirofilaria immitis*

Dirofilaria immitis (Spirurida: Onchocercidae) is a filarial nematode parasite and the causative agent of "canine heartworm" disease (Grieve et al. 1983). It is transmitted between vertebrate hosts by mosquitoes and requires the mosquito as an intermediate host for three stages of larval development. Prevalence of *D. immitis* infection in dogs can reach up to 45% in the United States and the American Heartworm Society was formed in 1974 to provide a forum for research (Boreham and Atwell 1988, Roberts and Janovy Jr. 2005). It has been reported in North and South America, the Caribbean, Europe, Africa, southeast Asia, and Australia, and although it was originally thought to persist only in warm coastal areas the range is spreading into temperate inland areas due to either

movement of infected dogs or increasing abundance of mosquito vectors, or both (Lok 1988).

Over 60 species of mosquito in the genera *Aedes*, *Anopheles*, *Culex*, and *Psorophora* have been implicated as vectors, and some of these are serious pests of man and domesticated animals (Lok 1988). A mosquito ingests first-stage *D. immitis* microfilariae when it takes a bloodmeal from an infected and competent vertebrate host. The microfilaria migrate to the mosquito Malpighian tubules, the entrance of which are located in the lumen of the pyloric valve between the midgut and hindgut (Thompson 1905), where they molt through their second and third stages. The third stage larvae then migrate to the mosquito mouthparts (specifically the lumen of the labium) and are introduced to a new host when the mosquito takes its next bloodmeal, whereupon they exit the labium and enter the host through skin ruptured by the mosquito bite (Grieve et al. 1983). This developmental process is temperature-dependent, but typically takes 12 - 14 days between mosquito ingestion of microfilaria and subsequent inoculation of a new host with infective third-stage *D. immitis* larvae. In the vertebrate host *D. immitis* larvae take 70 – 90 days to molt through their fourth and fifth stages of development and then reach sexual maturity once located in the heart and pulmonary arteries. At this point the cycle begins anew as females mate and release microfilariae into the host circulatory system (this paragraph was adapted from chapters in Boreham and Atwell 1988, and Foster and Walker 2009).

Although *D. immitis* or "heartworm disease" is most often associated with domestic dogs, it has been found in many wild and domestic animals, and evidence exists of a persistent sylvatic reservoir of the parasite in wild canines (e.g., coyotes) (Ciferri 1982, Abraham 1988, Lok 1988). It has been found in at least 10 species of canine other than dogs, 6 feline species including domestic cats, 20 other species of mammals including several that are regularly exhibited in zoos (e.g., otters, orangutans, seals), and man (Abraham 1988, Boreham 1988). *Dirofilaria immitis* infections in zoo animals have been associated with host death, and some represent first records in a particular host (e.g., penguins at the Tokyo Zoo) (Sano et al. 2005); some of the more notable cases are included in Table 2.2.

Once an animal is diagnosed with heartworms, treatment typically consists of chemotherapy using thiacetarsamide or levamisole (adulticides), followed by dithiazanine iodide or ivermectin (larvicides) with ongoing chemoprophylaxis using diethycarbamazine citrate or ivermectin (Courtney 1988). However, adulticides can be very dangerous to the host animal and often require the animal's movement to be severely restricted for several weeks following treatment (Courtney 1988, Kreeger et al. 1990). In heartworm endemic regions, heartworm infection should be included in the differential diagnosis for sudden death of exotic cats housed outdoors, non-human primates with cardiopulmonary disease, and human lung cancer (Ciferri 1982, Deem et al. 1998, Gamble et al. 1998). Continued screening of native and exotic zoo animals which can be definitive hosts of *D. immitis* was recommended after a study of heartworm

prevalence in Calgary, Alberta, Canada which included the Calgary Zoo (although no zoo animals tested positive) (Frimeth and Arai 1984). Additionally, *D. immitis* might serve as

Table 2.2. Selected reports of confirmed or putative *Dirofilaria immitis* infection in captive animals at zoos.

Country	Common Name	Linnaean Name	First report?[1]	Cause of death?[2]	Reference
Japan	Snow leopard	*Uncia uncia*	Yes	No	Murata et al. 2003
Korea	Eurasian otter	*Lutra lutra*	No	Yes	Matsuda et al. 2003
USA	Black-footed cat	*Felis nigripes*	Yes	Yes	Deem et al. 1998
USA	Pale-headed saki monkey	*Pithecia pithecia*	No	N/A[3]	Gamble et al. 1998
Japan	Humboldt penguin	*Spheniscus humboldti*	Yes[4]	Yes	Sano et al. 2005
USA	Wolverine	*Gulo luscus*	Unknown	Yes	Williams and Dade 1976
USA	California sea lion	*Zalophus californianus*	No	Yes	White 1975
USA	Red panda	*Ailurus fulgens fulgens*	No	Yes[5]	Neiffer et al. 2002
USA	Bengal tiger	*Panthera tigris*	Unknown	Yes	Kennedy and Patton 1981

1. Whether this is the first report in the literature of *D. immitis* infection in the particular animal.
2. Whether *D. immitis* was implicated as the cause of animal death upon post-mortem examination.
3. First premortem diagnosis of *D. immitis* infection in a non-human primate with subsequent successful treatment of infection, i.e., no animal death.
4. First report of *D. immitis* infection in a bird.
5. Treatment for parasite with melarsomine was putative cause of death.

an excellent proxy for studies on the epidemiology of human filariases; incidentally, it was the first filarial nematode transmitted by mosquitoes to have its life cycle determined (by Thomas Bancroft in Brisbane, Australia in 1901) (Grieve et al. 1983, Boreham and Atwell 1988).

Pyloric armature of mosquitoes

Distinct areas of armature composed of lightly and heavily sclerotized "teeth", have been noted along the interior of mosquito fore- and midguts (Trembley 1951). Most notable are those termed the cibarial, pharyngeal, and pyloric armature. These armatures might aid in mechanical hemolysis of mosquito host blood cells, or disruption leading to death of filarial parasites infecting the mosquito (Lyimo and Ferguson 2009). Additionally, migration of infective filarial larvae across the mosquito body cavity can be damaging (thus reducing fitness) to the adult mosquito (Perrone and Spielman 1986). Therefore, mosquito pecies susceptible to *D. immitis* infection would have an advantage by maintaining physical defenses against filarial infection (which are usually less costly than physiological defenses). Alternatively, species with less well-developed armature could be more susceptible to infection, as was the case in a study including well known *Aedes* sp. and *Culex* sp. vectors that had poorly developed armature (McGreevy et al. 1978).

The pyloric armature might aid in mechanical filtering and concentration of mosquito-host erythrocytes from serum and its structure might vary with structure of host erythrocytes (Vaughan et al. 1991, Lyimo and Ferguson 2009). Up to thirty-fold differences have been noted in the size of vertebrate red blood cells (ca. 2μm to 52 μm in diameter), with class level differences apparent (Snyder and Sheafor 1999). Therefore as a consequence of coevolution, and selection pressures to either specialize (e.g., on one host class, such as Aves) or generalize (i.e., opportunistically feed on the closest host available) armature structure might reflect known differences in host erythrocyte size and

shape. Differences in the number and type of teeth on mosquito maxillae have previously been related to host type (e.g., birds, "cold-blooded" animals, mammals including humans) (Lee and Craig 1983). And, because of the peristaltic action of the pylorus, the armature might also aid in hemolyzing host blood cells (Vaughn et al. 1991), a known function of the cibarial armature (Coluzzi et al. 1982, Chadee et al. 1996).

The foregut armature aids in shredding, and thus killing, filarial nematodes (e.g., *Wuchereria bancrofti*) ingested in mosquito bloodmeals (McGreevy et al. 1978). Additional evidence that armature elaboration tends to decrease as vector efficiency increases, as was the case in studies including well known *Aedes* spp. and *Culex* spp. vectors that had poorly developed cibarial and pharyngeal armature (Shoukry and Soliman 1995, McGreevy et al. 1978). Given the precedent set by the action of mosquito foregut armature, the pyloric armature might aid in killing of *Dirofilaria immitis* L1 larvae that migrate into the Malpighian tubules through openings in the pyloric valve (Dr. John McCall, personal communication 2011), specifically, where the Malpighian tubules open into the space between the midgut and ileo-colon valves that form the pyloric valve (Thompson 1905)– a strategy different from that of other filarioid nematodes that migrate across the midgut into the hemocoel (Macdonald and Ramachandran 1965). Disruption of the migration of infective *D. immitis* larvae could provide a fitness benefit as their passage and development can be damaging to the adult mosquito, and shortens mosquito lifespan (Kershaw et al. 1953). Accordingly, species with less well-developed armature would be more susceptible to filarial infection. Some mosquito species are capable of

ingesting *D. immitis* larvae, and harboring development until a certain stage at which they kill the mosquito (Sulaiman and Townson 1980). Therefore, some mosquitoes can be infected but are incompetent vectors, whereas other species can be infected and are competent vectors. However, wide variation in the number of microfilariae ingested by individual mosquitoes (Russell and Geary 1992), and low incidences of *Dirofilaria* spp. infection in most natural populations might render selection pressure by filarial nematodes on pyloric armature weak.

Thompson (1905) and de Boissezon (1930) state that the pyloric armature is lacking in mosquito larvae, and it is not mentioned in larvae by Christophers (1960), whereas other authors have suggested that it is 1) present (Clements 1963), and 2) aids mosquito larvae in disrupting the establishment of trichomycete fungal parasites (McCreadie and Beard 2003), and the armature could subsequently be carried over into the adult during metamorphosis; however, no work has been done documenting the fate the the larval pylorus in the adult mosquito (Clements 1963). Because the cuticular lining of the hindgut is shed during molting, the pyloric armature would likely not persist in the adult mosquito unless there was a function for it. Trichomycete fungi have been noted for having a mysterious "preference" for the larval mosquito rectum although the pH drop required for trichomycete sporangiospore formation begins in the pylorus (which has a neutral pH as opposed to the basic pH of the midgut) and sporangiospore formation has been noted in the mosquito pylorus (Horn 2001). The esophageal armature might aid in drawing a type II peritrophic matrix through the gut, and the pyloric armature might serve

a similar function, and aid in the disintegration, or backwards passage of type I peritrophic matrixes (Wigglesworth 1950). It might also aid in backwards passage of the peritrophic matrix encapsulated larval meconium. A previous study on the pyloric armature of sand flies suggested it could serve to facilitate disruption of undigested blood-meal residue and peritrophic matrix, and might influence *Leishmania* parasites that localize in the pylorus (Christensen 1971). One study on phlebotomine sand flies documented bloodmeal excretion into the hindgut occurring simultaneously with the breakdown of the peritrophic matrix, and noted unattached, motile flagellated *Leishmania* parasites in the lumen of the pylorus and Malpighian tubules (Walters et al. 1987).

A recent SEM study documented "cuticular ridges with tentacle-like appendages" in the pylorus or "hind triangle" of the sand fly, *Phlebotomus papatasi* (Warburg 2008). The cibarial, and pharyngeal, armature of mosquitoes have taxonomically meaningful characters (Chwatt 1945, Forattini and Sallum 1992) and the armature of spines and plates in pyloric intima of lepidopteran and black fly larvae have been noted for their taxonomic importance (Kim and Adler 2007, Byers and Bond 1971). Trembley (1951) suggested the pyloric armature of adult mosquitoes might have taxonomic utility.

CHAPTER THREE

HABITAT CHARACTERISTICS OF LARVAL MOSQUITOES IN ZOOS OF SOUTH CAROLINA, USA[1]

Zoos provide a variety of larval habitats and a wide range of blood-meal hosts for mosquitoes (Beier and Trpis 1981a, Derraik 2004, Nelder 2007). Mosquito vectors also can introduce pathogens to zoo animals (often of endangered or threatened species) from wild populations (Fix et al. 1988, McConkey et al. 1996, Alley et al. 2008). With an increase in zoo-based rehabilitation services for wild animals, including captive-breeding and reintroduction programs, previously naive animals could be released into the wild after becoming infected on zoo grounds (Brossy et al. 1999). Unique combinations of adventive and indigenous (*sensu* Frank and McCoy 1990) hosts, mosquitoes, and pathogens occur in zoos, as evident during the outbreak of West Nile Virus at the Bronx Zoo, NY, in 1999 (Ludwig et al. 2002), and when cardiac infection with *Dirofilaria immitis* (dog heartworm) was implicated in the death of a black-footed cat (*Felis negripes*) at a zoo in Florida (Deem et al. 1998). Zoos ideally should incorporate mosquito control into existing pest-management programs or implement programs if none currently exist (Derraik 2005).

Meaningful relationships between physicochemical variables of aquatic habitats and the presence or absence of mosquito larvae have been demonstrated (Senior-White 1926,

[1] Published in the J. Am. Mosq. Control Assoc. . 2011. 27(2): 111-119

Rejmankova et al. 1991, Muturi et al. 2007). Interactions between aquatic habitats and surrounding terrestrial ecology, including location of blood-meal hosts, can influence distributions of mosquito larvae (Vanwambeke et al. 2007, Yee and Yee 2007, Gu et al. 2008). At the proximate level, habitat parameters such as vegetation patterns and water chemistry can serve as cues for oviposition (Allan et al. 1987, Bentley and Day 1989, Blackwell and Johnson 2000). Effective mosquito abatement and control programs should begin with a survey of mosquitoes in a given area and a characterization of their habitats (WHO 1975). Because of differential distributions of adults and larvae, and inherent biases in methods, surveys of multiple life stages should be conducted (Minakawa et al. 2002, Silver 2008). Understanding larval mosquito ecology is relevant to understanding adult distributions (Gimnig et al. 2001) and implementing control through "habitat-based interventions" (Gu et al. 2008).

Zoos contain a novel juxtaposition of habitats. For instance, an aviary mimicking a tropical rainforest might be located next to an arctic penguin exhibit. These varied habitats could create partitioned breeding sites and blood-meal hosts, and therefore act as accidental yet informative choice experiments or represent wholly new environments. To exploit this aspect of zoos as a study system for mosquito behavior, I surveyed aquatic habitats for mosquito larvae and measured physicochemical variables in two South Carolina zoos. During a previous survey of adult mosquitoes at the same zoos, larval mosquito habitats were noted but not characterized (Nelder 2007). The purpose of my study was to test the hypothesis that the distribution of larvae is predictable on the basis

of selected habitat characteristics, and determine whether larval mosquito habitats in zoos differ from those reported previously in the literature. This study also provides information for zoo personnel, regarding mosquito management.

Materials and Methods

Study Locations and Sites. All sites sampled were in the Greenville Zoo (Greenville, Greenville Co., South Carolina, USA; GPS: N34° 50.493′ W82° 23.133′, elev. 266m) and Riverbanks Zoo and Garden (Columbia, Richland Co., South Carolina, USA; GPS: N34° 00.358′ W81° 04.280′, elev. 51m). The Greenville Zoo (GZ), located in the piedmont ecoregion, is approximately 4 hectares and is bordered by the Reedy River. The Riverbanks Zoo (RZ) (excluding the gardens which were not part of the study area), located in the sandhills ecogregion, is approximately 21 hectares and is bordered by the Broad and Congaree rivers. The two zoos are 152 kilometers apart. Each zoo was initially surveyed and all accessible water bodies were examined as potential mosquito habitats. Accessibility was determined by zoo restrictions (e.g., alligator ponds at both zoos were not examined), with emphasis on minimal disturbance to zoo animals and visitors. Some sites did not persist for the entire study.

Sites were defined as an individual larval habitats within the zoos, such as a container or pool, and were classified by origin (artificial or natural), type (container or pool), and disturbance (disturbed or undisturbed). Artificial sites were defined as having a discernible human origin. Containers were distinguished from pools by having a

perimeter, a substrate discontinuous with the surrounding landscape, and a surface area ≤0.30 m^2. Disturbance was determined as any habitat disruption to the site (e.g., weekly cleaning by zoo employees, regular flooding by road run-offs, sporadic treatment with *Bacillus thuringiensis israelensis* (Bti) pellets). For large sites (e.g., vernal ponds), a meter along the edge was randomly selected and used for both environmental and larval samples during the entire study. Sites were ordered in a circuit and for each visit a beginning site was chosen randomly. During each zoo visit, physicochemical parameters were measured in one full circuit in the morning, and larval samples were taken in a second full circuit in the afternoon. Collections during each visit spanned one or two days, depending on the number of sites with water. Monthly collections were conducted at each zoo in March, May – August, October (RZ) and November (GZ) 2008, and January 2009.

Environmental Measurements. The average weekly high and low air temperatures and precipitation amounts preceding the week of collections for each zoo were obtained from the Greenville (KGMU) and Columbia (KCUB) downtown airports. Continuous measurements, of water at each site, taken on-site, were conductivity (Horiba Conductivity meter B-173), dissolved oxygen and temperature (Extech Dissolved Oxygen meter 407510A), pH (Oyster pH/mV/temp meter), depth, and surface length and width or surface diameter. Categorical measurements (taken by visual estimation) included aquatic vegetation (submerged and emergent, scored as presence or absence), canopy cover (≤50% or >50%), and height of predominant shade (≤2m or >2m).

Collection equipment was cleaned with distilled water between sites, and all meters were primed with water from the site before taking measurements. Conductivity, dissolved oxygen, and pH meters were calibrated with reference standards before each visit. Approximately the same amount of time was spent taking environmental samples at each site (mean = 8.8 ± 2.5 minutes).

Larval Sampling and Identification. Larvae were sampled with a baster (21.0 ml), ladle (70.0 ml), or dipper (470.0 ml), depending on site depth and surface area. Initially, 8 sites were sampled with a net and larvae were pipetted from a white pan, but the procedure was time intensive and abandoned. Due to low water volume, five sites were sampled with a pipette (~2.8 ml). Sites were sampled until either 10 samples were taken or the water in the site was exhausted (yielding fewer than 10 samples). Sample water was strained through a mesh net that was then rinsed with distilled water into a 284-ml glass jar and transported to the laboratory. A sample of water was taken regardless of perceived larval presence or absence. Approximately the same amount of time was spent taking water samples at each site (mean = 6.8 ± 3.0 minutes). Late instars (3^{rd} and 4^{th}) were killed in warm water and fixed in 80% EtOH. Early instars were reared to either late instars or adults. Pupae were reared to adults. Mosquito larvae and adults were identified to species using the keys of Darsie and Ward (2004). Larvae from the first day (of two) of the initial visit at Riverbanks Zoo were fixed on site in 80% EtOH and all late instars were indentified. Representative specimens of larvae and adults were deposited in the Clemson University Arthropod Collection.

Statistical Methods. Conductivity, dissolved oxygen, pH, surface area and depth, water temperature, and weekly mean high and low air temperature and precipitation, were entered into a principal components analysis (PCA). Beforehand, conductivity, dissolved oxygen, pH, depth, and surface area were log transformed to approximate normality. The purpose of a PCA is to preserve useful variability in a dataset with highly correlated variables while eliminating collinearity. All PCs with eigenvalues >1.0 were used in place of original habitat variables in subsequent analyses (Stoops et al. 2007). The relationships between original variables and derived principal components (PCs) were interpreted using Spearman's rank correlations (Ciborowski and Adler 1990). Significant associations between habitat parameters and PCs with presence of mosquito larvae (regardless of abundance) were determined using stepwise multiple logistic regression with backward elimination (Chatterjee and Hadi 2006) in the SAS JMP 8 statistical platform (Sall et al. 2007). Species present at more than 10% of sites also were analyzed separately.

Parameters were initially tested for significance by univariate analysis. All significant variables ($p<0.25$ to avoid type II errors) were entered into the multiple regression (Hosmer and Lemeshow 2000). The parameters initially tested were PC1, PC2, aquatic vegetation (presence/absence), canopy cover (≤50% or >50%), origin (artificial or natural), shade height (≤2m or >2m), site disturbance (disturbed or undisturbed), type (container or pool), and zoo (GZ or RZ). After univariate screening, parameters were assessed for inclusion in the model, using significance of Chi-square scores (probability

to enter and leave were both set at $p \leq 0.10$ to avoid type I errors) (Udevitz et al. 1987). Final parameters were determined by comparing Aikaike information criterion (AIC) scores of the models. The model with the lowest AIC score was chosen (Chatterjee and Hadi 2006). The goal was to develop a model with the strongest predictive power, using the least number of parameters.

Final fitted models were tested for overall goodness of fit, and percentages of larvae correctly classified as either present or absent at a site were calculated (McCreadie and Adler 1999, Hamada et al. 2002). Additionally, an analysis was conducted in SAS (9.2) to determine if the effect of time of visit was having an impact on the development of the regression model. The analysis included a repeated measures approach by adding visit as a variable in the regression models (adjusted for using a random effects model) and also analyzing bivariate correlations between visit and environmental variables already in the model.

In addition to model creation, coefficients of pairwise associations between the four most abundant species across both zoos were calculated using Hurlbert's C_8 with Ratliff's correction, and tested for significance using chi-square analysis (Hurlbert 1969, Ratliff 1982). These associations were analyzed according to source of habitat (artificial or natural origin), habitat type (container or pool), and amount of shade (canopy cover ≤50% or >50%).

Results

In total, 59 sites were sampled repeatedly over the study period, 27 at the Greenville Zoo and 32 at the Riverbanks Zoo. Artificial containers (n = 19) included buckets, a birdbath, concrete cedar stumps, gutters, a pool in a tarp, plastic and metal pipes, trash bins, and a tire. Natural containers (n = 20) included treeholes and bamboo stumps. Artificial pools (n = 11) included garden ponds and puddles in tire tracks, and natural pools (n = 9) included vernal pools and a duck pond. Extra sites were sampled for larvae on an ad hoc basis (not included in regression analyses); positive sites included buckets, plant pots, puddles, standing water on a park bench (once), tarps, tires, treeholes, and unused snack carts.

A total of 1,630 larvae, representing 16 species in 7 genera, was collected and identified from 238 samples (Table 3.1). Of these, 653 were collected at the Greenville Zoo (91 samples) and 977 at the Riverbanks Zoo (147 samples). Mosquito larvae were found in all seasons at the Riverbanks Zoo, but no mosquito larvae were found during winter at the Greenville Zoo. Only one species, *Culex restuans* (Theobald), was found in all four seasons. Four species comprised 91.7% of all larvae collected at both zoos: *Aedes albopictus* (Skuse) (46.0%), *Ae. triseriatus* (Say) (23.6%), *Culex pipiens* complex (L.) (9.7%), and *Cx. restuans* (12.4%). *Aedes albopictus* was found in artificial and natural containers and artificial pools but not in natural pools (Table 3.2). *Aedes triseriatus* was

Table 3.1. Total number of individuals collected at Greenville (GZ) and Riverbanks (RZ) zoos, South Carolina, and percentage of aquatic habitats positive for larvae (by season), 2008-2009.

	GZ				RZ				
	No.	% sites[b]			No.	% sites			
Species[a]	larvae total	Spr. (n=36)	Sum. (n=40)	Fall (n=4)	larvae total	Spr. (n=52)	Sum. (n=54)	Fall (n=21)	Win. (n=20)
Ae. albopictus	472	25	62.5	25	278	15.4	25.9	19	0
Ae. triseriatus	71	19.4	20	0	314	19.2	13	14.3	0
Cx. restuans	22	5.6	5	0	180	17.3	9.3	4.8	15
Cx. pipiens complex	64	0	10	50	94	7.7	9.3	4.8	0
Cx. salinarius	0	0	0	0	27	3.8	0	0	0
Or. signifera	0	0	0	0	27	0	3.7	4.8	5
Cx. territans	20	8.3	10	0	6	7.7	1.9	4.8	0
Ae. vexans	0	0	0	0	21	7.7	3.7	0	0
An. punctipennis	1	0	2.5	0	8	0	7.4	0	0
Tx. rutilus	1	0	2.5	0	7	1.9	5.6	0	0
Cx. erraticus	0	0	0	0	7	1.9	7.4	0	0
An. crucians complex	2	2.8	0	0	1	0	1.9	0	0
Ps. ferox	0	0	0	0	3	0	1.9	0	0
An. quadrimaculatus complex	0	0	0	0	2	0	1.9	0	0
Ae. canadensis	0	0	0	0	1	0	0	0	5
Ps. ciliata	0	0	0	0	1	0	1.9	0	0
Total[c]	653	44.4	75	50	977	59.6	59.3	33.3	20.0[b]

[a]In descending order by total number of larvae across both zoos
[b]Number of sites sampled in parentheses; no mosquito larvae were collected at GZ in winter (11 sites sampled)
[c]Total number of larvae at each zoo, and percentage of total sites positive for mosquito larvae by respective season

found only in artificial and natural containers. *Culex pipiens* complex was found in all except natural containers. *Culex restuans* was found in artificial and natural containers and natural pools in one zoo (RZ) but in only artificial pools at the other zoo (GZ).

The variables entered into the PCA resulted in two PCs with eigenvalues >1.0 and these explained 58.2% of the variability in the environmental measurements (Table 3.3). Water temperature, and weekly mean high and low air temperature and precipitation, were

Table 3.2. Percentage of aquatic habitat types positive for larvae at Greenville (GZ) and Riverbanks (RZ) zoos, South Carolina, 2008-2009.

Species	GZ				RZ			
	AC (n = 29)	NC (n = 36)	AP (n = 25)	NP (n = 1)	AC (n = 40)	NC (n = 36)	AP (n = 46)	NP (n = 25)
Ae. albopictus	34.5	52.8	24.0	0	40.0	27.8	0	0
Ae. triseriatus	17.2	27.8	0	0	12.5	41.7	0	0
Ae. vexans	0	0	0	0	5.0	0	6.5	4.0
An. crucians complex	0	0	4.0	0	2.5	0	0	0
An. punctipennis	0	0	4.0	0	2.5	0	4.4	4.0
An. quadrimaculatus	0	0	0	0	0	0	2.2	0
Cx. erraticus	0	0	0	0	2.5	0	8.6	0
Cx. pipiens complex	0	0	24.0	0	15.0	0	4.4	4.0
Cx. restuans	0	0	16.0	0	17.5	5.6	0	36.0
Cx. salinarius	0	0	0	0	0	2.8	0	4.0
Cx. territans	0	2.8	24.0	0	2.5	2.8	2.2	12.0
Or. signifera	0	0	0	0	0	11.1	0	0
Ps. ferox	0	0	0	0	0	0	0	4.0
Ps. ciliata	0	0	0	0	0	0	2.2	0
Tx. rutilus	0	2.8	0	0	2.5	8.3	0	0
Oc. c. canadensis	0	0	0	0	0	0	0	4.0

[a]Alphabetically ordered

significantly positively associated with PC1. Dissolved oxygen was significantly negatively associated with PC1. Surface area and depth were significantly positively associated with PC2. Conductivity and pH were significantly negatively associated with PC2.

The final model correctly predicted presence and absence of mosquito larvae at 72.6% of sites (Table 3.4). The model included PC1, PC2, origin, disturbance, aquatic vegetation, canopy cover, and shade height. Larval presence was significantly ($p<0.05$) positively associated with PC1, natural habitats, and absence of aquatic vegetation. It was weakly ($p<0.10$) positively associated with PC2, undisturbed habitats, and shade height ≤2m. Separate logistic regression analyses also were conducted for *Ae. albopictus* and *Ae. triseriatus* because they were present at more than 10% of sites.

Table 3.3.. Minimum, maximum, and mean values of continuous variables used in principal components (PCs) analysis, with Spearman's rank correlation coefficients for each variable and associated PCs. Environmental measurements taken from aquatic habitats at Greenville and Riverbanks zoos, South Carolina, 2008-2009.

Parameter	Values			Principal Component			
	Min	Max	Mean	PC1	p	PC2	p
Ambient high (C°)	12.00	31.00	21.32	0.878	<0.0001*	0.117	0.146
Ambient low (C°)	-2.00	27.00	13.46	0.863	<0.0001*	0.058	0.472
Precipitation (cm)	0.00	1.30	0.42	0.402	<0.0001*	-0.098	0.223
Water temp (C°)	2.00	28.80	16.99	0.875	<0.0001*	0.036	0.654
DO (mg/L)	0.50	20.90	4.01	-0.658	<0.0001*	0.107	0.181
pH	4.50	9.66	6.90	0.059	0.464	-0.608	<0.0001*
Conductivity (µS/cm)	19.00	7700.00	681.52	0.088	0.273	-0.812	<0.0001*
Surface Area (m^2)	$4.91x10^{-4}$	15.00	1.06	-0.096	0.230	0.747	<0.0001*
Depth (cm)	0.50	66.00	10.41	0.075	0.351	0.684	<0.0001*
Variance explained (%)							
Total				35.5		22.7	
Cumulative				35.5		58.2	

[a]Asterisk indicates significance at the 0.05 level

The final model for *Ae. albopictus* correctly predicted presence and absence at 79.5% of sites. The model included PC1, zoo, type, and shade height. *Aedes albopictus* was significantly ($p<0.001$) positively associated with PC1 and container habitats and weakly ($p<0.10$) associated with the Greenville Zoo and shade height ≤2m. The final model for *Ae. triseriatus* correctly predicted presence at 84.6% of sites. The model included PC1, origin, and shade height. The type category (container or pool) could not be included in the model for *Ae. triseriatus* because it was a perfect predictor (i.e., *Ae. triseriatus* was found only in container habitats) and caused model instability. *Aedes triseriatus* was significantly ($p<0.001$) positively associated with natural containers and shade height ≤2m and weakly ($p<0.10$) positively associated with PC1. Time of visit was found to be a non-significant variable in the regression models and bivariate analyses, and the repeated measures analysis based on time of visit did not significantly improve or change the form of the final models chosen.

Coefficients of interspecific association (C_8) were obtained by analyzing sites from both zoos as one data set (Table 3.5); there was no zoo associated difference in interspecific associations among larvae when zoos were analyzed separately. *Aedes albopictus* and *Ae. triseriatus* were significantly positively associated in artificial and natural habitats, and in shaded habitats. *Aedes albopictus* was also significantly positively associated with *Cx. pipiens* complex in pools, and completely (i.e., the two species were never found together) negatively associated with *Cx. restuans* in natural habitats. *Aedes triseriatus* was not significantly associated with the other two species.

Table 3.4. Logistic regression on association between habitat variables and principal components with mosquito larval presence in aquatic habitats sampled at Greenville (GZ) and Riverbanks (RZ) zoos, South Carolina, 2008-2009.

Parameter	Regression coefficient	SE	X^2	p	Reg.Coeff. Lower 95%	Reg. Coeff. Upper 95%
Aedes albopictus						
(R^2 = 0.3103; % correctly classified: 79.5; Goodness of fit = 0.8206; Observations = 156)						
Intercept	-1.467	0.355	17.03	<0.001*	-2.262	-0.834
PC1	0.682	0.145	22.09	<0.001*	0.412	0.984
Zoo[GZ/RZ]	0.417	0.217	3.69	0.055	-0.006	0.851
Type[C/P]	1.127	0.336	11.22	<0.001*	0.532	1.890
ShdHght[≤2m/>2m]	0.384	0.229	2.82	0.093	-0.065	0.837
Aedes triseriatus						
(R^2 = 0.2471; % correctly classified: 84.6; Goodness of fit = 0.995; Observations = 156)						
Intercept	-1.465	0.264	30.80	<0.001*	-2.020	-0.975
PC1	0.268	0.155	3.00	0.084	-0.027	0.587
Origin[N/A]	0.949	0.271	12.22	<0.001*	-1.521	-0.445
ShdHght[≤2m/>2m]	1.064	0.264	16.27	<0.001*	0.568	1.613
All Species						
(R^2 = 0.2642; % correctly classified: 72.6; Goodness of fit = 0.2680; Observations = 156)						
Intercept	-0.169	0.321	0.28	0.598	-0.818	0.452
PC1	0.585	0.132	19.67	<0.001*	0.340	0.861
PC2	0.354	0.201	3.10	0.078	-0.032	0.762
Origin[N/A]	0.627	0.277	5.14	0.0234*	-1.191	-0.099
Disturbed[U/D]	0.536	0.287	3.49	0.062	-0.020	1.111
AqVeg[No/Yes]	0.964	0.315	9.39	0.0022*	0.370	1.614
CanCov[>50%/≤ 50%]	0.345	0.237	2.13	0.144	-0.826	0.107
ShdHght[≤2m/>2m]	0.476	0.257	3.44	0.064	-0.016	0.996

[a] AqVeg = aquatic vegetation (both submerged and emergent); CanCov = amount of canopy cover above habitat >50%/≤ 50%; Disturbed: D = disturbed, U = undisturbed; Origin: A = artificial, N = natural; ShdHt = height of predominant shade source above habitat ≤2m or >2m; Type: C = container, P = pool

Table 3.5. C_8 coefficients of pairwise associations, within three habitat categories, between four most common mosquito species sampled as larvae at Greenville and Riverbanks zoos, South Carolina, 2008-2009. A value of 1 indicates complete positive association (always found together), and -1 indicates complete negative association (i.e., never found together). Artificial, container, and shaded values above the diagonal, and natural, pool, and unshaded values below.

Species[a]	*Aedes albopictus*	*Aedes triseriatus*	*Culex pipiens* complex	*Culex restuans*
		Artificial vs. Natural		
Ae. albopictus	–	0.13*	0.10	0.08
Ae. triseriatus	0.21*	–	0.00	0.12
Cx. pipiens complex	-1.00	-1.00	–	0.30*
Cx. restuans	-1.00	-0.64	-1.00	–
		Container vs. Pool		
Ae. albopictus	–	0.11	0.01	-0.15
Ae. triseriatus	NA[b]	–	NA[b]	NA[b]
Cx. pipiens complex	0.25*	NA[b]	–	0.42**
Cx. restuans	0.10	NA[b]	-0.25	–
		Shaded vs. Unshaded		
Ae. albopictus	–	0.19*	0.05	-0.04
Ae. triseriatus	0.09	–	-1.00	-0.08
Cx. pipiens complex	0.02	0.04	–	0.17
Cx. restuans	-0.20	0.00	0.18*	–

[a]Significance of chi-square statistic, *p<0.05, **p<0.001, [b]*Ae. triseriatus* never found in pools

Discussion

Mosquitoes oviposit in a variety of aquatic habitats at the Greenville and Riverbanks zoos, and the presence of their larvae is predictable. The most common species across both zoos, *Ae. albopictus*, is of particular importance from a zoo-based perspective. It bites people during the day, causing a nuisance to zoo visitors and employees (unpublished data). It also bites animals and can transmit arboviruses and the causative agent of dog heartworm, *Dirofilaria immitis* (Gratz 2004). Both zoos have attempted mosquito control in the last three years: two "mosquito magnet" traps at the Greenville

Zoo (currently not being used), and unmonitored and sporadic Bti use at the Riverbanks Zoo (ongoing). The other dominant species (*Ae. triseriatus*, *Cx. pipiens* complex, and *Cx. restuans*) are also of medical and veterinary concern (Foster and Walker 2009).

The 16 species collected as larvae in the zoos were previously represented in a statewide survey of adults from 1996 to 1998 in which 34 species were collected (Wozniak et al. 2001). During my study 23.5% of those species were found at the Greenville Zoo and 47.1% at the Riverbanks Zoo. Comparisons also were made with 2008 adult monitoring records from the South Carolina Department of Health and Environmental Control (SCDHEC) (C.L. Evans, SCDHEC, personal communication). Of the four species collected by SCDHEC in Greenville County, three were found as larvae in the zoo. In Richland County also, the mosquito larvae represented a subset of the local mosquito population and 15 of the 16 species (with the exception of *Tx. rutilus*) collected in the Riverbanks Zoo were collected as adults at non-zoo locations; 20 species were collected as adults in Richland County that were not collected as larvae in the zoo (most notably, *Cq. perturbans* was present in adult but not larval collections).

Aedes albopictus oviposits in large and small artificial and natural containers and pools (e.g., bromeliads, tin cans, treeholes, and water drums) and less commonly in large natural water bodies such as trenches and ground pools (Chan et al. 1971, Moore et al. 1988). It can persist in an environment with no human-created water bodies, but can

flourish in human-altered environments such as parks and gardens (Moore 1999). These characterizations are consistent with the results of my study.

Aedes triseriatus, the second most abundant species in my study, oviposits almost exclusively in shaded containers (e.g., tires, treeholes) (Beier et al. 1983, Williams et al. 2007), and in my study, larvae of *Ae. triseriatus* were significantly associated with natural containers and shade height ≤2m. Shade might prevent the desiccation of container habitats during periods of drought (Kitron et al. 1989). Additionally, understory canopy can contribute to increased microbial respiration in natural containers, leading to an increase in mosquito production, and exhibit a reduction in secondary metabolites, possibly reducing metabolic costs in detritivorous mosquito larvae (Strand et al. 1999).

Overall, habitat use in the two zoos did not differ from previous accounts of immature mosquito distributions. One aspect not investigated in my study is that the overall abundance of mosquitoes could be higher in zoos due to a larger population of captive hosts, compared with non-zoo areas (e.g., city parks, abandoned lots, sylvan habitats) or, alternatively, lower because of insecticide treatment of captive animals.

Additionally, according to C_8 values calculated, habitat partitioning or competition between species for oviposition sites, or between larvae, could be occurring in the zoos. *Aedes triseriatus* was not significantly associated with the other two species but C_8 values between it and *Cx. restuans* in shaded habitats, and *Cx. pipiens* complex in unshaded

habitats were very similar to values calculated in a 1981 study in Indiana tire yards, although significance of associations differed between my study and the previous one (Beier et al. 1983). Additionally, the C_8 value between *Cx. pipiens* complex and *Cx. restuans* in unshaded habitats differed marginally from Beier et al. although the species' association was significant in my study. However, although some association values were similar to the Beier et al. study others were different, indicating that more comparisons are required to determine if within zoo species associations differ significantly from those in habitats outside the zoos.

Zoo employees should receive semi-annual training in larval mosquito habitat recognition, and eliminate or ameliorate container habitats (e.g., fill with sand, utility foam, or overturn when not in use) (Shimonski 2009). If containers are an integral part of the zoo environment (e.g., artificial tree stumps), they can be flushed or treated regularly with mosquito larvicides. If larvicides are used, they need to be monitored by maintaining a database of when and where they are used. Gutters at both zoos were a frequent source of mosquito larvae. If gutters are unnecessary they should be removed, but if required, they should be cleaned regularly to prevent standing water. Shade sources ≤2m in height over larval habitats should be eliminated when possible or receive special attention as they are associated with mosquito larvae.

Both the Greenville and Riverbanks zoos regularly flush most artificial pools in animal enclosures and the Riverbanks Zoo stocks most artificial pools with G*ambusia* spp.

(larvivorous fish) and incorporates a flowing water design in most artificial pools that creates a strong current at the pool edges. These practices probably prevent mosquito development in artificial pools. In general, it seems that the mosquito problem in both zoos is due to unrecognized (and hence, uncontrolled) container habitats and natural pools, not zoo aquatic exhibits. However, if an exhibit pool lacks flowing water, regular flushing, larvicides, and mosquito predators it is likely mosquito larvae will develop there. Natural pools (e.g., vernal ponds) are a control concern; for example, they were the source of most *Cx. restuans* larvae at the Riverbanks Zoo. Zoos will need to decide if the cost to eliminate, modify, or regularly treat these habitats is worthwhile.

Absence of aquatic vegetation was significantly associated with larval presence, possibly because few anophelines were found during the study. However, increasing shade from growing aquatic vegetation can render habitats unsuitable as breeding sites (Munga et al. 2006). Other species, such as *Cq. perturbans*, associated with plants were probably missed by the sampling methods used. An independent study of mosquito larvae in a nearby zoo (e.g., Atlanta Zoo) should be undertaken to assess the validity of the model.

The results of my study can aid zoo employees in recognizing larval mosquito habitats, and remediating and designing zoo displays with the prevention of larval mosquito development in mind. Additionally, this and previous studies (Beier and Trpis 1981b, Derraik 2005, Nelder 2007) indicate that although zoos might not provide novel breeding habitats, mosquito populations within zoos are representative of populations, or subsets

of populations, outside of them. Research collaboration between zoos and medical entomologists can start with mosquito monitoring in zoological parks and training of zoo employees in mosquito-habitat recognition, but it has the potential to evolve into well-designed studies on mosquito, pathogen, and host interactions, and testing of primers for mosquito blood-meal identification using banked sera in zoos (e.g., an exotic animal in a zoo is a native animal elsewhere in the world). By providing a heterogeneous landscape with habitats simulating vastly different environments and novel assemblages of hosts, zoos are natural experiments that can be used to study mosquito ecology, behavioral plasticity, and vector potential.

CHAPTER FOUR

MOSQUITO HOSTS IN SOUTH CAROLINA ZOOS

Zoos are unique environments in which to study mosquito foraging behavior and to use strong hypothesis testing to elucidate the host adaptations and preferences of mosquitoes. For instance, they can be used to investigate the role of genetic components versus developmental or environmental parameters in shaping mosquito host choices, or nestedness of ectoparasite and host networks, two recently suggested goals of current medical and veterinary entomology research (Graham et al. 2009, Chaves et al. 2010). Species of captive animals represented in mosquito bloodmeals can be compared with those available in a particular zoo, and information such as flight distances from hosts (Ejiri et al. 2011) and larval mosquito habitats can be acquired. Zoos are also excellent experimental environments for addressing another recently suggested goal of research, that being how environmental factors alter or shape mosquito assemblages (Beketov et al. 2010). If the results of studies in zoos are representative of non-zoo environments, then they provide the power to predict mosquito distributions and host-usage patterns in areas not feasible for field studies.

Additionally, zoos have epidemiological consequences for captive and wild animals and humans. Mosquitoes transmit pathogens that have resulted in the deaths of captive birds and mammals, including endangered species (Beier and Trpis 1981a, Adler et al. 2011). *Culex pipiens pallens* was documented as a vector of avian *Plasmodium* spp. at a zoo in

Japan (Ejiri et al. 2011). If mosquitoes are interrupted during feeding on a zoo animal, the diversity of potential second hosts nearby is higher than it would be in most non-zoo environments because of human-mediated groupings of animals. Mosquitoes in zoos could have a feeding advantage because hosts might not have coevolved behavioral defenses. Alternatively, mosquitoes, especially adventive species, could be at a feeding disadvantage because some zoo animals sharing the same historical distribution as the mosquitoes might have coevolved defenses. Veterinary hospitals at zoos present the problem of sequestered and sick, and possibly restrained, animals that mosquitoes could access and might prefer (Klowden and Lea 1979, Hurd et al. 1995).

Finally, zoos have high host heterogeneity that might contribute to increases in pathogen prevalence, leading to epizootics (Kilpatrick et al. 2006a), or cause a dilution of biting rates on susceptible hosts, thereby decreasing pathogen incidence in the general population (Bradley and Altizer 2007). Zoos are ideal for addressing these competing hypotheses because mosquitoes are present, hosts and their movements are known, animals are under regular observation, and wild and captive animals are in the same area. In an era of shrinking global borders, and re-emerging pathogens previously sequestered in a sylvan cycle, zoos could act as pathogen buffers in increasingly disturbed and urbanized spaces.

My objectives in this study were to investigate 1) feeding patterns and hosts of mosquitoes in zoos; 2) distributions of mosquitoes after feeding; and 3) prevalence of dog

heartworm, *Dirofilaria immitis*, in bloodfed mosquitoes. I tested the hypotheses that mosquito bloodmeals 1) degrade with time; 2) represent captive animals, humans, and wildlife; 3) include examples of mixed-species; and 4) conform to previous known mosquito-host class associations (e.g., mammals, birds).

Materials and Methods

Site selection and mosquito sampling. Mosquitoes were collected from the Greenville Zoo (Greenville County) and Riverbanks Zoo (Richland County), South Carolina, USA, from May 2009 to October 2010, once or twice a month, with gravid traps and backpack and hand-held aspirators. In 2009, mosquitoes were collected with gravid traps, transported alive to a lab, fixed at -70C in an ultralow freezer, identified (Darsie and Ward 2005), and separated by gonotrophic condition according to Sella's (1920) stages. The head plus thorax of each bloodfed female mosquito was separated from the abdomen, with a razor blade on a fresh Kimwipe, and placed in an autoclaved and UV-sterilized 1.5-ml centrifuge tube. The razor and forceps were immersed in alcohol and flame-sterilized for at least 30 seconds after each mosquito was cut. The same procedures were used in 2010, except mosquitoes were collected using hand-held and backpack aspirators and killed on dry ice in the field. All collections were stored at -70C, and later moved to -20C before further processing. Latex gloves were worn during sorting and processing.

In April 2009, 15 gravid-trap sites were selected at the Greenville Zoo and 19 at the Riverbanks Zoo. Selected sites had little human traffic, partial shade, and protection from

wind and artificial lighting. Gravid-trap infusion water was based on that of Jackson et al. (2005). Once a month, five locations at each zoo were selected randomly for gravid-trap placement, with the caveat that all traps be at least 50 m apart to ensure independence (Allan et al. 1987, Reiter 2007). Traps were turned on between 1600 and 1700 and retrieved between 0800 and 0900. Two traps were run for three days per location, resulting in 30 trap nights per zoo per month. Both zoos were sampled from June through September 2009. The Greenville Zoo also was sampled in April and the Riverbanks Zoo in May. All catch containers were cleaned with ethanol between trap days to prevent experimenter contamination of the traps.

In April 2010, 13 aspiration sites were selected at the Greenville Zoo and 17 at the Riverbanks Zoo; if new sites later were noted, they were added on an *ad hoc* basis. Both zoos were sampled 1-4 times per month for 1-3 day periods from May to September. The Riverbanks Zoo also was sampled in April 2010 and February 2011. In 2009, resting boxes were placed inside and outside animal habitats at both zoos. During 2009 and 2010, creeping ground cover at both zoos was sampled with a backpack aspirator. Starting in May 2010, bloodfed females were collected in Richland County (including the Riverbanks Zoo). They were fixed on dry ice in the field; the heads and thoraxes were not separated from the abdomens.

Representative voucher specimens of each mosquito species are deposited in the Clemson University Arthropod Collection, South Carolina.

Mosquito handling and preservation. Genomic DNA was extracted from the heads plus thoraxes, and the abdomens of bloodfed mosquitoes, using a DNAzol BD Direct Extraction Kit (Molecular Research Center, Cincinnati, OH, USA), according to manufacturer's instructions, with slight modifications. Briefly, 50 µl DNAzol BD solution was added to a 1.5 ml tube containing the respective mosquito divisions and homogenized by crushing with a pipette tip. Then, 200 µl DNAzol BD was added, the solution was vortexed, and left to sit at room temperature (RT) for 30 – 60 minutes. Subsequently, 125 µl was removed to a new tube and the original mosquito material was stored in the remaining DNAzol BD in a freezer at -20°C. Then, 50 µl isopropanol was added to the transferred 125 µl DNAzol BD-mosquito homogenate. It was shaken and vortexed for ca. 1 minute and left at RT for ca. 60 minutes, then centrifuged at 6,000g for six minutes. The supernatant was removed and 62.5 µl DNAzol BD added, vortexed until the DNA pellet dispersed, and centrifuged at 6,000g for 5 minutes. The supernatant was removed and 125 µl 75% EtOH added, then centrifuged at 6.000g for 5 minutes. Then, the ethanol was carefully decanted and pipetted out, and the tubes were left upside down on Kimwipes to allow remaining alcohol to evaporate. The pellet was dissolved in 25 µl 8mM NaOH, left at RT for 5 minutes, then shaken and vortexed until the pellet dissolved. Finally, 4 µl of HEPES was added. The DNA extract was stored at 4°C for up to one week but transferred to -20°C for longer periods. Genomic DNA was extracted from mosquitoes in batches of 20-30 individuals, with an initially empty sterilized 1.5 ml tube as a negative control in each batch of extractions to control for extraction contamination

(i.e., extracted gDNA from one tube is accidentally transferred to another, or there is a source of bench contamination).

The DNAzol BD is the least expensive kit on the market and has been used successfully for genomic DNA extractions from mosquito bloodmeals and filarioid nematodes in the family Onchocercidae (Molaei et al 2008 & 2009, Watts et al. 2009, Neary et al. 2010). Before processing experimental samples, methods were refined using bloodfed mosquitoes with known hosts procured from colonies at The Ohio State University, Columbus, OH, USA (*Anopheles gambiae* and *Culex pipiens*),Clemson University (*Cx. pipiens*), and the NIH Filariasis Research Reagent Resource (FR3) at the University of Georgia in Athens, GA, USA (*Aedes aegypti* infected with *Dirofilaria immitis*)

Bloodmeal analysis. Genomic DNA extracts from mosquito abdomens were amplified by PCR on a Bio-Rad iCycler, using order-specific primers for birds and mammals, and universal vertebrate primers (Table 1). All primers amplified segments of the mitochondrial cytochrome *b* gene and have been previously used in studies analyzing mosquito bloodmeals. A 25 µl reaction mixture containing 12.5 µl GoTaq Colorless Master Mix (Promega), 1 µl forward and 1 µl reverse (premixed), 1 µl gDNA, and 9.5 µl nuclease-free water (provided with GoTaq) was used. Negative and positive controls were included in every PCR. Negative controls consisted of distilled, autoclaved water, and positive controls were domestic dog gDNA (from blood obtained at the FR3) for mammal-specific and universal vertebrate primers, and chicken gDNA (from bloodfed

Cx. pipiens from a colony at Ohio State University) for avian primers. The same gDNA used as positive controls was used to optimize PCR cycling conditions for each primer (Table 4.1). Our strategy was to first attempt amplification with order-specific primers for birds and mammals. If neither of these reactions yielded a product for a given sample, that sample was then subjected to another PCR with universal vertebrate primers. Successful amplifications were determined by visualizing PCR products on a 1.5% agarose gel with EDTA followed by ethidium bromide staining and UV trans-illumination. Gels were documented digitially on a Bio-Rad Gel Doc System and archived.

PCR products were purified out of successful reaction mixtures, using an "Exo-AP" protocol. Briefly, a master "Exo-AP" mix was made by diluting (with DNA-grade H_2O) Exonuclease I at 1:100, and Antarctic phosphatase at 1:10 in the same PCR tube. Then, 1 µl of the Exo-AP mix was added to 1 µl of PCR product. The resultant mixture was placed in a PCR cycler and the following thermal profile was used to purify PCR products: 37°C for 30 minutes, 80°C for 15 minutes, and a 4°C hold. Subsequently, either a forward or reverse primer was added (depending on the best performing primer for sequencing in test trials) to wells and purified products were sent to the Clemson University Genomics Institute for Sanger sequencing on an ABI 3130 (Applied Biosystems). Primer sequences were removed from trace file results and the remaining sequences were edited with BioEdit 7.0.5.3 freeware (Hall 1999). The sequences were run through the GenBank nucleotide (nr) database, using the BLASTN 2.2.25+

algorithm, and vertebrate hosts identified (Altschul et al. 1997). Results with the highest "Max score" were recorded along with the "Max ident" percent values. For each analyzed bloodmeal, the highest percent identity that was geographically reasonable is presented, with lower percent identities (e.g., <95%) indicating questionable results. If there were two or more similar percentages the discrepancy is discussed. Common and Latin names follow those of the International Ornithologist's Union for birds (www.worldbirdnames.org), Wilson and Reeder's Mammal Species of the World 3rd ed. on-line searchable database for mammals (http://www.bucknell.edu/msw3), and the IUCN Red List of Threatened Species for reptiles and amphibians (www.iucnredlist.org).

To screen for experimenter contamination that would lead to false-positive human identifications (Malmqvist et al. 1999), sample sequences from successful amplifications with the mammal-specific primer set were checked against the sequence of the same amplicon from gDNA isolated from the experimenter (HT), using the CAP Contig Assembly program in BioEdit (7.0.5.3) (Hall 1999), with parameters of a 1-base minimum match and 85% overlap. Other conspecific sample sequences were checked against each other in the same way to ensure all non-human results were not due to bench contamination.

Dirofilaria screen. Genomic DNA extracts from mosquito heads plus thoraxes and abdomens were amplified by PCR on a Bio-Rad iCycler, using a "pan-filarial" primer set that amplifies gDNA from at least nine species of filarioid nematodes including

Acanthocheilonema reconditum, *Dirofilaria immitis*, and *D. repens* (Table 4.1). The resultant amplicons can be distinguished to species with a gel separation; *A. reconditum* produces bands at 578bp, *D. immitis* at 542bp, and *D. repens* at 484bp. Genomic DNA from *D. immitis*-infected dog blood and *D. immitis*-infected mosquitoes (obtained at the FR3) was used to optimize PCR cycling conditions. Genomic DNA from uninfected mosquitoes (also from the FR3) was used as a negative control. To ensure that genomic DNA was extracted from heads plus thoraxes, a control PCR was performed with a universal insect primer that amplifies a portion of the insect 12s rRNA gene (Table 1). All PCR products and gels were treated as for bloodmeals.

Statistical Analyses. All analyses were conducted in JMP 9 (SAS Institute, Cary, NC, USA). Mosquito-host forage ratios (Hess et al. 1968) were used to determine if mosquitoes exhibited host biases. Forage ratios were obtained by dividing the percent of a particular host represented in mosquito bloodmeals by the percent of that host type in the general population. Minimum flight distances were calculated, using Google Earth, for mosquitoes with bloodmeals from captive hosts, based on the locations of mosquito captures and hosts. Because most animal enclosures were irregularly shaped, the shortest and longest distances between mosquito captures and enclosure boundaries were estimated. These distances were pooled across zoos and tested separately by host type (bird versus mammal), Sella stage, and mosquito species, and if there were no differences between short and long distances, the average of the two distances was used. All analyses were conducted in JMP 9 (SAS Institute, Cary, NC, USA).

Table 4.1. Primers used to amplify mosquito-host genomic DNA and *Dirofilaria immitis* genomic DNA.

Primer	Sequence (5'-3')	Target	Product (bp)	Dena-turation	Anneal-ing	Exten-sion	No. cycles	Ref.
Avian	GACTGTGACAAAATCCCNTTCCA GGTCTTCATCTYHGGYTTACAAGAC	Cytb	508	94	55	72	33	Ngo and Kramer 2003
Mammal	CGAAGCTTGATATGAAAAACCATCGTTG TGTAGTTRTCWGGGTCHCCTA	Cytb	772	94	50	72	35	
Universal vertebrate	AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA	Cytb	307	94	50	72	40	Kocher et al. 1989
Pan-filarial	AGTGCGAATTGCAGACGCATTGAG AGCGGGTAATCACGACTGAGTTGA	Cuticular antigen gene	578 (*A. reconditum*) 542 (*D. immitis*) 484 (*D. repens*)	94	60	72	32	Rishniw et al. 2006
Universal insect	AAACTAGGATTAGATACCCTATTA AAGAGCGACGGGCGATGTGT	12S	400	94	50	72	32	O'Neill et al. 1992

Results

Mosquito collections. Sixteen species of mosquitoes were collected from both zoos: 13 from the Greenville Zoo and 14 from the Riverbanks Zoo, with 11 in common (Table 4.2). In total, 2873 individuals were collected at Greenville and 1476 at Riverbanks. Of these, 106 (2.4%) were bloodfed, 34 (1.2%) at Greenville and 72 (4.9%) at Riverbanks (Table 3). Five species were bloodfed at Greenville, and nine at Riverbanks, with no species at Greenville that were not also at Riverbanks: *Aedes albopictus* (Skuse 1895), *Ae. triseriatus* (Say 1823), *Anopheles punctipennis* (Say 1823), *Anopheles quadrimaculatus* complex (Say 1824), *Culex erraticus* (Dyar and Knab 1906), *Cx. pipiens* complex (L. 1758), *Cx. restuans* (Theobald 1901), *Cx. territans* (Walker 1856), and *Psorophora columbiae* (Dyar and Knab 1906).

Genomic DNA Amplifications and Identifications.

Hosts were successfully identified from 63.2% of bloodmeals, 32.4% at Greenville and 77.8% at Riverbanks. Host identity was not obtained from the single *Ps. columbiae* bloodmeal. Two multiple bloodmeals were detected from mosquitoes at Riverbanks, one wild bird and captive mammal in *An. punctipennis* and one wild bird and reptile (wild/captive status undetermined) in *Cx. pipiens* complex. One of the human results that was removed due to possible experimenter contamination might have been part of a mixed human and wild bird bloodmeal in a female of *Cx. pipiens* complex from Greenville. Overall, four human sequences had high homology with the experimenter sample (1 *Cx. erraticus* from Riverbanks, 3 *Cx. pipiens* complex from Greenville).

Because the primer set was universally mammal-specific, the four excluded sequences might not have been from the experimenter but, rather, were legitimate; nonetheless, the four data points were not included in statistical analyses or in tables (their inclusion in analyses did not alter the significance of statistical test results).

The likelihood of extraction success was significantly lower for *Cx. restuans* than for *Ae. albopictus*, *An. punctipennis*, *Cx. erraticus*, *Cx. pipiens* complex, and *Cx. territans*, and for *Cx. pipiens* complex and *Cx. territans* than for *An. punctipennis* and *Cx. erraticus* (chi-square, $G=30.442$, $df=5$, $p<0.0001$). The overall extraction success rates by Sella's stages were 81.0% (30/37) for Sella II, 81.0% (17/21) for Sella III, 81.8% (9/11) for Sella IV, 33.3% (1/3) for Sella V, and 26.3% (5/19) for Sella VI. Product success declined significantly with increasing Sella stage and, as a group, II, III, and IV were significantly different from VI, while V was not included because it was a lone data point (chi-square, $G=21.414$, $df=4$, $p<0.0003$). Additionally, Sella stage was independent of host type.

No significant differences were found for host ($F=0.2499$, $df=2$, $p=0.7796$), Sella stages ($F=2.1386$, $df=3$, $p\leq0.1068$ (Sella V not included because it was a lone data point)), mosquito species ($=1.9916$, $df=3$, $p=0.1246$ (excluding species with only one or two bloodmeals)), or zoo ($t=-0.16624$, $df=14.06339$, $p=0.8703$), in the maximum percent sequence identity between sequences GenBank and sample sequences (due to low sample size, mosquitoes were pooled across zoos, except when comparing across zoos) (Table 4.3).

Table 4.2. Mosquito species collected with hand and backpack aspiration, light traps, and (primarily) gravid traps at the Greenville and Riverbanks zoos, South Carolina, from 2009-2011. Species presented alphabetically rather than in order of prevalence. GZ = Greenville Zoo, RZ = Riverbanks Zoo.

All Species	Total GZ	% GZ	Total RZ	% RZ	Total (GZ + RZ)	% (GZ + RZ)
Ae. albopictus	281	9.78	261	17.68	542	12.46
Ae. canadensis canadensis	0	0.00	1	0.07	1	0.02
Ae. japonicus japonicus	7	0.24	0	0.00	7	0.16
Ae. triseriatus	22	0.77	11	0.75	33	0.76
Ae. vexans	2	0.07	12	0.81	14	0.32
An. punctipennis	1	0.03	54	3.66	55	1.26
An. quadrimaculatus complex	1	0.03	5	0.34	6	0.14
Cx. erraticus	0	0.00	64	4.34	64	1.47
Cx. pipiens complex	1707	59.42	860	58.27	2567	59.03
Cx. pipiens/restuans	132	4.59	11	0.75	143	3.29
Cx. restuans	700	24.36	108	7.32	808	18.58
Cx. spp	1	0.03	22	1.49	23	0.53
Cx. territans	17	0.59	64	4.34	81	1.86
Or. signifera	1	0.03	0	0.00	1	0.02
Ps. ferox	0	0.00	1	0.07	1	0.02
Ur. sapphirina	1	0.03	2	0.14	3	0.07
Total	2873	100.00	1476	100.00	4349	100.00

Flight Distances. Overall, minimum flight distances (dispersal) from host locations ranged from 15.5 m to 327.0 m with a mean and standard error of 94.1 m ± 13.4 m. Flight distances did not differ significantly between host types (bird versus mammal) (Welch's ANOVA, WF=4.2395, df=1, p=0.0527) (Table 4.3) (three reptile bloodmeals were not included because mosquito flight distances were identical). No significant differences were found in flight distances among *An. punctipennis* (n=9), *Cx. erraticus* (n=10), and *Cx. pipiens* complex (n=10)(F=2.2438, df=2, p=0.1262) (*Ae. triseriatus* and *An. quadrimaculatus* not included because each had n=2). Average flight distance for Sella

stage III was significantly longer than Sella II, but neither was different from IV ($F=3.8099$, $df=2$, $p \leq 0.0344$).

Mosquito Hosts (Table 4.4). Of the four species with more than 5 bloodmeals across both zoos, *Cx. pipiens* complex and *Cx. erraticus* fed on a significantly different ratio of avian to mammalian to reptilian hosts in captivity than did *An. punctipennis* (chi-square=14.848, df=4, $p<0.005$) (Fig. 4.1). No significant differences were found among the four species in the wild category. Bloodmeals from humans were included in the wild category (removing them did not change significant differences). *Culex pipiens* complex and *Culex erraticus* showed a slight bias for birds and *An. punctipennis* showed a strong bias for mammals (Table 4.5). *Aedes albopictus* fed on only wild animals (including one human); of the five *Ae. albopictus* bloodmeals, 3 were birds and 2 were mammals. Three bloodmeal identifications of wild bird hosts of *Cx. erraticus* (two European starlings, one Grey catbird) and one of *Cx. pipiens* complex (Grey catbird) were not the highest percent hits returned by GenBank but were the highest hits that made sense geographically (i.e., hosts with higher percent hits do not occur in North America and were not known zoo residents).

Collections with more than four identified bloodmeals at the Riverbanks Zoo did not show a trend toward increased use of captive animals in the zoo interior versus exterior (Fig. 4.2); however, the only human bloodmeals were recorded in a more interior location (i.e., within the perimeter of the outer customer walkway) in the zoo. Differences were

Table 4.3. Average GenBank BLAST percent identity (between sample sequence and sequence in GenBank), percent of host genomic DNA amplification success (number mosquito bloodmeals successfully amplified out of total number sampled), and minimum flight distances (inferred from known location of captive hosts and location of mosquito capture) for each zoo and overall total by mosquito species and Sella stage.

Bloodfed Species.	Sella Stage	Avg. GenBank BLAST % identity ± SE (n)			Amp. Success (# successful/total tested)	Avg. flight distance ± SE (m) (n)		
		GZ	RZ	Total	Total	GZ	RZ	Total
Ae. albopictus	unknown	96 (1)	93 ± 7 (3)	93.8 ± 5 (4)	80.0 (4/5)	na	na	na
	II	99 (1)	na	99 (1)	100 (1/1)	na	na	na
Ae. triseriatus	II	na	99 ± 0 (2)	99 ± 0 (2)	100 (2/2)	na	41±18 (2)	41±18 (2)
An. punctipennis	unknown	na	99 (1)	99 (1)	100 (1/1)	na	80 (1)	80 (1)
	II	99 (1)	99.4 ± 0.2 (8)	99.3 ±0.2 (9)	90 (9/10)	76 (1)	54±12 (7)	57±11 (8)
	VI	na	95 (1)	95 (1)	50 (1/2)	na	na	na
An. quadrimaculatus complex	III	na	100 (1)	100 (1)	100 (1/1)	na	55 (1)	55 (1)
	IV	na	99 (1)	99 (1)	100 (1/1)	na	68 (1)	68 (1)
Cx. erraticus	II	na	95 ± 2 (10)	95 ± 2 (10)	90.9 (10/11)	na	106±46 (5)	106±46 (5)
	III	na	95 ± 2 (11)	95 ± 2 (11)	91.7 (11/12)	na	249±79 (2)	249±79 (2)
	IV	na	99 ± 1 (2)	99 ± 1 (2)	66.7 (2/3)	na	110±84 (2)	110±84 (2)
	V	na	95 (1)	95 (1)	100 (1/1)	na	93 (1)	93 (1)
Cx. pipiens complex	unknown	na	na	na	0 (0/5)	na	na	na
	II	100 (1)	98 ± 2 (6)	98 ± 1.5 (7)	100 (7/7)	16 (1)	85 ± 18 (2)	62 ± 25 (3)
	III	99 (1)	93 ± 7 (4)	94 ± 5 (5)	62.5 (5/8)	30 (1)	201±34 (2)	144±60 (3)
	IV	99.5 ± 0.5 (2)	99 (3)	99.2 ± 0.2 (5)	83.3 (5/6)	53 (1)	121±34 (3)	104±29 (4)
	V	na	na	na	0 (0/1)	na	na	na
	VI	92 ± 5 (4)	na	92 ± 5 (4)	28.6 (4/14)	na	na	na
Cx. restuans	II	na	na	na	0 (0/1)	na	na	na
	IV	na	93 (1)	93 (1)	100 (1/1)	na	na	na
	V	na	na	na	0 (0/1)	na	na	na
	VI	na	na	na	0 (0/3)	na	na	na
Cx. territans	II	na	99 (1)	99 (1)	20 (1/5)	na	na	na
Ps. columbiae	na	na	na	na	0 (0/1)	na	na	na

not found in the percentages of mosquito-host classes (i.e., bird, mammal, reptile) between the zoo interior and exterior. However, of the four reptile hosts identified, three came from mosquitoes captured within 37 m of their hosts, captive giant tortoises. A bloodmeal from an amphibian host (American green tree frog) came from *Cx. territans* taken near an alligator pond. Of the two species with the most hosts, *Cx. pipiens* complex and *Cx. erraticus*, a seasonal shift was detected in the use of birds versus mammals (e.g., early-season bird feeding) when host identities were summed across zoos and years (Fig. 4.3).

Hosts identified from mosquitoes collected in Richland County, but not in the Riverbanks Zoo, from May to July 2010 included *Canis lupus baileyi* (Mexican wolf) (likely *Canis lupus familiaris*) for *Ae. albopictus*; *Canis familiaris* (domestic dog), *Odocoileus hemionus hemionus* (mule deer) (likely *Odocoileus virginianus*) for *Cx. erraticus*; and *Canis lupus* (Grey wolf, n=2) (likely *Canis lupus familiaris*) for *Cx. pipiens* complex. One bloodmeal identified (93% GenBank sequence identity) from a mosquito trapped outside the zoo at the State Park Health Center in Richland Co. had two competing and equally unlikely host identifications (both with 92% max. ident.): Anderson's flapshell turtle (*Lissemys punctata andersoni*) and the Desert monitor (*Varanus griseus*). A turtle or monitor bloodmeal could be from a captive or released pet, as there is an exotic reptile show in Richland Co. every year. But no exotic reptile farms are in the surrounding area. Two soft-shelled turtle species, *Apalone spinifera* and *A.*

ferox, are found in South Carolina (www.texasturtles.org/Trionychidae) and both have entries in GenBank.

Dirofilaria immitis screening. When tested with *D. immitis*-infected mosquitoes, the extraction protocol had an 80% success rate. Of the 67 mosquitoes with an identified host, the heads plus thoraces and abdomens of 59 were tested separately for *D. immitis*. Of the 59 heads plus thoraces tested, 45 had positive bands when amplified with the Insect 12s extraction control primers. No samples were positive for *D. immitis*.

Table 4.4. Bloodfed mosquito species collected at Greenville and Riverbanks zoos, South Carolina, 2009 - 2011. If more than one mosquito was positive for a given host, the separate mosquitoes are indicated in parentheses with zoo and GenBank BLAST percent identities. C = captive, H = human, W = wild; A = avian, M = mammal, R = reptile.

Bloodfed Spp.	# hosts ID/total GZ (%)	# hosts ID/total RZ (%)	# hosts ID/total (%)	Host Spp. (Zoo, GenBank BLAST % identity)	C:H:W GZ	C:H:W RZ	A:M:R GZ	A:M:R RZ
Ae. albopictus	2/2 (100)	3/5 (60)	5/7 (71)	**Avian**: Northern cardinal (*Cardinalis cardinalis*) (R, 79), Carolina chickadee$^{\Delta}$ (*Poecile carolinensis*) (R, 100), Mourning dove$^{\Delta}$ (*Zenaida macroura*) (R, 100). **Mammal**: Virginia opossum (*Didelphis virginiana*) (G, 96), Human (*Homo sapiens*) (G, 99).	0:1:1	0:0:4	0:2:0	3:0:0
Ae. triseriatus	0/0 (na)	2/2 (100)	2/2 (100)	**Avian**: Common ostrich$^{\Delta}$ (*Struthio camelus*) (R, 99). **Mammal**: Brown bear$^{\Delta}$ (*Ursus arctos*) (R, 99)	NA	2:0:0	NA	1:1:0
An. punctipennis	1/2 (50)	10/10* (100)	11/12 (92)	**Avian**: Summer tanager$^{\Delta}$ (*Piranga rubra*) (R, 99†), Common ostrich$^{\Delta}$ (R, 99). **Mammal**: Auroch (i.e., cow) (*Bos Taurus*) (R, 99; R, 99†), Goat (*Capra hircus*) (G, 99), Spotted hyena$^{\Delta}$ (*Crocuta crocuta*) (R, 100), Horse (*Equus caballus*) (R, 100; R, 100; R, 99; R, 99), Human (R, 95).	1:0:0	8:1:1	0:1:0	2:8:0
An. quadrimaculatus complex	0/0 (na)	2/2 (100)	2/2 (100)	**Avian**: Common ostrich$^{\Delta}$ (R, 100). **Mammal**: Brown bear$^{\Delta}$ (R, 99).	NA	2:0:0	NA	1:1:0
Cx. erraticus	0/0 (na)	24/28 (86)	24/28 (86)	**Avian**: Grey crowned crane$^{\Delta, \text{¥}}$ (*Balearica regulorum*) (R, 97), Northern cardinal (R, 99; R, 99), Grey catbird (*Dumetella carolinensis*) (R, 88), Carolina chickadee (R, 89), American flamingo$^{\Delta}$ (*Phoenicopterus ruber*) (R, 99; R, 95), Keel-billed toucan$^{\Delta}$ (*Ramphastos sulfuratus*) (R, 100), Common ostrich$^{\Delta}$ (R, 100), Common starling (*Sturnus vulgaris*) (R, 89; R, 89), Mourning dove (R, 100; R, 99; R, 99). **Mammal**: Horse (R, 100), Human (R, 95; R, 88; R, 82), Raccoon (*Procyon lotor*) (R, 99; R, 90), Ring-tailed lemur$^{\Delta}$ (*Lemur catta*) (R, 95). **Reptile**: Galápagos tortoise$^{\Delta, \text{¥}}$ (*Chelonoidis nigra*) (R, 98; R, 98, R, 98).	NA	10:3:11	NA	14:7:3
Cx. pipiens complex	8/24 (33)	13/19* (68)	21/42 (50)	**Avian**: Wreathed hornbill$^{\Delta}$ (*Rhyticeros undulatus*) (G, 100), Northern cardinal (G, 100; R, 99), Yellow-throated warbler (*Dendroica dominica*) (R, 72†), Grey catbird (R, 89), Tufted titmouse (*Baeolophus bicolor*) (G, 77), American flamingo$^{\Delta}$ (R, 100; R, 100), Carolina chickadee (G, 99), Toco toucan$^{\Delta}$ (*Ramphastos toco*) (R, 100), Common ostrich$^{\Delta}$ (R, 99), Carolina wren (*Thryothorus ludovicianus*) (R, 99), Northern red-billed hornbill$^{\Delta}$ (*Tockus erythrorhynchus*) (R, 99), Mourning dove (R, 100; R, 99). **Mammal:** Auroch (R, 99), Spotted hyena$^{\Delta}$ (*Crocuta crocuta*) (R, 99), Human (G, 100; R, 95), Ring-tailed lemur$^{\Delta}$ (G, 99), Siamang (*Symphalangus syndactylus*) $^{\Delta, \Phi}$ (G, 99). **Reptile**: American box turtle (*Terrapene carolina*) $^{\text{¥}}$(R, 99†).	3:1:4	7:0:6	5:3:0	10:2:1
Cx. restuans	0/4 (0)	1/2 (50)	1/6 (17)	**Avian**: Northern cardinal (R, 93).	NA	0:0:1	NA	1:0:0
Cx. territans	0/2 (0)	1/3 (33)	1/5 (20)	**Amphibian**: Green treefrog (*Hyla cinerea*) (R, 99).	NA	0:0:1	NA	0:0:0
Ps. columbiae	0/0 (na)	0/1 (0)	0/1 (0)	na	NA	NA	NA	NA
Total	11/34 (32)	56/72 (78)	67/106 (63)		4:2:5	29:4:24	5:6:0	32:19:4

$^{\Delta}$ Novel host record (results with <95% identity not evaluated); *1 mosquito with mixed bloodmeal; †Mixed bloodmeal; ¥IUCN 2.3 "Vulnerable"; $^{\Phi}$IUCN 2.3 "Endangered"

Table 4.5. Forage ratios of three mosquitoes in South Carolina zoos on captive animals. A forage ratio >1 indicates preference for that host type because the mosquito is taking more bloodmeals from that host type than is found in the standing population. Ratios are presented for the current study, and a previous study in the Riverbanks Zoo. Additionally, percentages from previous studies outside of zoos are presented for the purposes of comparison.

Species	Current Study (n)		Current + Previous study* (n)		Previous study (n)	
***Cx. pipiens* complex**	Avian (6)	Mammal (4)	Avian (24)	Mammal (5)	Avian (18)	Mammal (1)
% from literature†	68.5	31.5				
% in Bloodmeals	60.0	40.0	83.0	17.0	95.0	5.0
% in Zoo Population^	52.0	48.0	52.0	48.0	52.0	48.0
Forage Ratio	**1.2**	**0.8**	**1.6**	**0.4**	**1.8**	**0.1**
Cx. erraticus	Avian (5)	Mammal (2)	Avian (8)	Mammal (2)	Avian (3)	Mammal (0)
% from literature	39.0	61.0				
% in Bloodmeals	71.0	29.0	80.0	20.0	100.0	0.0
% in Zoo Population^	52.0	48.0	52.0	48.0	52.0	48.0
Forage Ratio	**1.4**	**0.6**	**1.5**	**0.4**	**1.9**	**0.0**
An. punctipennis	Avian (1)	Mammal (8)				
% from literature	5.0	95.0				
% in Bloodmeals	12.5	87.5				
% in Zoo Population^	52.0	48.0				
Forage Ratio	**0.2**	**1.8**				

*Nelder 2007

†Contact author for dataset and list of publications used to obtain literature numbers.

^Estimate of percent of individuals in each class exposed to mosquitoes at zoos summed across both zoos

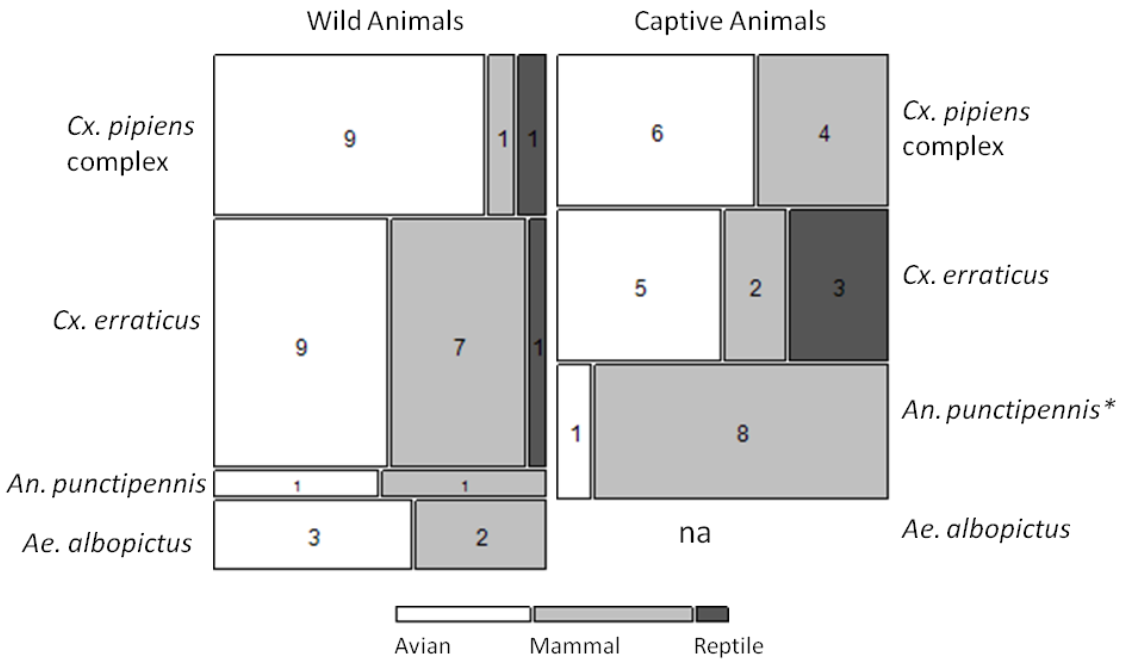


Figure 4.1. Identity of mosquito bloodmeals during 2009-2011, across two South Carolina zoos, by captive versus wild status. Numbers in boxes refer to number of hosts, and size of boxes represents different host numbers.Wild category includes human bloodmeals for *An. punctipennis* (n=1), *Cx. erraticus* (n=1) or *Cx. pipiens* complex (n=3). **An. punctipennis* significantly different than two other species in captive category ($p \leq 0.05$).

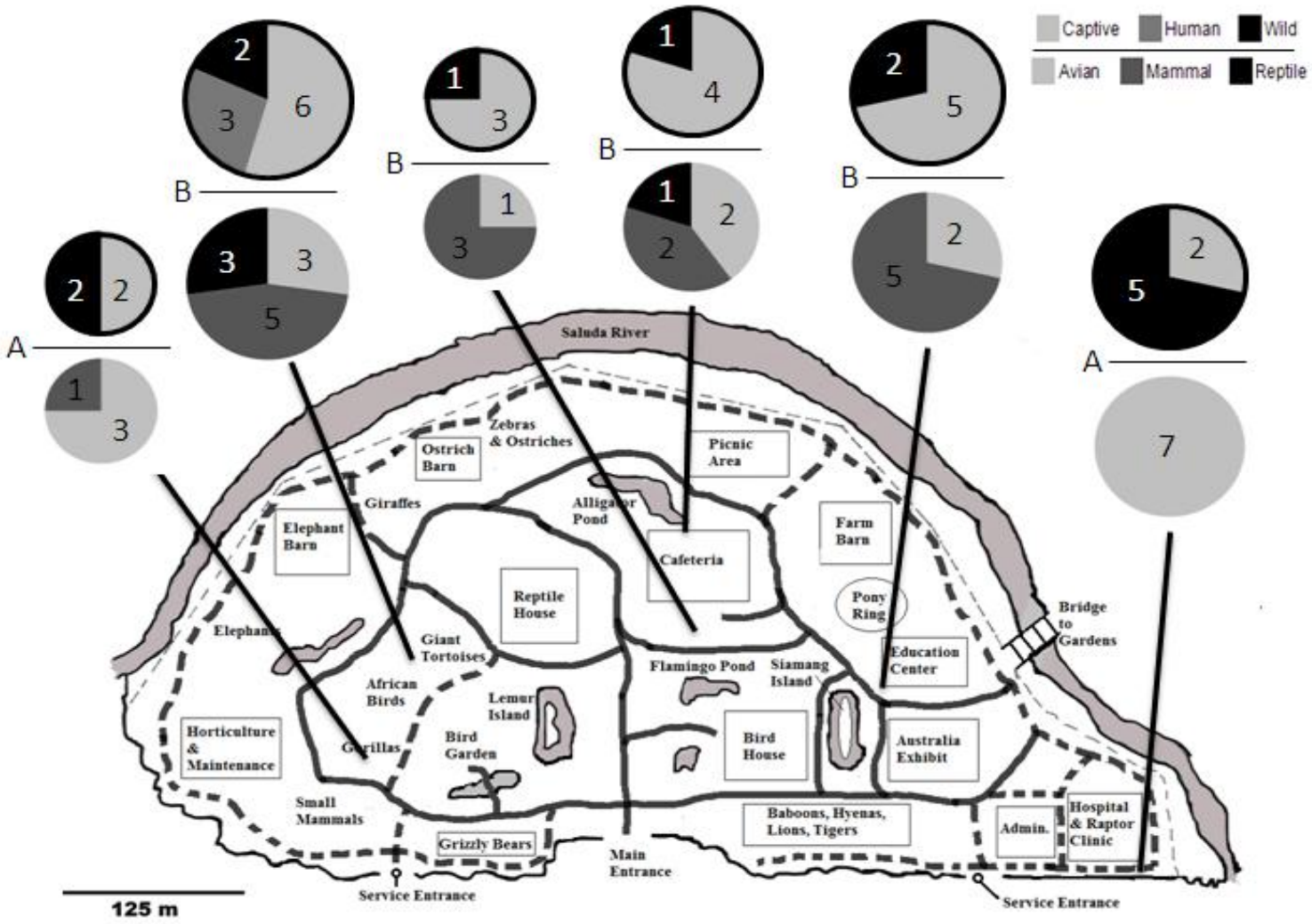


Figure 4.2. Mosquito hosts (C:H:W above line, A:M:R below line) in Riverbanks Zoo 2009-2011. "A" indicates a gravid trap site, while "B" is a hand aspiration site. Dashed lines indicate boundary of zoo and employee access roads. Solid lines are customer walkways within zoo.

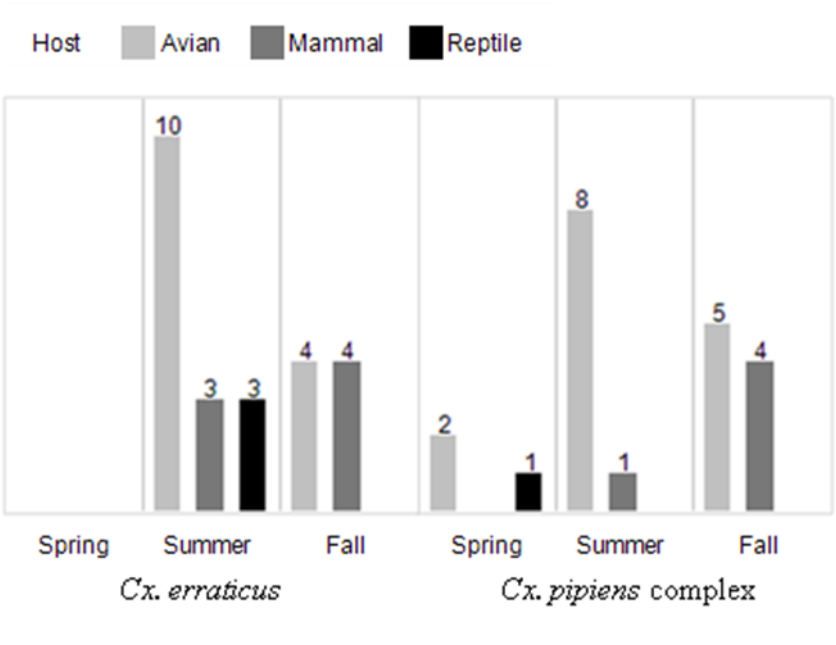


Figure 4.3. Host types for two mosquito species summed across years 2009-2011 and zoos. Total number of hosts given at top of the bar.

Discussion

The success rate (63.2%) for bloodmeal identifications and percentage of multiple bloodmeals (3%) is within the range of previous studies using similar methods and species (Molaei et al. 2008 & 2009, Ejiri et al. 2011). The success rates of bloodmeal identification for Sella stages were similar to those found by Ejiri et al. (2011), where "full-fed"=Sella II, "partial-fed"=Sella III, "half-gravid"=Sella IV-V, and "gravid"=Sella VI-VII. The declining success rate of extractions likely was due to decreasing bloodmeal volume (and hence, decreasing amounts of DNA) in the mosquito abdomen (Ejiri et al. 2011) due to the digestive action of bloodmeal nucleases. Although the likelihood of obtaining gDNA decreased with increasing Sella stage, if gDNA was obtained, the

quality of the amplicon was similar across stages, suggesting that regardless of bloodmeal age, a successful identification is likely if host genomic DNA is recovered.

Host DNA extractions from mosquitoes collected at the Riverbanks Zoo were more successful than those at the Greenville Zoo. I think this is because the Greenville samples were subjected to one more thaw-freeze cycle (due to transport) than were the Riverbanks samples. Although the magnitude of extraction failure was greater for the Greenville samples, the failure trend across species did not differ between zoos.

Although 63.2% of bloodmeals from mosquitoes in zoos were successfully identified, only 27% (6/22) of SC-DHEC collections outside the zoo and 30% (3/10) inside the zoo were successful. I believe this was due to an inhibitory effect of the mosquito heads on PCR efficiency (Lardeux et al. 2008). The successful identifications by species were 50% for *Ae. albopictus* (4/8) and 75% for *Cx. erraticus* (3/4), but only 13% for *Cx. pipiens* complex (2/15). No hosts were identified for *An. punctipennis* (0/1), *Cx. restuans* (0/3), or *Ps. columbiae* (0/1), suggesting that the inhibitory effect might not be as strong in *Ae. albopictus* and *Cx. erraticus* as in other species.

The relative proportion of captive hosts in the current study was lower than that in a study by Nelder (2007) at the Riverbanks Zoo: 41.7% versus 71.4% for *Cx. erraticus*, and 47.6% versus 62.5% for *Cx. pipiens* complex. In this study, 1 amphibian species, 16 bird species, 10 mammal species, and 2 reptile species were identified, which is comparable to the 17 bird species and 7 mammal species in the study by Ejiri et al. (2011), with one

mammalian host (*Bos taurus*) and one avian genus (*Parus*) in common. Two of the most prevalent mammals (horses and humans) and three of the most common birds (American robin, grey catbird, and northern cardinal) in my study also were reported among the most common mammals and birds in a previous meta-analysis of 12 bloodmeal studies conducted primarily in the eastern United States (Chaves et al. 2010).

Of the species with more than 5 bloodmeals from captive animals across both zoos, *Cx. pipiens* complex and *An. punctipennis* showing host-class usage similar to that reported previously in the literature, with *Culex erraticus* showed a reverse of previous literature reports (i.e., bias for birds in this study). This reversal was seen previously in *Cx. erraticus* bloodmeals collected at the Riverbanks Zoo (Nelder 2007). *Aedes albopictus* also showed avian associations more so than previously reported in the literature.

The apparent differences in host use by *Ae. albopictus* and *Cx. erraticus* inside, as opposed to outside, zoos merit further investigation. *Aedes albopictus* is rarely reported as having avian hosts but, being opportunistic and ground-associated, it might take blood meals from any hosts it encounters during appetitive flights (Dennett et al. 2007). Zoos possibly represent predator-limited areas for wild birds, especially urban-associated passerines, which might forage more often on the ground where *Ae. albopictus* would encounter them. Alternatively, they might forage more often on the ground because of less competition in zoos from ground-dwelling mammals (e.g., chipmunks) that are subject to pest control programs. Northern cardinal (*Cardinalis cardinalis*) has been

reported once as a host of *Ae. albopictus* (Richards et al. 2006), but to my knowledge the other two avian hosts of *Ae. albopictus* in this study are novel records. *Culex erraticus* has been described as both ornithophilic and opportunistic, and it might preferentially feed on large birds with lowered defenses (e.g., nesting birds) that occur in high abundances (Hassan et al. 2003, Unnasch et al. 2006, Mackay 2007). Five of the fourteen *Cx. erraticus* bird hosts in my study were captive and large (e.g., flamingo), indicating that this mosquito might exploit noticeable and vulnerable hosts. Of the previous authors reporting host usage in zoo mosquitoes, Nelder (2007) found a similar avian association in *Cx. erraticus*, and Ejiri et al. (2011) reported *Ae. albopictus* feeding on four humans, one rat, and one Black-necked swan.

Bird-feeding in zoos merits further investigation given the possibility for avian malaria transmission to wild and captive birds by mosquitoes in zoos (Ejiri et al. 2011), and the transmission of West Nile virus from birds (which act as natural amplification reservoirs) to humans (Kilpatrick et al. 2006b). The first fully sequenced strain of West Nile virus (WNv) from North America was isolated from a flamingo at the Bronx Zoo (Lanciotti et al. 1999). *Aedes albopictus*, *Ae. triseriatus*, *Cx. erraticus*, *Cx. pipiens* complex, and *Cx. restuans* have been implicated as vectors of Eastern Equine Encephalitis virus, LaCrosse Encephalitis virus, and West Nile virus (Wozniak et al. 2001, Dennett et al. 2007, Kilpatrick et al. 2007) and my study showed some species of mosquitoes feeding on WNv "super-spreader" bird species, such as American robin (*Turdus migratorius*) (Hamer et al. 2009). Eight mammal hosts from the current study are susceptible to WNv

(Brown bear, cow, goat, horse, human, lemur, opossum, raccoon) (Blitvich 2008). And *An. punctipennis* has been implicated as a vector of *Dirofilaria immitis* in Georgia, USA (Licitra et al. 2010). *Dirofilaria immitis* can infect large carnivores and has been implicated in the deaths of zoo animals (Adler et al. 2011). *Culex territans* is a vector of reptile and amphibian trypanosomes (Bartlett-Healy et al. 2009). Although they often do not receive much attention in the wild animal literature, many zoos house rare and exotic reptiles and amphibians that could be vulnerable to mosquito-borne pathogens.

Although Ejiri et al. (2011) found a significantly longer flight distance for gravid females than for full-fed, partial-fed, and half-gravid females, no differences were noted in flight distances among the 33 mosquitoes with known Sella stages in the present study. This difference could be due to the availability of oviposition sites in the two zoos.

Overall, mosquito behaviors conform to what has been previously recorded outside of zoos, but differ enough to merit further investigation. And, the study of mosquito blood feeding ecology in zoos will be of medical and veterinary benefit. My results demonstrate that by engaging zoos as experiments on mosquito behavior, further investigations will add to the growing literature on the developmental, environmental, and genetic aspects of host choice in mosquitoes.

CHAPTER FIVE

PYLORIC ARMATURE OF MOSQUITOES

Distinct armature composed of groups of lightly and heavily sclerotized, sometimes toothed, chitinous spines (also called spicules or microspines) are borne on the cuticular lining of the anterior hindgut, or pylorus (i.e., ileo-colon or pyloric ampulla), in mosquitoes and other insect taxa, including larvae of Simuliidae and Lepidoptera, and adults of Ephemeroptera, Diplopoda, and phlebotomine Psychodidae (Trembley 1951, Byers and Bond 1971, Christensen 1971, Elzinga 1998, Kim and Adler 2009). Adult mosquitoes have armature in three areas: the cibarium and pharynx of the foregut (McGreevy et al. 1978) and the pylorus of the hindgut. The pyloric armature of mosquitoes is a collection of chitinous spines lining the intima of the pylorus that project posteriorly, and are located just posterior to the pyloric valve. Less is known of it than the other mosquito armature.

The mosquito pyloric armature has been briefly mentioned (Eysell 1905, Thompson 1905, de Boissezon 1930, Richins 1938, Snodgrass 1959, Christophers 1960, Clements 1963), and although species differences have been noted (Trembley 1951, Vaughan et al. 1991), no quantitative analysis has been published. Eysell (1905) called the spines of the pyloric armature “chitin-nadeln” or chitin-needles and noted they “projected downward” and were deposited in a “regular” formation (possibly referring to them being in rows). Thompson (1905) described the “ileo-colon” as being a pumping apparatus “roughened

by bristle-like chitinous papillae which point caudad" and described the armature as a "hirsute belt". De Boissezon (1930) described spines in the pyloric armature as "poils chitineux hérissés" or bristly chitinous hairs. Richens (1938) described the pylorus as having "rough spines projecting caudad into the lumen." Snodgrass (1959) stated that "the inner wall of the pyloric funnel is armed in some species with numerous small spines directed posteriorly". Christophers (1960) said "the epithelium [of the pyloric ampulla] has a fine cuticular lining which carries backwardly projecting spinous processes". Additionally, Christophers noted that "the spines are not unlike those seen on the larval cuticle in some situations, namely a thorn-like base which is continued into from four to six fine spines projecting in a horizontal plane". Clements (1963) noted "numerous backward-pointing spines" on the inner surface of the pylorus of larvae (but only cites Trembley 1951 so this could be inaccurate) and adults.

The most comprehensive investigation to date, including the only published light microscope pictures of the armature, was by Trembley (1951). She found "pyloric spines" of 6 – 16 μm arranged in "irregular rows" that changed from "fine and comblike" to "heavier" in an anterior to posterior direction, in *Ae. aegypti*. She reported pyloric spines in both sexes of *Ae. aegypti*, *Ae. atropalpus*, *Ae. albopictus*, *Ae. triseriatus*, *An. quadrimaculatus*, *An. freeborni*, *An. albimanus*, *An. aztecus*, *Cx. pipiens, and Cx. quinquefasciatus*. She reported differences in spines among genera and species. Vaughan et al. (1991) also reported differences among different *Anopheles* species but did not present quantitative data. Two scanning electron micrographs of the pyloric spines of *Ae.*

aegypti were previously published as part of a larger study on the alimentary canal (Dapples and Lea 1974).

Because "spines" is the most commonly used term in the literature, is used in the two most comprehensive works to date (Trembley 1951, Vaughan et al. 1991), and is used by one well-established authority (Snodgrass), this is the term that will be used in the current work to describe the individual spiculate projections lining the cuticular intima of the mosquito pylorus.

The pyloric armature might aid in mechanical filtering and concentration of mosquito-host erythrocytes from serum and its structure might vary with size and shape of host erythrocytes (Vaughan et al. 1991, Lyimo and Ferguson 2009). Because of the peristaltic action of the pylorus, the armature might also aid in hemolyzing host blood cells (Vaughn et al. 1991), a known function of the cibarial armature (Coluzzi et al. 1982, Chadee et al. 1996). The foregut armature aid in shredding, and thus killing, filarial nematodes (e.g., *Wuchereria bancrofti*) ingested in mosquito bloodmeals (McGreevy et al. 1978), and the pyloric armature possibly aids in killing of *Dirofilaria* spp. L1 larvae. These larvae migrate into the Malpighian tubules through openings in the pyloric valve (Dr. John McCAll, UGA-Athens, personal communication 2011), specifically, where the Malpighian tubules open into the space between the midgut and ileo-colon valves that form the pyloric valve (Thompson 1905) – a strategy different from that of other filarioid

nematodes that migrate across the midgut into the hemocoel (Macdonald and Ramachandran 1965).

A quantitative and descriptive understanding of mosquito pyloric armature can potentially elucidate mechanisms behind mosquito vector competence and host choice and aid taxonomy. The objectives of this study were to document, describe, and compare the pyloric armature of mosquitoes. The hypothesis was that there would be significant differences in spine structure among species.

Materials and Methods

Mosquito Collections and Preparation. Mosquitoes were obtained from June to September 2009 with gravid and light traps at the Greenville (Greenville Co.) and Riverbanks (Richland Co.) zoos, and April to May 2011 at the Clemson University Cherry Farm Insectary, South Carolina. Zoo samples were stored in a -20C freezer prior to dissection while Cherry Farm Insectary samples were dissected fresh from the insect traps. If insects were previously frozen, they were placed in a 10% Alconox solution in a refrigerator for 1-3 days to rehydrate before dissection. Mosquitoes were sexed and identified to species beforehand.

Mosquito Dissections. Each individual was dissected in a small drop of Phosphate Buffered Saline on a microscope slide. The mosquito was oriented to a lateral view and, using the aid of a dissecting microscope, a pin was placed through the center of the

thorax. With the pin holding the mosquito in place, the eighth abdominal segment was gently pinched with a pair of fine-tipped forceps. The forceps were gently pulled away from the mosquito body, while the gut could be viewed exiting the body cavity. The gut would either be pulled out whole or break at the midgut and hindgut junction just anterior to the pyloric armature. If the fore- and midguts were also obtained, dissecting pins were used to separate the hindgut from the rest of the alimentary tract.

By means of a dissecting pin inserted into the still-attached eighth abdominal segment, the entire hindgut was dragged across the slide into a drop of 10% KOH. The gut was left to clear in this solution for 3-4 hours at room temperature, with periodic refreshments made to compensate for evaporation. After 3-4 hours, the gut was then dragged by the eighth abdominal segment into a drop of acetic acid on the slide. The terminalia were severed from the gut with a dissecting pin and removed from the slide. A coverslip was placed on top of the drop containing the gut.

Pylorus Images and Measurements. Pyloric armature was viewed and photographed at 50x, 125x, 250x, 500x, and 1250x (oil immersion) magnifications with a compound microscope (Olympus BH-2) with a camera (ProgRes Speed XT core 5, Jenoptik). Measurements were made on pictures of the armature in the ImageJ software program (U. S. National Institutes of Health) (Abramoff et al. 2004). Measurements were made of pylorus lengths, spine base width, spine stem width, spine length, tooth length, and number of teeth (Fig. 5.1). Spine measurements were taken for up to five spines in each

of the first (i.e., proximad) and second (i.e., middle) third of the pylorus. Additionally, as many distances between spine tips as possible were measured across the whole pylorus (Fig. 5.2). Anterior and middle spines were also scored for whether they had 1) a straight (teeth flush at the same point) or irregular (variation in tooth attachment line) base widths; 2) barbed (i.e., flared at the base like a spearhead) teeth; and 3) pointed and closed (i.e., proximal portion of spine coming to a complete tip), pointed and open (i.e., tip approaching a point but not complete) or truncate (i.e., no noticeable tip, rather proximad portion of spine similar in width to base) tips (Fig.5. 2).

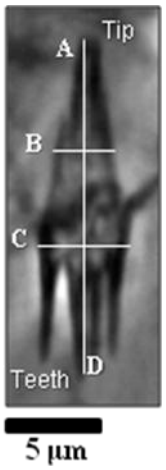


Figure 5.1. Up to four measurements were taken of each spine. A) Spine length: the length from the tip to line C; B) Stem width: the width of the spine at the midpoint of A; C) Base width: the width of the spine where the outside teeth meet the spine body; and D) Tooth length: the length of the tooth closest to the intersection of A and C. Spines were also scored for whether 1) the line where teeth bases met the spine body was straight or irregular; 2) the teeth were barbed (i.e., the tooth base or whole tooth were darkened with bases thicker than tips) or needlelike (i.e., no darkening and little to no difference in width along length

Respectively, the mean lengths and widths were compared for anterior and middle spines. In general, posterior spines were less elaborate than those in the proximad or middle portions, often having only 1 or 2 teeth or being toothless spicules. Therefore, the posterior spines were not compared among species. Male specimens were measured for two species, four *Ae. albopictus* and two *Ae. triseriatus*.

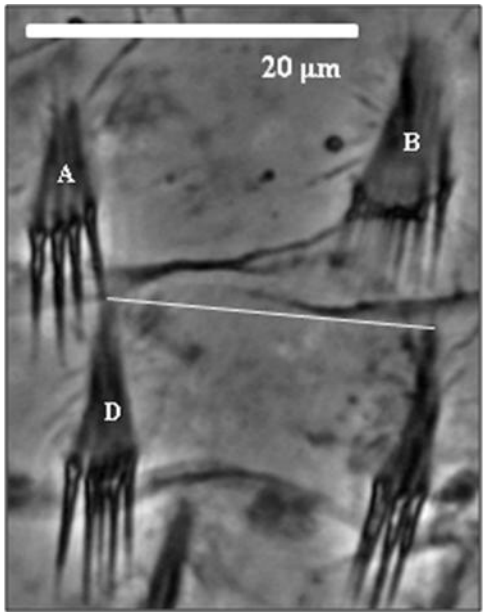


Figure 5.2. Tip to tip spine distance represented by white line. Spine "A" has a straight base width, barbed teeth, and a truncate tip; "B" has an irregular base width, needlelike teeth, and a pointed but open tip; and "C" has a straight base width, barbed teeth, and a closed tip.

The preparation method resulted in either one of two distinct slide mounts, one in which the pylorus popped open, rendering a top-down view of the pylorus interior, and another in which the pylorus laid flat on its side, rendering a top to bottom view of the pylorus exterior (Fig.5.3).

Pyloruses were scored for whether 1) spines in the pylorus were sparse (distance between spines > one spine width apart), regular (distances between spines ≤ one spine width apart, but not overlapping), or dense (overlapping spines); 2) spines were in rows; and 3) spines were untoothed or bifurcated (e.g., 0-2 teeth), toothed (e.g., ≥ 3 teeth), or progressing from posteriorly toothed to anteriorly untoothed. In some cases, spine

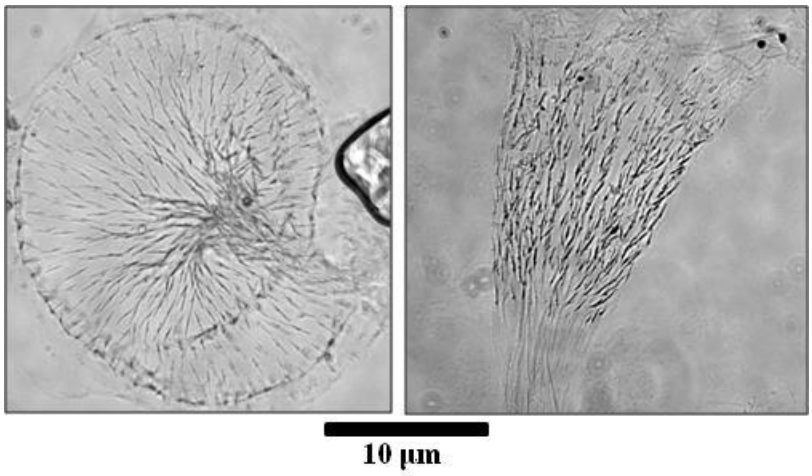


Figure 5.3. Two different results of slide-mounting. The left picture is a popped-open top down view of the pylorus interior, whereas the right picture is a top to bottom view of the pylorus exterior. Spines were measured in both types.

characters could not be measured because of preparation artifacts or quality – characters were only measured if they were clearly visible. All images taken are deposited on CD in the Clemson University Arthropod Collection with voucher specimens.

Over 600 male and female mosquitoes of eleven species were examined. Pictures and measurements were taken of four *Ae. albopictus* females and four males, five *Ae. j. japonicus* females, two *Ae. triseriatus* females and two males, three *An. punctipennis* females, four *Cx. pipiens* complex females, five *Cx. restuans* females, one *Or. signifera* female, and one *Tx. rutilus* female. No males of *Ae. j. japonicus* and *Or. signifera* were collected, and *Cx*. spp. males were collected but could not be identified to species. Pictures were also taken of outgroups consisting of two Ceratopogonidae females, three females and one male of Corethrellidae, one Psychodidae female, and one Mycetophilidae female.

Statistics. The means of spine lengths, widths, and tip to tip distances were compared among *Ae. albopictus*, *Ae. j. japonicus*, *Ae. triseriatus*, *Cx. pipiens* complex, and *Cx. restuans* without and with corrections for within species pseudoreplication (i.e., multiple spine measurements within each individual within each species) were compared using an Analysis of Variance (ANOVA). The model was a simple one-factor model with a term for species. Data were checked for conformation to the ANOVA assumptions of normality and homoskedasticity. If necessary, data were transformed prior to analysis. If transformation did not achieve normality and homoskedasticity, then a non-parametric Kruskal-Wallis one-way ANOVA was used. When the assumption of equality of variances was violated Welch's ANOVA was used. If the results of Kruskal Wallace test and/or the Welch's test did not differ from those of a traditional ANOVA, the results of the ANOVA were reported because of ease of interpretation and means comparison tests.

Results

Armature Structure within Species (Fig.5. 4)

Ae. albopictus females

Pylorus. One of four was scored as regular, while three of four were dense. Two were in obvious rows, and two were not. Three were visibly more elaborate anteriorly as opposed to posteriorly.

Proximad spines. Four of twenty spines had straight tooth bases, and sixteen were irregular. All teeth were barbed. Seven had a closed tip, seven had an open but pointed tip, and six had a truncated tip.

Middle spines. Two of twenty middle spines had straight tooth bases, and eight were irregular. All teeth were barbed. Six had a pointed, closed tip, and four had pointed, open tips.

Ae. albopictus males (not included in Fig.5.4)

Pylorus. Two of four were sparse, and the other two dense. Three were not in obvious rows, while one was. Two were uniformly simple, one was uniformly elaborate, and two were anteriorly elaborate grading to posteriorly simple.

Proximad spines. Six of twenty had straight tooth bases, four had irregular. Ten were barbed, and five were needlelike.

Middle spines. Six of twenty spines had straight bases, three had irregular. Eight had barbed teeth, five had needlelike. Eighteen had closed tip, one had truncate.

Ae. j. japonicus

Pylorus. All five were dense. All five were in obvious rows and uniformly elaborate anterior to posterior.

Proximad spines. Nineteen of twenty-three spines had regular tooth bases, four were irregular. One tooth was scored as barbed, twenty-one scored as needlelike. Fifteen had a closed tip, three had an open but pointed tip, and five had a truncated tip.

Middle spines. Fifteen of twenty-four middle spines had straight tooth bases, four had irregular. Nineteen were barbed. Seventeen had pointed, closed tip, one had pointed, open tip, one had truncate.

Ae. triseriatus females

Pylorus. One was regular, and one dense. Both were in obvious rows. One was anteriorly elaborate grading to posteriorly simple.

Proximad spines. Five of ten spines had straight tooth bases, five were irregular. All ten teeth were barbed. Three had a closed tip, one had an open but pointed tip, and six had a truncated tip.

Middle spines. Nine of ten spines had straight tooth bases, and one had irregular. All ten were barbed. Seven tips were pointed and closed, while three were truncate.

Ae. triseriatus males (not included in Fig.5.4)

Pylorus. One of two was regular, and the other one dense. One was not in obvious rows, while one was. Both were uniformly elaborate.

Proximad spines. Eight of ten had straight tooth bases, two had irregular. Nine of ten were barbed, and one was needlelike.

Middle spines. Four of ten had straight tooth bases. Three had barbed teeth. Four had pointed tips.

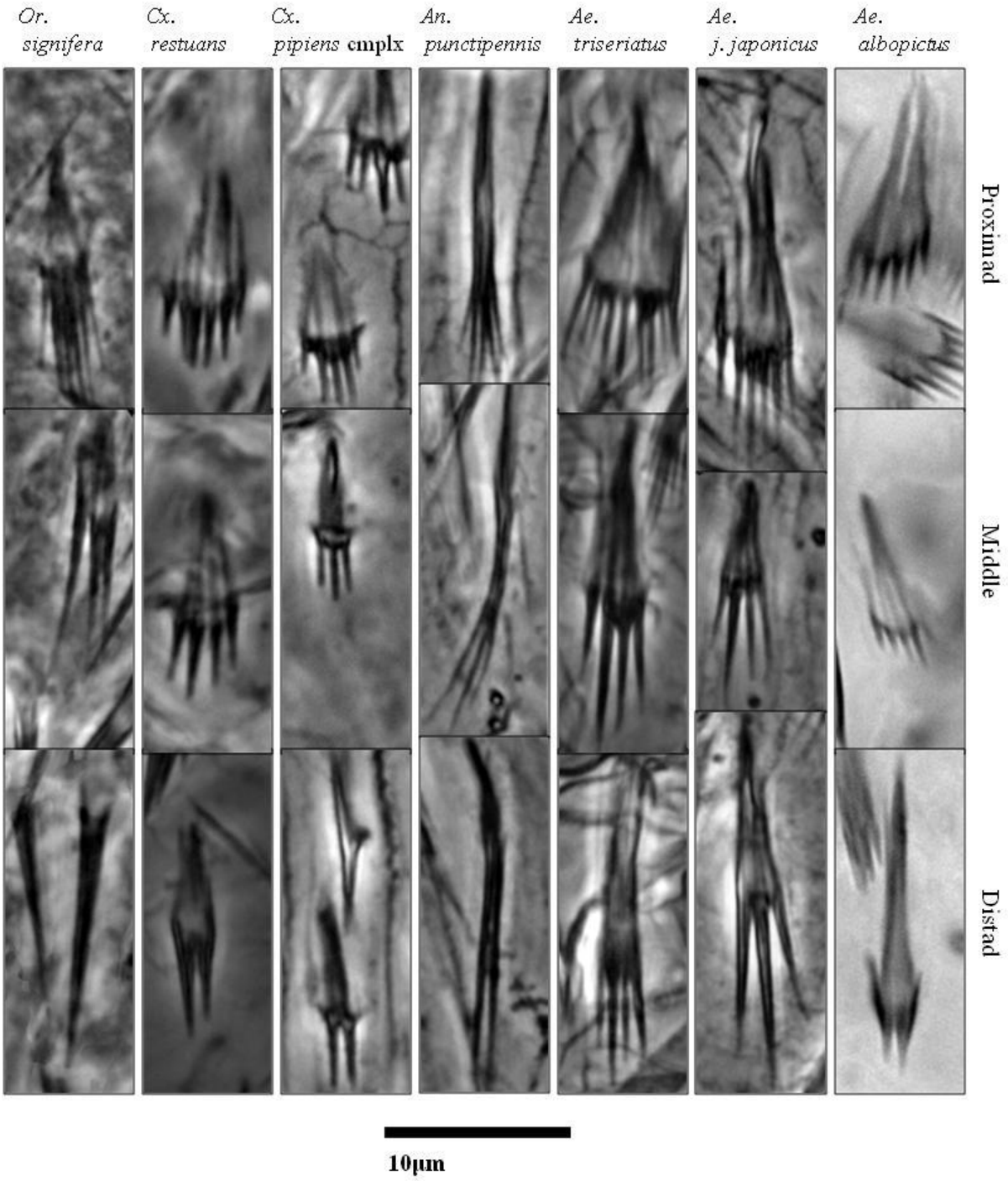


Figure 5.4. Spines of the pyloric armature in seven species of mosquitoes. Pictures are representative of the hundreds of spines found in an individual pylorus. Topographic distinctions generally exist in spine size and shape between anterior third (i.e., proximad), middle third (i.e., middle) and posterior third (i.e., distad) portions of pylorus. Spines are oriented as they would be in the mosquito pylorus with a single tip anterior and, generally, several teeth posterior. When observed in the sagittal plane, posterior portions of spines project into the pyloric ampulla while anterior portions are flush with the intima. Two teeth on *Ae. albopictus* distad spine might be artifact of preparation and represent a split spine end.

An. punctipennis This species was difficult to measure (e.g., teeth often hard to distinguish) because teeth were long and wispy, and seemed much more hair like than spiculate, compared to the other species, giving the impression of paintbrushes. The measurements presented here document variation but are not considered representative of the species and likely greatly underestimate the extent of variation. The spines that were measured happened to be pressed out so that individual teeth could be distinguished.

Pylorus. One of three was sparse, and the other two regular. None were in obvious rows. All three were uniformly simple.

Proximad spines. Eight of ten had straight tooth bases, two had irregular. Nine of ten were barbed, and one was needlelike. Measured spine width ranged from 1.60 - 3.38 μm, stem width ranged from 0.56 - 2.30 μm, spine length ranged from 5.22 - 16.54 μm, and tooth length ranged from 3.82 - 8.20 μm. The number of proximad teeth was 0 to 5.

Middle spines. Four of fifteen had closed tips, one had open but pointed tip. The middle spine width ranged from 2.41 - 2.89 μm, stem width ranged from 1.18 - 1.86 μm, spine length ranged from 3.18 - 5.34 μm, and tooth length ranged from 3.33 - 3.34 μm. The number of middle teeth ranged from 0 to 5.

Cx. pipiens complex

Pylorus. All four were dense, in obvious rows, and anteriorly elaborate grading to posteriorly simple.

Proximad spines. Nineteen of twenty spines had a straight tooth bases, one was irregular. All twenty teeth were barbed. Eleven had a closed tip, eight had an open but pointed tip, and one had a truncated tip.

Middle spines. Thirteen of twenty middle spines had straight tooth bases, one had irregular. Fourteen of twenty were barbed. Thirteen of twenty tips were pointed and closed, while two were pointed and open.

Cx. restuans

Pylorus. One was sparse, other four were dense. All five were in obvious rows. Four were anteriorly elaborate grading to posteriorly simple, one was unscored.

Proximad spines. Eight of twenty-five spines had straight tooth bases, sixteen were irregular. Twenty of twenty-five teeth were barbed, four were not. Ten had a closed tip, two had an open but pointed tip, and twelve had a truncated tip.

Middle spines. Seven of twenty-two middle spines had straight tooth shoulder, while six had irregular. Thirteen of twenty-five were barbed. Twelve tips closed and pointed, one open and pointed, two truncate

Or. signifera

In terms of quantitative analysis this species was similar to *An. punctipennis*. The one pylorus measured was dense, not in obvious rows, anteriorly elaborate grading to uniformly simple, and four of five measured spines had straight tooth shoulders, while one was irregular. All visible teeth were needlelike. The proximad spine width ranged

from 3.32 - 6.34 μm, stem width ranged from 2.33 - 4.11 μm, spine length ranged from 7.64 - 11.59 μm, and tooth length ranged from 5.26 - 10.15 μm. The number of proximad teeth ranged from 6 to 10. No measurements were taken for middle spines.

Tx. rutilus (not included in Fig.5.4)

Pylorus. Dense, not in obvious rows, with elaborate barbed spines throughout (Fig. 5.5). No measurements were made of teeth because of poor specimen quality.

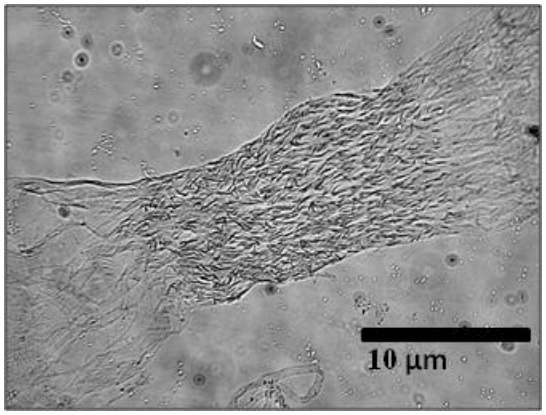


Figure 5.5. Female *Tx. rutilus* pylorus. Anterior section on right, posterior on left.

Outgroups Two ceratopogonid females were examined. One in the blood feeding *Piliferous* sp. group did not have visible pyloric armature, but the other, a predaceous *Atrichopogon* sp. did have spinous spicules in the pylorus. Three corethrellid females had armature with anteriorly elaborate and posteriorly simple spines, but the three males did not have visible spines. Two non-blood feeding psychodids and one mycetophilid examined did not have visible spines. None of the spines examined were as elaborate as those in the Culicidae.

Spine Comparisons (Tables 5.1 & 5.2)

Proximad spines. Among species, mean base widths (F=1.4473, df=4, p=0.2683) or stem width (F=1.2029, df=4, p=0.3512) were not significantly different. Mean spine lengths were significantly different, with *Ae. j. japonicus* having a significantly longer mean spine length than *Ae. albopictus*, *Cx. restuans*, and *Cx. pipiens* complex (F=4.5729, df=4, p≤0.0132). Also, *Ae. j. japonicus* and *Ae. triseriatus* had significantly longer mean tooth lengths than *Ae. albopictus*, *Cx. restuans*, and *Cx. pipiens* complex (F=7.2278, df=4, p≤0.0019). Additionally, *Ae. albopictus* had significantly more teeth than *Ae. j. japonicus*, *Cx. restuans*, and *Cx. pipiens* complex, *Ae. triseriatus* had significantly more than *Cx. restuans* and *Cx. pipiens* complex, and *Ae. j. japonicus* had significantly more than *Cx. pipiens* complex (F=5.9826, df=4, p≤0.0047) (on data transformed to meet normality assumptions by raising to the power of ½).

Middle spines. Among species, the mean base widths (F=2.6447, df=4, p=0.0960) and stem widths (F=1.5025, df=4, p=0.2740) were not significantly different. Spine length was significantly different, with *Ae. j. japonicus* and *Ae. triseriatus* having longer mean spine lengths than *Cx. restuans* and *Cx. pipiens* complex (F=4.2834, df=4, p≤0.0281) (*Ae. albopictus* did not differ from any species). Also, *Ae. j. japonicus* and *Ae. triseriatus* had significantly longer mean tooth lengths than *Cx. pipiens* complex and *Cx. restuans*, and all four were significantly longer than *Ae. albopictus* (F=15.2117, df=4, p≤0.0004). Additionally, the mean number of teeth was significantly different, with *Ae. albopictus*

having more than all other species except *Ae. j. japonicus*, and *Ae. j. japonicus* having more than both *Cx.* spp. ($F=6.6045$, $df=4$, $p \leq 0.0069$).

Spine tips. The mean distances between spines were not significantly different ($F=1.2079$, $df=4$, $p=0.3554$).

Quantitative Differences between Females and Males

Ae. albopictus Four males and four females were compared. The mean width of proximad spine bases and stems, and mean tooth lengths were not significantly different between males and females. Females did have significantly more teeth than males ($F=6.6841$, $df=1$, $p \leq 0.0415$). The mean width of middle spine bases and stems, mean tooth lengths, and mean number of teeth were not significantly different between males and females.

Ae. triseriatus Two males and two females were compared. The mean width of proximad and middle spine bases and stems, mean tooth lengths, and mean number of teeth were not significantly different between males and females.

Table 5.1. Means ± SE (μm) and ranges (in parentheses) of female mosquito spines comprising the pyloric armature. Proximad spines found in ca. the anterior third of pylorus, middle spines located in ca. the middle third. Spines in the posterior third not measured. Base width is where base of the spine teeth meet the body of the spine, stem width is the width of the spine at the middle of the body length, length is the tip of the spine to the base of the teeth. Different letters in superscript indicate significant differences between means at the 0.05 level of significance.

Species (no. spines measured)*	Base Width	Stem Width	Length	Length: Base Width ratio†	Base Width: Stem Width ratio†	Length of teeth	No. teeth	No. teeth (median)	Pylorus length (no. pyloruses measured)	Distance between spine tips (no. spines measured)
				Anterior					**Both Sections**	
Aedes albopictus (20)	4.94 ± 0.48 (1.34-9.90)	3.01 ± 0.41 (0.92-7.13)	7.97 ± 0.41^{B} (5.87-12.13)	1.96 ± 0.28 (0.80-6.55)	1.75 ± 0.10 (1.21-2.71)	3.84 ± 0.20^{B} (2.10-5.41)	7.75 ± 0.46^{A} (4-11)	8	213.78-244.90 (2)	18.24 ± 4.57 (13)
Aedes j. japonicus (23)	4.59 ± 0.27 (2.62-7.23)	2.46 ± 0.20 (1.34-4.66)	10.63 ± 0.65^{A} (5.22-16.24)	2.41 ± 0.15 (1.08-4.09)	1.98 ± 0.11 (1.29-3.07)	6.37 ± 0.33^{A} (3.56-9.26)	5.78 ± 0.37^{B,C} (3-9)	6	392.37-462.18 (2)	19.60 ± 7.19 (124)
Aedes triseriatus (10)	5.57 ± 0.42 (3.79-8.57)	3.56 ± 0.47 (1.37-6.10)	9.04 ± 0.71$^{A, B}$ (6.23-13.25)	1.71 ± 0.18 (0.92-2.55)	1.77 ± 0.24 (1.11-3.64)	6.20 ± 0.52^{A} (4.12-9.63)	6.90 ± 0.59^{A,B} (5-11)	6.5	308.24 (1)	19.40 ± 1.09 (63)
Culex pipiens complex (20)	3.57 ± 0.16 (2.38-5.33)	2.11 ± 0.12 (0.95-3.12)	6.70 ± 0.25^{B} (5.13-8.75)	1.96 ± 0.12 (0.96-2.93)	1.76 ± 0.09 (1.18-2.88)	4.58 ± 0.34^{B} (2.77-10.07)	4.10 ± 0.29^{D} (3-8)	4	195.45-240.40 (2)	18.15 ± 4.75 (109)
Culex restuans (24)	4.33 ± 0.35 (2.01-9.01)	3.15 ± 0.36 (1.00-8.36)	6.78 ± 0.24^{B} (4.65-9.58)	1.80 ± 0.14 (0.52-3.10)	1.52 ± 0.07 (1.06-2.47)	4.53 ± 0.26^{B} (1.84-7.16)	4.63 ± 0.27^{C,D} (3-8)	4.5	191.89 (1)	13.65 ± 4.25 (82)
				Middle						
Aedes albopictus (10)	3.91 ± 0.30 (2.39-5.45)	2.13 ± 0.14 (1.45-2.68)	9.53 ± 0.92^{A,B} (5.76-14.18)	2.52 ± 0.25 (1.48-3.66)	1.84 ± 0.09 (1.44-2.34)	3.23 ± 0.27^{C} (1.88-4.88)	5.60 ± 0.45^{A} (4-8)	5.5	na	na
Aedes j. japonicus (19)	4.22 ± 0.29 (2.60-7.42)	2.44 ± 0.22 (1.53-5.62)	12.24 ± 0.59^{A} (8.26-16.30)	3.15 ±0.25 (1.30-4.85)	1.79 ± 0.09 (1.14-2.63)	7.10 ± 0.29^{A} (4.90-9.39)	4.42 ± 0.30^{A,B} (3-7)	4	na	na
Aedes triseriatus (10)	3.48 ± 0.24 (2.06-4.50)	1.74 ± 0.18 (1.06-2.87)	1.23 ± 0.65^{A} (9.48-15.15)	3.65 ± 0.28 (2.23-4.72)	2.14 ± 0.24 (1.13-3.85)	7.73 ± 0.54^{A} (5.78-10.17)	3.60 ± 0.31^{B,C} (2-5)	3.5	na	na
Culex pipiens (14-15)	2.69 ± 0.19 (1.76-3.76)	1.48 ± 0.10 (0.89-2.34)	7.92 ± 0.47^{B} (5.37-11.15)	3.09 ± 0.26 (2.10-6.05)	1.87 ± 0.17 (1.18-3.28)	4.99 ± 0.29^{B} (3.64-7.60)	2.53 ± 0.26^{C} (0-4)	3	na	na
Culex restuans (13-15)	2.85 ± 0.25 (1.54-4.84)	1.81 ± 0.21 (0.95-3.54)	7.79 ± 0.26^{B} (6.68-9.77)	2.80 ± 0.26 (1.48-4.36)	1.65 ± 0.09 (1.21-2.38)	5.10 ± 0.28^{B} (3.32-6.61)	2.80 ± 0.36^{C} (0-5)	3	na	na

*Top row are mean ± SE (μm), bottom (in parentheses) are ranges

†Means not compared

Table 5.2. Means ± SE (μm) and ranges (in parentheses) of male mosquito spines comprising the pyloric armature. Proximad spines found in ca. the anterior third of pylorus, middle spines located in ca. the middle third. Spines in the posterior third not measured. Base width is where base of the spine teeth meet the body of the spine, stem width is the width of the spine at the middle of the body length, length is the tip of the spine to the base of the teeth.

Species (no. spines measured)*	Base Width	Stem Width	Length	Length: Base Width ratio†	Base Width: Stem Width ratio†	Length of teeth	No. teeth	No. teeth (median)	Pylorus length (no. pyloruses measured)
				Anterior †					**Both Sections**
Aedes albopictus (5-20	4.74 ± 1.48 (2.97-6.97)	1.48 ± 0.66 (0.87-3.56)	7.99 ± 1.22 (6.44-9.24)	2.26 ± 0.46 (1.76-2.91)	2.06 ± 0.67 (1.22-3.55)	2.40 ± 0.33 (2.10-2.87)	2.90 ± 3.13 (0-8)	2	273.38 ± 190.82 (4) (126.80-554.02)
Aedes triseriatus (10)	4.46 ± 0.71 (2.85-5.43)	2.35 ± 0.41 (1.80-2.96)	11.38 ± 2.06 (7.07-14.08)	2.57 ± 0.35 (1.76-2.96)	1.92 ± 0.32 (1.38-2.37)	4.55 ± 1.00 (3.09-5.92)	7.00 ± 1.25 (4-9)	7	152.88 (1)
				Middle†					
Aedes albopictus (9-19)	3.61 ± 1.01 (1.80-5.16)	1.48 ± 0.52 (0.84-2.91)	10.01 ± 2.14 (7.26-13.56)	3.07 ± 1.42 (1.82-5.67)	2.15 ± 0.68 (1.54-3.47)	3.55 ± 1.11 (2.50-5.94)	2.16 ± 2.69 (0-8)	0	na
Aedes triseriatus (4)	3.74 ± 0.84 (2.61-4.53)	1.84 ± 0.41 (1.50-2.43)	11.68 ± 2.36 (8.88-14.63)	3.19 ± 0.68 (2.51-4.04)	2.07 ± 0.53 (1.54-2.79)	5.14 ± 0.83 (4.40-6.22)	5.75 ± 1.26 (4-7)	6	na

*Top row are mean ± SE (μm), bottom (in parentheses) are ranges

†Means not compared

Discussion

Significant differences exist among species in quantitative measurements of spines. These differences roughly follow phylogenetic relationships, with the two *Cx.* spp. being most similar to each other, *Ae. j. japonicus* and *Ae. triseriatus* being more similar to each other than to *Ae. albopictus*, and *An. punctipennis* and *Or. signifera* being most similar to each

other. However, although *Tx. rutilus* is more closely related to the *Anopheles* and *Orthopodomyia* genera, its spine structure is more similar to the *Aedes* and *Culex* genera (Harbach 2007). But, only one rehydrated *Tx. rutilus* specimen was observed. Future studies could optimize spine characters onto phylogenies to determine if they have utility in phylogenetic studies.

The mosquitoes with different morphologies also generally display different host affinities. The two *Cx.* spp. are ornithophagic (i.e., bird feeding) and the *Ae.* spp. are mammalophagic (i.e., mammal feeding). *Anopheles punctipennis* feeds on birds and mammals, and *Or. signifera* on amphibians, birds, and mammals (refs for all). The differences in structure might relate to differences in host erythrocyte structure. Average erythrocyte cell size for mammals is 62.1 ± 22.2 μm^3, for birds 168.9 ± 28.5 μm^3, and for reptiles 398.2 ± 121.4 μm^3 (Hawkey et al. 1991), with considerable variation within classes (Wintrobe 1933). It might benefit mosquitoes to concentrate erythrocytes in species with lower densities of red blood cells, an aspect that changes by an order of magnitude between mammals ($7.77 \pm 2.86 \times 10^{12}$/l), birds ($2.79 \pm 0.53 \times 10^{12}$/l), and reptiles ($0.75 \pm 0.32 \times 10^{12}$/l).

Mosquito physiological reactions to bloodmeals could alter properties of the peritrophic matrix (Romoser et al. 1975, Berner et al. 1983); if it fluctuates with predominant host types in different mosquito species, then some might have more robust armature to deal with consequences to the matrix (e.g., thicker matrix). Finally, variation in spine shape,

spine density, or tooth number might be greater in mosquito species that switch between avians, humans, and mammals as opposed to specializing on one host, as suggested by Lyimo (2010). Further investigation with more mosquito species could reveal significant associations between erythrocyte structure (e.g., size, shape) and mosquito pyloric spine structure.

Parasites in bloodmeals could also alter properties of the peritrophic matrix, or parasites might exert a direct selection pressure on the female pyloric armature. Of the seven species in this study, all but *Or. signifera* are vectors of *D. immitis*. However, they vary in vector efficiency. These differences in efficiency might be related to armature differences. The armature might be lethal to all filarial parasites; for example, if parasites are displaced to the posterior portion of the pylorus during blood feeding, then the backwards projecting spines might disrupt subsequent parasite migration to the mosquito Malpighian tubules or flight muscles. If this is the case, pyloric spines would be expected in all mosquito species exposed to filarial parasites and their morphological differences might be correlated with differences in parasite structure (e.g., width, cuticle strength).

The presence of elaborate spines in male mosquitoes and a *Tx. rutilus* female, and lack of significant differences between males and females could be due to several factors. As suggested for sand flies, this could be a relic of a time when both males and females were putative blood feeders, a suggestion never definitively demonstrated (Christensen et al. 1971). Christophers (1960) mentions observing male *Ae. aegypti* feeding on diuretic fluid

of females, and elaborated pyloric spines could benefit males engaging in this behavior (although the behavior could be purely opportunistic and an artifact of the laboratory setting). But neither of these hypotheses would account for the presence of a robust armature in *Tx. rutilus*. If the pyloric armature aids in backward passage and disintegration of the peritrophic matrix (Wigglesworth 1950), males and non-blood feeding species might benefit as well as blood-feeding females, but an explanation is still needed for the variation among species. The armature also would be present in both sexes and non-blood feeders if it aided in the backward passage of the meconial peritrophic matrix, which has variable presence in different mosquito species (Romoser et al. 2000).

If the evolved function of the pyloric armature was to aid in backward passage and disintegration of the meconial peritrophic matrix, the armature might have been exapted by female mosquitoes to aid in bloodmeal processing or concentration, or to protect against parasites. Pharyngeal armature occurs in both male and female mosquitoes but has been implicated in damage to ingested microfilariae (McGreevy et al. 1978). If the pyloric armature does cause damage to microfilariae, more variation should exist in males than in females, which was not apparent in this study.

Trembley (1951) reported "groups of five to eight" spines in *Ae. aegypti* and Vaughan et al. (1991) reported "diamond-shaped spicules arranged in rosettes" in three *Anopheles* spp. , but neither of these patterns were seen in the current specimens. However, Vaughan et al. (1991) noted one species with spines arranged in "rows" (albeit an *Anopheles* sp.),

as was noted in several species in this study (no anophelines). Some of the posterior spines in a few species were untoothed and could be interpreted as "diamond-shaped". Although Vaughan et al. (1991) were not clear what they meant by spine size, their report of spine sizes of 3-7 μm, 3-9 μm, and 14-18 μm are similar to spine body lengths and total spine lengths (i.e., body length + tooth length). In some cases, the spines looked similar to oral armature (Buse and Kuhlow 1979, Somboon et al. 2009), and in particular *Ae. triseriatus* spines looked similar to the comb scales of immature *Ae*. spp. Females and males had both pointed and truncate spine tips; if these differences are due to tip breakage over time, they could lead to a method for age-grading female and male mosquitoes. An SEM study could verify whether the truncate shape of some spines was legitimate or due to breakage.

CHAPTER SIX

CONCLUSION

Mosquitoes display a diverse array of breeding and host-seeking behaviors. Depending on the species, oviposition can occur in natural or artificial containers, which are entire habitats or microhabitats associated with larger areas. Interactions between aquatic larval habitat and surrounding terrestrial ecology might influence mosquito population distribution and more study is needed in this area (Vanwambeke et al. 2007, Yee and Yee 2007). Even though an environment might contain optimal larval habitats, larval density could be low if the terrestrial environment is not advantageous to the adult female (Yee and Yee 2007). 'Bad mother' decisions made by herbivorous insects in which the adult female optimizes her longevity by placing larvae in suboptimal habitats close to the adult food source might also apply to mosquitoes (Mighthew 2001, Reiskind and Wilson 2004).

Host-seeking behavior and oviposition can occur contemporaneously during crepuscular hours (Reddy 2007). More adult mosquitoes occur in the vicinity of aquatic breeding grounds and dispersal of mosquitoes is influenced by both proximity to breeding grounds and hosts (Le Menach et al. 2005). And local mosquito abundance should increase with increasing larval habitat availability (Shaman et al. 2002, Reiskind and Wilson 2004). Studies of larval ecology are becoming more common as larval population dynamics are increasingly considered necessary to understanding fluctuations in, and distributions of,

adult populations (Gimnig et al. 2001). An optimal environment would provide adults access to sugar and blood meals, mates, and oviposition and larval development sites.

An advantage of studying mosquitoes in zoos is that mosquito larval development sites can be evaluated in relation to hosts. For instance, different mosquito species metabolize bloodmeal components with different efficiencies and fates, depending on female size (related to nutritional quality of larval habitat), bloodmeal size (related to time spent at host), and host species (Hurd et al. 1995); therefore, some mosquito species (e.g., strong competitors as larvae, catholic in adult feeding habits) could be at a distinct advantage or disadvantage in the zoo environment. Different mosquito species can have behavioral, physical, and physiological adaptations for different hosts and thus be expected to exhibit host preferences of varying specificities. For example, differential human erythrocyte concentration correlating with host usage has been shown in some Anopheline species (Vaughan et al. 1991). Zoos are an excellent environment in which to use strong hypothesis testing to elucidate the host and oviposition adaptations and preferences of different mosquito species. Results of such testing can provide us with the power to predict mosquito species distributions and host usage patterns in non-zoo environments.

We could also learn more about host avoidance in mosquitoes by analyzing the zoo species they do not feed on; for instance, might be there is a preferential avoidance of species with low blood levels of isoleucine, an amino acid essential to oogeneesis (Hurd et al. 1995). Adult female mosquitoes do not always display “gonotrophic concordance”

(i.e., one egg batch per bloodmeal), are known to take multiple bloodmeals during one gonotrophic cycle if hosts are readily available, and are more likely to take multiple bloodmeals if larvae developed in low-quality habitats (Hurd et al. 1995). A dilution effect on pathogen transmission might occur in zoos if mosquitoes feed on more species of hosts than they would outside of zoos. Alternatively, there could be an amplification of pathogen transmission if preferred or vulnerable hosts are present, or infected or sick hosts are confined and concentrated (e.g., such as in hospitals).

Collaboration between entomologists and zoo personnel can be beneficial to captive and wild animals, and the human zoo workers and attendees. The original study suggesting the importance of mosquitoes as vectors of pathogens in zoos was precipitated by an ongoing problem at the Baltimore Zoo, Maryland, USA, with avian malaria in the outdoor penguin exhibit (Beier and Trpis 1981a). A host of studies conducted since then on the inevitable problem of avian malaria when penguins are housed outdoors (penguins lack coevolved defenses against both mosquito vectors and *Plasmodium* spp.) generated a large body of knowledge on disease pathology and potential vectors before wild endangered Galapagos penguins were found to have avian malaria for the first time in 2009 (Levin et al. 2009). Potentially, many other animals of conservation concern currently housed in zoos could benefit by this type of epidemiological investigation. Zoos regularly trade animals for breeding and other purposes, and through these routes a local mosquito could acquire a foreign pathogen. For instance, animals are regularly quarantined in hospitals after transfer, and mosquitoes might have access to animals in

hospitals because mosquito exclusion is not a routine part of quarantine. Studying the relationships between mosquitoes, hosts, and pathogens in zoos can provide wildlife researchers and ecologists with early warning systems (e.g., identification of naïve and vulnerable hosts) for the management of mosquito-borne diseases in an era of global climate change (Reiter 2008), and provide medical entomologists and epidemiologists with information on mosquito-vector potential, pathogen plasticity, and host-learned defenses.

Additionally, communication between entomologists and zoo workers can provide insight into both disciplines. For instance, after presenting my research to the Zoological Association of America in November 2010 I learned that some zookeepers have noticed Capuchin monkeys rubbing green onion juice on their fur when bothered by mosquitoes, and others think animals might lie on warm mulch to keep biting flies away. These two anecdotes hint at intriguing possibilities for studying natural mosquito-avoidance behavior in zoo animals. And, as a direct result of my work, the South Carolina Department of Health and Environmental Control began running mosquito traps in the Riverbanks Zoo as part of its annual statewide WNv monitoring project.

I placed resting boxes in areas of high mosquito biting activity in the zoos, as reported by keepers. Oddly, mosquitoes were rarely found in the resting boxes possibly because there were much better sites nearby. The Riverbanks Zoo has a large water catchment basin underthe zoo that is connected by numerous sewer pipes. Drains in the floors of exhibits

lead to this basin. I have seen mosquitoes flying out of drains and I think they may be traveling between exhibits through the sewer pipes and also resting in them. Keepers report high mosquito biting in areas associated with the drains and it would be worthwhile to place rubber mats over the drains when not in active use to see if mosquito biting activity is reduced, and also place eclosion traps over the drains to capture any mosquitoes leaving them.

When the new Red-necked wallaby exhibit was installed at the Riverbanks Zoo, I was able to consult with them on potential mosquito breeding habitats in the exhibit. There were changes enacted in some of the problem areas I noted, during the course of my research, in my annual reports to the zoos. For example, a pump house station located in the wall of the bear exhibit of the Riverbanks Zoo (and directly across from an outdoor aviary) was an area where multiple species of bloodfed mosquitoes were found resting during my research and previous work (Nelder 2007). After the zoo repainted the walls, cleaned the floor of debris, moved objects located close to the walls (e.g., buckets) and (probably most importantly) put a door on the room, mosquito resting activity dropped to zero. Additionally, gutters at both zoos were either removed or are now being regularly inspected and cleaned. And, finally, areas where mosquitoes could rest after biting (for instance, shaded areas with creeping groundcover and ivy-covered walls) were stripped of vegetation and shade, and repainted. I also checked some indoor areas that might be trouble areas but could be overlooked, for instance bromeliads, bamboo stumps, other standing water, and ground vegetation inside the bird exhibits of the indoor aviary at the

Riverbanks Zoo, but never found mosquitoes in unusual places. The keepers at the Greenville Zoo did report to me that they've seen mosquito larvae in elephant footprints in dried mud. There are likely some novel mosquito breeding habitats in zoos that haven't been recorded before (e.g., *Ae. albopictus* in elephant footprints) but a lack of access to some animal habitats prevented me from investigating them.

Before robust inferential hypothesis-based works can be undertaken in zoos, a descriptive basis must be laid down. The next step is to more rigorously test hypotheses related to mosquito foraging in relation to oviposition sites and host locations in zoos and dilution/amplification hypotheses regarding pathogens. For instance, could we use zoos or mini-zoos as diluters in areas of high human pathogen transmission? Or will zoos serve as the focus of epizootics? We can address these and many other questions about mosquito biology by conducting experiments in the unique milieu of zoological parks. The zoo-as-experiment scenario is advantageous, as zoo habitats are replicated worldwide (e.g,. "Africa" exhibit, "rainforest" exhibit), many aspects of habitat design and input are controlled through necessity of operation (e.g., type of plants in an exhibit), long-term health records are kept on animals (including serobanking), and animals are under routine surveillance by zookeepers. Additionally, my work and the work of others (Beier and Trpis 1981b, Derraik 2004, Huijben et al. 2003) has shown that mosquito dynamics within zoos are similar enough to use as proxies for environments outside of zoos.

Some examples of work that can be done in the unique experimental milieu of zoos are as follows

Within-species host preference in mosquitoes. We can use DNA profiling to identify individual animals in exhibits from mosquito bloodmeals. Through this we can ask questions such as "Do mosquitoes preferentially feed on the young of this species" and "Does mosquito host preference change through time" Or, if an exhibit has problems with bird blood parasites we can ask questions such as "Do mosquitoes prefer or avoid parasitized hosts" or "Are some hosts at higher risk for parasitism because of mosquito within-host preferences" because we will know the identity of each parasitized host. We can also sex the host from which the bloodmeal was obtained.

Mosquito-host coevolution. We can determine if adventive species of mosquitoes (e.g., *Ae. j. japonicus*) are preferentially feeding in themed areas (e.g., "Asia Exhibit") with animals representative of their geographic origins. The opportunity also exists to study mosquito sugar-feeding behavior in zoos to determine if mosquitoes preferentially use native or exotic (e.g., found as part of the landscapting in animal habitats) plants.

Mosquito microhabitats and dispersal. Because hosts and oviposition sites can be known in great detail in zoos, this knowledge would facilitate an in-depth investigation of mosquito dispersal between feeding and oviposition sites and the controversial issue of possible home range memory in mosquitoes (Service 1997).

Zoo effects on mosquito populations. If zoos provide mosquitoes with abundant generational requirements (e.g., blood and sugar hosts, mates, oviposition and resting sites), gene flow out of the zoo might be inhibited, facilitating selection for zoo-associated traits.

More than 600 million people visits zoos each year, equivalent to 10% of the global human population (Adler et al. 2011), and zoo animals are particularly susceptible to parasites and their pathogens (Nelder 2007, Adler et al. 2011). Additionally, the licensing body for United States zoos, the American Zoological Association, does not have any specific requirements regarding mosquito control. However, a 2004 US congressional report on the National Zoo in Washington, DC, mandated that the zoo governance create and fill a pesticide program management position (Adler et al. 2011). Given that zoos are areas where animals, humans, and pathogens commingle without regulatory oversight, we cannot deny that the ecology of mosquitoes in zoos should necessarily be studied.

My research demonstrates that mosquito behaviors in zoos do not differ so much from non-zoo environments that zoos cannot be used as experimental environments; however, they differ enough to merit further investigation. Additionally, my work has demonstrated that a holistic investigation of mosquito oviposition and blood-feeding behavior, vector status, and anatomy can be undertaken in zoos.

Public Summary

Mosquitoes in zoos represent a potential public health threat and biting nuisance. They can transmit pathogens causing disease in zoo animals, and possibly, zoo visitors and workers. However, it is essential that potential threats be investigated before far-reaching conclusions lead to expensive control measures. From 2008 to 2011 I investigated the significance of mosquitoes at the Greenville and Riverbanks zoos in South Carolina.

I studied habitats where mosquito larvae are found, analyzed mosquito bloodmeals to determine hosts, and tested mosquitoes for agents of dog heartworm. Additionally, I investigated aspects of mosquito anatomy that are possibly related to blood feeding and mosquito resistance to pathogens. I discovered that mosquitoes are breeding on zoo properties and biting captive and wild animals on zoo grounds, including humans. However, no mosquitoes I tested were positive for dog heartworm. Additionally, I found significant differences in mosquito anatomy but cannot currently determine whether these differences affect bloodfeeding or pathogen presence.

My research indicates that mosquitoes do not appear to behave differently in zoos than they do outside of them. Therefore, mosquitoes are a manageable problem in zoos if proper control measures are taken. Additionally, my results indicate that zoos could be optimal experimental environments for the study of mosquito behavior; for instance, when field studies might not be affordable or feasible. Finally, because of my research I was able to give zoos recommendations on reducing the number of larval habitats of mosquitoes, and hence, the number of biting adults, and identify hosts potentially at risk of mosquito-borne pathogens in zoos.

APPENDICES

Appendix A

Pictures of larval mosquito habitats at the two zoos

Site 1

Site 2

Site 3

Site 4

Site 5

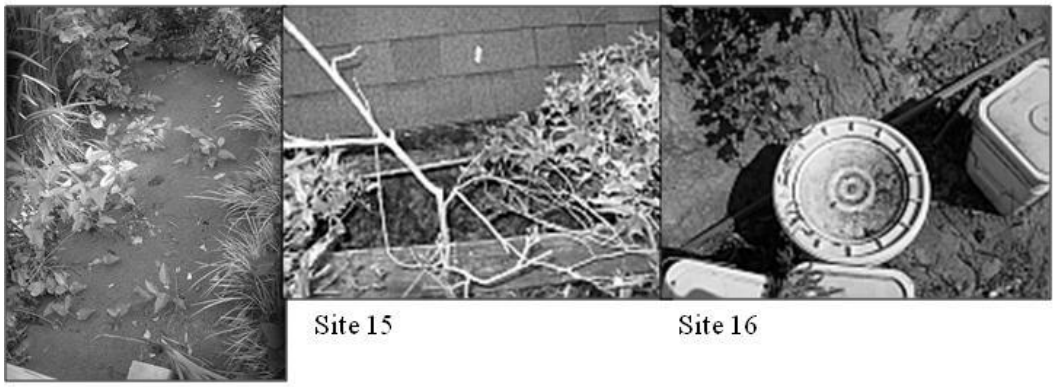
Site 6

Site 7

Site 8

Site 9

Site 10 Site 11 Site 12



Site 15 Site 16

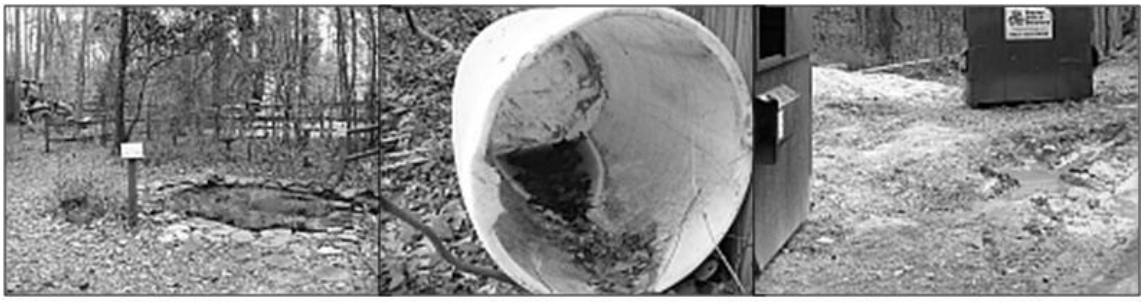
Site 13/14

Site 17 Site 18 Site 19

Site 20 Site 21 Site 22

Site 23 Site 24 Site 25

Site 26 Site 27 Site 28



Site 29 Site 30 Site 31

Site 32 Site 33 Site 34

Site 35 Site 36 Site 37

Site 38 Site 39 Site 40

Site 41 Site 42 Site 43

Site 44 Site 45 Site 46

Site 56 Site 57 Site 58

Site 59 Site 60

Appendix B

Pictures of gravid collection sites at the two zoos

Site 1

Site 2

Site 3

Site 4

Site 5

Site 6

Site 7

Site 8

Site 9

Site 10

Site 11

Site 12

Site 13

Site 14

Site 15

Site 16

No Picture

Site 17

Site 18

Site 19

Site 20

Site 21

Site 23

Site 24

Site 25

Site 26

No Picture

Site 27

Site 28

No Picture

Site 29

Site 30

Site 31

No Picture

Site 32

No Picture

Site 33

Site 34

Site 35

Appendix C

Pictures of aspiration sites at the two zoos

Site 1

Site 2

Site 3

Site 4

Site 5

Site 6

Site 7

Site 8

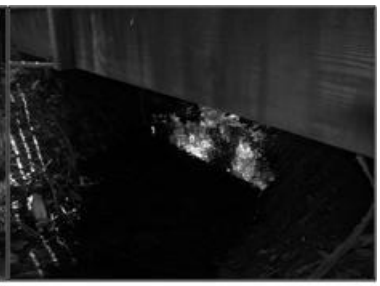
Site 9

Site 10 Site 11 Site 12

Site 13

Site 14

Site 15



Site 16

Site 17

Site 18

Site 19

Site 20

Site 21

Site 22

Site 23 Site 24 Site 25

No Picture

Site 26 Site 27 Site 28

Site 29 Site 30 Site 31

Appendix D

GPS coordinates and descriptions of mosquito collection locations at the two zoos

(All coordinates measured with a Garmin eTrex GPS unit)

Larval Collection Locations

Site	GPS North	GPS West	Description
			Greenville Zoo
1	34.84690	82.38733	Treehole near base of oak tree behind lemurs
2	34.84701	82.38742	Top of pvc pipe between picnic tables and shed
3	34.84643	82.38854	Pumphouse behind S. America exhibit
4	34.84614	82.38798	Holly bush behind S. America exhibit
5	34.84673	82.38884	Wheelbarrow tire by orangutans
6	34.84577	82.38843	Tallest pipe on backside of duck pond
7	34.84577	82.38843	Shortest pipe on backside of duck pond
8	34.84600	82.38862	End of runoff ditch before it drains into duck pond
9	34.84590	82.38851	Pipe at end of fence demarcating edge of site #8
10	34.84639	82.38912	Pool at mouth of runoff ditch
11	34.84564	82.38836	Fake concrete tree stump between flamingo and garden pond
12	34.84577	82.38820	Edge of flamingo pond by porch
13	na	na	Another edge of bog-Combined with 14
14	34.84564	82.38836	Garden pond by alligator viewing house
15	34.84674	82.38686	Gutter on restroom building next to owl cage
16	34.84801	82.38610	White bucket top near machine shed
17	34.84745	82.38623	Fake concrete tree stump by operations office
18-28	34.84661	82.38893	Bamboo stumps (n=11) in grove between waterfall and backside of orangutan enclosure
			Riverbanks Zoo
29	34.00858	81.07459	Garden pond near ponies and raptors
30	34.00834	81.07413	Overturned white 50g bucket near dumpsters
31	34.00780	81.07431	Mud puddle by dumpsters
32	34.00778	81.07343	Oak treehole behind ostrich cage
33	34.00879	81.07113	5g sunken bucket closest to Ndoki house (in old canal)
34	34.00877	81.07128	5g sunken bucket closest to elephant barn (in old canal)
35	34.08680	81.07127	Oak treehole on edge of canal
36	34.00865	81.07082	Oak treehole by Ndoki house
37	34.00895	81.07102	Hole in rock by site #34

38	34.00927	81.07054	Oak treehole in bog
39	34.00940	81.07048	One section of largest vernal pond
40	34.00910	81.07129	Vernal pool 1; one closer to maintenance shed
41	34.00911	81.07027	Vernal pool 2; one closest to road
42	34.00960	81.07496	Garden pool in front of Australia house
43	34.00924	81.07468	Metal birdbath in front of educational building
44	34.00883	81.07422	Edge of stream by pony ring
45	34.00893	81.07370	Oak treehole in pony ring
46	34.00881	81.07354	Metal cigarette bucket behind Kenya café
47	34.00838	81.07359	Water bog next to alligator pond
48	34.00845	81.07354	Small glass bowl within bog area next to alligator pond
49	34.00832	81.07258	Gutter on tortoise house
50	34.00875	81.07225	Mud puddle behind reptile house
51	34.00875	81.07225	Black plastic pool behind reptile house
52	34.00875	81.07225	Large metal pool behind reptile house
53	34.00941	81.07259	Back edge of lemur pool closer to reptile house
54	34.00991	81.07259	Bamboo stump behind bear exhibit
55	34.08900	81.07146	Water at edge of elephant enclosure by viewing deck
56	34.00986	81.07030	Pump housing by machine shop
57	34.00990	81.06901	Mud puddle by landscaping shed
58	34.00925	81.06982	Drainage ditch near vernal pools
59	34.00923	81.07026	Sunken stumphole in ground
60	34.00970	81.06250	Tarp around marshmallow roasting structure

Gravid Trap Locations

Site	GPS North	GPS West	Description
			Greenville Zoo
1	34.84734	82.38658	Behind building. next to employee break room
2	34.84865	82.38806	At perimeter fence, near alligators, clump of Holly bushes
3	34.84565	82.38829	Beside alligator viewing house
4	34.84626	82.38858	Behind shack at back of flamingo pond
5	34.84648	82.38823	Side of bathroom building. by waterfall
6	34.84687	82.38849	Backside of fence behind children's playground
7	34.84701	82.38766	Backside of fence between picnic tables and shed
8	34.84712	82.38722	Front of education building. behind bushes
9	34.84637	82.38797	Under bushes next to employee walkway to S. America
10	34.84679	82.38733	Behind reptile house
11	34.84801	82.38601	Back corner of machine shop under eaves
12	34.84759	82.38615	Behind fence behind animal hospital
13	34.84757	82.38637	Behind zoo offices
14	34.84755	82.38659	Behind fence next to garage
15	34.84731	82.38685	Bamboo between elephant enclosure and education building.
			Riverbanks Zoo
16	34.00999	81.07036	Behind machine shop, across from vernal pool
17	34.00978	81.07169	Side of gorilla viewing building. near front door
18	34.00950	81.07188	Against fence behind gorilla building.
19	34.00993	81.07204	Behind "Gorilla Goodies" stand
20	34.01003	81.07243	Behind bear exhibit, by bamboo stumps
21	34.00960	81.07350	Backside of Starbucks
22	na	na	Side of aviary, across from Baboons - INACCESSIBLE
23	34.00919	81.07484	Front of 3D Adventure theater
24	34.00965	81.07558	Behind merry-go-round wall
25	34.01027	81.07564	Front of raptor clinic, facing parking lot
26	34.00813	81.07440	Behind fence, by dumpsters near Safari station
27	34.00871	81.07464	Behind pony ring
28	34.00806	81.07218	Side of reptile house across from cafeteria
29	34.00865	81.07257	Side of tortoise shelter, against building
30	34.00878	81.07368	Side of cafeteria, facing zoo train depot
31	34.00996	81.07552	Corridor in front of vet clinic
32	34.00930	81.06963	Back corner of storage shed by landscaping, in ivy
33	34.00938	81.07086	Between back of Ndoki lodge and largest vernal pool
34	34.00908	81.07248	Front corner of reptile house by tortoises
35	34.00836	81.07354	Corridor between ostrich enclosure and safari station

Aspiration Locations

Site	GPS North	GPS West	Description
			Greenville Zoo
1	34.84632	82.38871	Tree stump in woods behind duck pond
2	34.84562	82.38820	Crepe myrtle bushes beside alligator house
3	34.84731	82.38685	Ivy covered area behind education building
4	34.84679	82.38733	Ivy covered wall behind reptile house
5	34.84679	82.38733	Ivy covered hill behind reptile house
6	34.84634	82.38803	Pumphouse behind S. America exhibit
7	34.84629	82.38804	Bushes and ivy behind S. America exhibit
8	34.84639	82.38793	Employee trail behind S. America exhibit
9	34.84649	82.38776	Ivy covered hill behind barnyard animals
10	34.84685	82.38847	Underside of playground porch
11	34.84625	82.38858	Shed behind duck pond
12	34.84578	82.38825	Underside of duck pond porch
13	34.84672	82.38741	Gutters behind reptile house
			Riverbanks Zoo
14	34.00933	81.07501	Ivy on wall of education building
15	34.00859	81.07224	Wall of metal pool behind reptile house
16	34.00875	81.07395	Underside of walkway to train
17	34.00922	81.07256	Underside of platform by old hippo pool
18	34.00923	81.07452	Front wall of education building behind garden
19	34.00988	81.06953	Bunker inside landscaping shed
20	34.00919	81.07391	Wall of aviary across from train
21	34.01012	81.07541	Sewer grates behind storage sheds by clinic
22	34.00992	81.07530	Outside bathroom by merry-go-round
23	34.00951	81.07436	Large fan unit at employee access to ape island
24	34.01003	82.07036	Pumphouse behind bears
25	34.00870	81.07354	Underside cafeteria
26	34.00992	81.07169	Underground pumphouse near “Gorilla Goodies”
27	34.00990	81.07031	Pumphouse area at machine shop
28	-	-	Under ape island (overnight Siamang habitat)
29	34.00980	81.07141	Employee area, Research Conservation Outpost
30	34.00905	81.07354	Groundcover and bushes on back of aviary
UV-A	34.00878	81.07458	CDC-miniature light trap (CO_2–baited) behind pony ring (used one time in May 2010)

Appendix E

Annual updates to the two zoos

Mosquito collections at the Greenville Zoo March 2008 to October 2008: Annual Report

Holly Tuten
Department of Entomology, Soils & Plant Sciences
Clemson University

Overview

Immature mosquitoes were collected from habitats at the Greenville zoo from March to October 2008. Samples were taken once a month, excepting April and September. All samples were taken within the zoo's perimeter fence. In December 2007 and January 2008 the entire zoo property was examined for potential mosquito breeding habitats. Twenty-eight study sites were selected based on three main criteria: 1. non-disturbance to animals, 2. the likelihood of retaining water throughout the study period, and 3. accessibility. These sites were then sampled on each subsequent visit, if they contained water. The average number of study sites positive for larvae per visit, over the entire sampling period, was 64 percent. This means that for each visit approximately two of every three sites sampled had mosquito larvae.

Eight species of mosquito were collected as larvae on zoo property. These species are:

Species	Feeding Behavior	Larval Habitat(s)
▪ *Aedes albopictus*	▪ Avian, mammal, reptile	▪ Standing water
▪ *Anopheles crucians*	▪ Mammal	▪ Ponds, rain pools, swamps
▪ *Anopheles punctipennis*	▪ Mammal	▪ Ditches, slow streams, swamps, tire ruts
▪ *Culex pipiens*	▪ Avian, mammal	▪ Foul water in ditches, large containers, pools
▪ *Culex restuans*	▪ Mammal	▪ Standing water
▪ *Culex territans*	▪ Amphibian, reptile	▪ Grassy margins of clean water
▪ *Ochlerotatus triseriatus*	▪ Avian, mammal	▪ Tires, treeholes, some artificial containers
▪ *Toxorhynchites rutilus*	▪ No blood meal (nectar feeder)	▪ Tires, treeholes

Breeding habitats of concern

Data collected over the six sampling visits indicate that some sites, or site types, have consistently high numbers of immature mosquitoes but can be controlled or altogether eliminated. The most notable of these are:

1. In the area behind the main office there at least one container every visit had hundreds of immature mosquitoes. Containers included garbage bins, overturned buckets, and the tops of closed 50g buckets. Prevention of mosquitoes breeding in these containers requires that they be overturned, periodically tipped, or drained. Some of the worst nuisance species breeding at these sites are *Aedes albopictus, Culex restuans*, and *Culex pipiens*.

2. Bamboo stumps in the bamboo grove consistently contained water and larvae. Although each individual stump will not produce large numbers of mosquitoes each month, their combined volume will. The stumps need to be either drained or eliminated.

3. The flamingo pond. This is the most problematic site at the zoo. Thousands of mosquitoes emerged from it over the course of the study, with numbers increasing over the summer. Mosquito species collected were *Culex restuans, Culex pipiens,* and *Culex territans*.

4. The gutter on the bathrooms beside the owl cage. This site (and probably other gutters) has a tendency to get clogged with leaves. The result is water stagnation and breeding by *Aedes albopictus* and *Ochlerotatus triseriatus*.

5. The pump housing behind small primates consistently had high numbers of multiple species of immature mosquitoes.

Control of mosquito breeding on zoo property

Although many breeding sites cannot be controlled reliably (e.g., treeholes, ephemeral puddles) many others can be (e.g., pump house, buckets, tarps). To foster effective control on zoo grounds all employees should look for potential breeding sites and eliminate them when they can. When sites cannot be eliminated *Bti* treatments should be used in a regulated manner. *Bti* pellets could be dispensed in the manner of a prescription. There would be a point person for each area in the zoo responsible for picking up pellets from management each month. This person would also be tasked with treating sites identified in their respective area. In this way a monthly check would show which departments have picked up *Bti* pellets. Additionally, these point people could receive instruction in how to recognize potential breeding sites and how to monitor those sites for mosquito activity. The ideal situation would be for one zoo employee to work a monthly maintenance day where they walk around the zoo and treat sites with *Bti,* tip over buckets, pick up trash, and identify potential mosquito pest problems.

Employee reports of mosquito biting activity

Throughout the study employees offered anecdotal information on mosquito biting activity. Additionally, a map of the zoo installed in the employee break room served as a reporting station for mosquito bites. Based on these sources of information, the areas of highest biting activity are 1. behind primates, 2. behind the bamboo grove, 3. behind small primates, and 4. in front of the lion cage.

Conclusions

Currently, at the zoo, the best method for reducing the number of mosquito bites received by employees and visitors is to reduce the number of mosquito breeding habitats. The best remedy is for employees to know what breeding habitats look like and how to eliminate or treat them when habitats are recognized. Additionally, two "mosquito magnet" machines are installed at the zoo. They currently require a refill of carbon dioxide for operation. I have spoken with the installer, Mr. Weeks, and we agreed I would empty and replace the nets whenever I visit the zoo.

Although I did not sample every potential breeding site at the zoo, the survey conducted was comprehensive enough to provide a basis for determining which sites should be managed and which are not problems. The bias inherent in my sampling scheme (e.g., limited access to animal enclosures) calls for vigilance on the part of zoo employees regarding identification of potential mosquito breeding sites. Additionally, I spoke with employees who work near breeding sites but do not know what immature mosquitoes look like. To ameliorate this, I would like to give a 30-minute seminar at the zoo sometime in May to educate employees about mosquitoes. This seminar could be repeated two times over the course of one day to include as many employees as possible. The seminar will include information which can facilitate recognition of mosquito breeding sites.

Mosquito collections at the Riverbanks Zoo March 2008 to October 2008: Annual Report

Holly Tuten
Department of Entomology, Soils & Plant Sciences
Clemson University

Overview

Immature mosquitoes were collected from habitats at Riverbanks Zoo from March to October 2008. Samples were taken once a month, excepting April and September. Sampling was also conducted in areas adjacent to the zoo (e.g., treeholes near the river) but most samples were taken within the zoo's perimeter fence. In January and March 2008 the entire zoo property was examined for potential mosquito breeding habitats. Thirty-two study sites were selected based on three main criteria: 1. non-disturbance to animals, 2. the likelihood of retaining water throughout the study period, and 3. accessibility. These sites were then sampled on each subsequent visit, if they contained water. The average number of study sites positive for larvae per visit, over the entire sampling period, was 63 percent. This means that for each visit approximately two of every three sites sampled had mosquito larvae.

Fifteen species of mosquito were collected as larvae on zoo property. These species are:

Species	Adult Feeding Behavior	Larval Habitat(s)
▪ *Aedes albopictus*	▪ Avian, mammal, reptile	▪ Standing water
▪ *Aedes vexans*	▪ Large mammals	▪ Grassy Ditches, rain pools, tire ruts
▪ *Anopheles crucians*	▪ Mammal	▪ Ponds, rain pools, swamps
▪ *Anopheles punctipennis*	▪ Mammal	▪ Ditches, slow streams, swamps, tire ruts
▪ *Anopheles quadrimaculatus*	▪ Large mammals	▪ Marshes, lake margins
▪ *Culex erraticus*	▪ Amphibian, avian, mammal, reptile	▪ Lake margins, slow streams
▪ *Culex pipiens*	▪ Avian, mammal	▪ Foul water in ditches, large containers, pools
▪ *Culex restuans*	▪ Mammal	▪ Standing water
▪ *Culex salinarius*	▪ Avian, mammal	▪ Water with rotting vegetation
▪ *Culex territans*	▪ Amphibian, reptile	▪ Grassy margins of clean water
▪ *Ochlerotatus triseriatus*	▪ Avian, mammal	▪ Tires, treeholes, some artificial containers
▪ *Orthopodomyia signifera*	▪ Avian	▪ Treeholes, artificial containers

▪ *Psorophora ferox* ▪ *Psorophora ciliata* ▪ *Toxorhynchites rutilus*	▪ Avian, mammal, reptile ▪ Mammal ▪ No blood meal (nectar feeder)	▪ Temporary and woodland pools ▪ Woodland pools ▪ Tires, treeholes

Breeding habitats of concern

Data collected over the six sampling visits indicate that some sites, or site types, have consistently high numbers of immature mosquitoes but can be controlled or altogether eliminated. The most notable of these are:

1. In the area behind Kenya café at least one container every visit had hundreds of immature mosquitoes (these containers are probably sources of biting adults in the pony ring). Containers included drink carts, large cooking pots with old food scraps, cigarette buckets, and various kitchen containers. Prevention of mosquitoes breeding in these containers requires that they be overturned, periodically tipped, or drained. Some of the worst nuisance species breeding at these sites are *Aedes albopictus*, *Culex restuans*, and *Culex pipiens*.

2. The frame for the marshmallow roaster stored on perimeter road. During the months when this is not being used it is covered with a tarp. The tarp develops many rain-filled pockets ranging from 1L to 5L in volume. Hundreds of adult mosquitoes were emerging from this site every month. At one point during the study the tarp was treated with *Bti* granules but did not receive a thorough application. Because of this, pockets nearest perimeter road did not have immature moquitoes but those furthest away were still producing the same number of adults as before the application.

3. The vernal ponds located between the back of the horticulture house and Ndoki lodge. These pools were dry from June to October but thousands of adult mosquitoes emerged from March to May. Mosquito species collected from these sites during the active months were *Culex restuans*, *Culex pipiens*, *Culex salinarius*, and *Culex territans*. Personnel in the reptile department told me that they deliberately flood these habitats during winter months (to provide habitat for salamanders).

4. Although not on the zoo grounds, there is an extensive bog adjacent to the perimeter road fence. This is probably a significant source of adults during the months that it is flooded. During this study it was flooded in March, May, and October.

5. During the course of the study thousands of immature mosquitoes of multiple species were observed in puddles and tire tracks along the length of perimeter road. Although some puddles are ephemeral (and therefore hard to treat) others held water during all but one month (July) of the study. The most notable of these are beside the dumpsters and the horticulture building. Additionally, garbage located along perimeter road provided

productive breeding habitats (e.g., old lamp posts, drink bottles, broken plastic containers).

6. The pump housing located on the side of the machine shop consistently had high numbers of multiple species. I was told by an employee that it cannot be drained and is not being treated with *Bti* pellets.

Control of mosquito breeding on zoo property

Although many breeding sites cannot be controlled reliably (e.g., treeholes, ephemeral puddles) many others can be (e.g., pumphouse, permanent puddles, buckets, tarps). To foster effective control on zoo grounds all employees should look for potential breeding sites and eliminate them when they can. When sites cannot be eliminated *Bti* treatments should be used in a regulated manner. Dr. Tiffany Moore suggested that *Bti* pellets could be dispensed to different departments in the zoo in the manner of a prescription. There would be a point person in each department responsible for picking up *Bti* pellets from the animal hospital each month. This person would also be tasked with treating sites identified in their respective area. In this way a monthly check would show which departments have picked up *Bti* pellets each month. Additionally, these point people could receive instruction in how to recognize potential breeding sites and how to monitor those sites for mosquito activity. The ideal situation would be for one person to have a monthly maintenance day where they walk around the zoo and treat sites with *Bti*, tip over buckets, pick up trash, and identify potential mosquito pest problems.

Employee reports of mosquito biting activity

Throughout the study employees offered anecdotal information on mosquito biting activity. Additionally, a map installed in the employee break room served as a reporting station for mosquito bites. Based on these sources of information, the areas of highest biting activity are 1. the pony exhibit, 2. the area between the Siamangs and the merry-go-round, and 3. the area behind the bird garden and lemur exhibit.

Employees reported that mosquitoes at pony rides are particularly bad on humid days and on some weekends they use more than one can of aerosol mosquito repellent. They are sharing this spray with zoo visitors and spraying the ponies.

Conclusions

Currently, at the zoo, the best method for reducing the number of mosquito bites received by employees and visitors is to reduce the number of mosquito breeding habitats. The best remedy is for employees to know what breeding habitats look like and how to eliminate or treat them when habitats are recognized.

Although I did not sample every potential breeding site at the zoo, the survey conducted was comprehensive enough to provide a basis for determining which sites should be managed and which are not problems. The bias inherent in my sampling scheme (e.g., limited access to animal enclosures) calls for vigilance on the part of zoo employees regarding identification of mosquito breeding sites. Additionally, I spoke with many employees who work near breeding sites but do not know what immature mosquitoes look like. To ameliorate this, I would like to give a 30-minute seminar at the zoo sometime in May to educate employees about mosquitoes. This seminar could be repeated three times over the course of one day to include as many employees as possible.

Mosquito collections at the Greenville Zoo January 2009 to September 2009: Annual Report

Holly Tuten
Department of Entomology, Soils & Plant Sciences
Clemson University

Overview

Habitats were sampled for immature mosquitoes at the Greenville Zoo in January (none were found). Adults were collected 1-2x per month June – September 2009 (Table One). In June 2009 the entire zoo property was examined for potential adult trapping sites. Fifteen study sites were selected based on four main criteria: 1. non-disturbance to animals, employees, and zoo visitors, 2. accessibility, 3. ground cover and canopy vegetation, and 4. nearby wind breaks (e.g., walls or fences) (Table Two). These sites were then sampled with adult traps which use water attractive to certain species of mosquitoes as egg-laying habitats.

Additionally, statistical analyses were performed on data collected in 2008 – 2009 on the environmental variables of habitats with immature mosquitoes. These results of the 2008 – 2009 study were presented at the annual meeting of the Entomological Society of America in December 2009, are the subject of a publication submitted to the J. Med. Entomol. , and will be presented later in this report.

Species	Adult Feeding Behavior	Larval Habitat(s)
▪ *Aedes albopictus*	▪ Avian, mammal, reptile	▪ Standing water
▪ *Aedes triseriatus*	▪ Avian, mammal	▪ Tires, treeholes, some artificial containers
▪ *Culex pipiens*	▪ Avian, mammal	▪ Foul water in ditches, large containers, pools
▪ *Culex restuans*	▪ Mammal	▪ Standing water
▪ *Culex territans*	▪ Amphibian, reptile	▪ Grassy margins of clean water
▪ *Orthopodomyia signifera*	▪ Avian	▪ Treeholes, artificial containers

Table One. Species of mosquito collected as adults in 2009 at the Greenville Zoo, with the previously recorded host preferences of adult females, and habitats where larvae are found.

Site number	Location description
1	Behind bldg. next to employee break room
2	At perimeter fence, near alligators, clump of Holly bushes
3	Beside alligator viewing house
4	Behind shack @back of Flamingo pond
5	Side of bathroom bldg. by waterfall
6	Backside of fence behind children's playground
7	Backside of fence between picnic tables and shed
8	Front of education bldg. behind bushes
9	Under bushes next to back walkway to S. America
10	Behind reptile house
11	Far back corner of machine shop under eaves
12	Behind fence behind animal hospital
13	Behind Jeff's office
14	Behind fence next to trash can & rain barrels by garage
15	Bamboo between elephant enclosure and education bldg.

Table Two. Sites for placement of adult traps during 2009 and 2010

Results of 2008 – 2009 study on habitats with mosquito larvae

Objectives:

1. Survey the mosquito species present as larvae in the Greenville Zoo
2. Determine environmental factors associated with larval presence
3. Determine seasonality of species
4. Provide control and monitoring recommendations to the zoo

Results:

1. 653 larvae collected representing 8 species
2. 4 species comprised 96% of collections
    a. *Aedes albopictus*: 72%
    b. *Aedes triseriatus*: 11%
    c. *Culex pipiens* complex: 10%
    d. *Culex restuans*: 3%
3. Mosquito larvae were most abundant in summer (75.0% of sites sampled had larvae) and fall (50.0% of sites sampled had larvae), followed by spring (44.4% of sites had larvae), then winter (no sites sampled had larvae)
4. Mosquito larvae were found in artificial and natural containers, and artificial and natural pools.
5. The most abundant species, *Aedes albopictus*, was 6.3 times more likely to be found in container habitats than in other habitat types.

6. The second most abundant species, *Aedes triseriatus*, was 3.6 times more likely to be found in natural habitats, and 3.5 times more likely to be found when the shade source for the habitat is less than or equal to 2 meters (e.g., low bushes or shrubs).
7. Mosquito larvae, regardless of species, were 1.4 times more likely to be found in natural habitats, and 1.7 times more likely when aquatic vegetation was absent from the habitat.
8. Presence of mosquito larvae was positively correlated with temperature and precipitation.

Recommendations:

1. Empty or eliminate container and natural habitats or remediate (e.g., fill with sand).
2. Regularly clean or flush all container and pool habitats that are integral to animal exhibits.
3. Eliminate shade sources less than 2 meters above aquatic habitats.
4. Use mosquito larvicides for habitats that cannot be eliminated, remediated, or regularly flushed.
5. Provide a yearly training seminar or educational video for zoo employees on habitat and larval recognition, and control strategies, before peak mosquito abundance.

Control of mosquito breeding on zoo property

Although many breeding sites cannot be controlled reliably (e.g., treeholes, ephemeral puddles) many others can be (e.g., pump house, buckets, tarps). To foster effective control on zoo grounds all employees should look for potential breeding sites and eliminate them when they can. When sites cannot be eliminated *Bti* treatments should be used in a regulated manner. *Bti* pellets could be dispensed in the manner of a prescription. There would be a point person for each area in the zoo responsible for picking up pellets from management each month. This person would also be tasked with treating sites identified in their respective area. In this way a monthly check would show which departments have picked up *Bti* pellets. Additionally, these point people could receive instruction in how to recognize potential breeding sites and how to monitor those sites for mosquito activity. The ideal situation would be for one zoo employee to work a monthly maintenance day where they walk around the zoo and treat sites with *Bti*, tip over buckets, pick up trash, and identify potential mosquito pest problems.

Specific breeding habitats of concern at the Greenville Zoo

Data collected over the six sampling visits indicate that some sites, or site types, have consistently high numbers of immature mosquitoes but can be controlled or altogether eliminated. The most notable of these are:

1. In the area behind the main office there at least one container every visit had hundreds of immature mosquitoes. Containers included garbage bins, overturned buckets, and the tops of closed 50g buckets. Prevention of mosquitoes breeding in these containers requires that they be overturned, periodically tipped, or drained. Some of the worst nuisance species breeding at these sites are *Aedes albopictus, Culex restuans,* and *Culex pipiens*.

2. Bamboo stumps in the bamboo grove consistently contained water and larvae. Although each individual stump will not produce large numbers of mosquitoes each month, their combined volume will. The stumps need to be either filled (e.g., with concrete or sand), drained, or eliminated.

3. The flamingo pond. This is the most problematic site at the zoo. Thousands of mosquitoes emerged from it over the course of the study, with numbers increasing over the summer. Mosquito species collected were *Culex restuans, Culex pipiens,* and *Culex territans*.

4. The gutter on the bathrooms beside the owl cage. This site (and probably other gutters) has a tendency to get clogged with leaves. The result is water stagnation and breeding by *Aedes albopictus* and *Aedes triseriatus*.

5. The pump housing behind small primates consistently had high numbers of multiple species of immature mosquitoes.

Conclusions

Currently, at the zoo, the best method for reducing the number of mosquito bites received by employees and visitors is to reduce the number of mosquito breeding habitats. The best remedy is for employees to know what breeding habitats look like and how to eliminate or treat them when habitats are recognized.

Although I did not sample every potential breeding site at the zoo, the survey conducted was comprehensive enough to provide a basis for determining which sites should be managed and which are not problems. The bias inherent in my sampling scheme (e.g., limited access to animal enclosures) calls for vigilance on the part of zoo employees regarding identification of mosquito breeding sites. Additionally, I spoke with many employees who work near breeding sites but do not know what immature mosquitoes look like. To ameliorate this, **I would like to give a 30-minute seminar at the zoo in April 2010 to educate employees about mosquito breeding habitat recognition and control**. This seminar could be repeated three times over the course of three days to include as many employees as possible.

Mosquito collections at the Riverbanks Zoo January 2009 to September 2009: Annual Report

Holly Tuten
Department of Entomology, Soils & Plant Sciences
Clemson University

Overview

Immature mosquitoes were collected from habitats at Riverbanks Zoo in January and June 2009 (Table One). Adults were collected 1-2x per month June – September 2009. In June 2009 the entire zoo property was examined for potential adult trapping sites. Nineteen study sites were selected based on four main criteria: 1. non-disturbance to animals, employees, and zoo visitors, 2. accessibility, 3. ground cover and canopy vegetation, and 4. nearby wind breaks (e.g., walls or fences) (Table Two). These sites were then sampled with adult traps which use water attractive to certain species of mosquitoes as egg-laying habitats.

Additionally, statistical analyses were performed on data collected in 2008 – 2009 on the environmental variables of habitats with immature mosquitoes. These results of the 2008 – 2009 study were presented at the annual meeting of the Entomological Society of America in December 2009, are the subject of a publication submitted to the J. Med. Entomol. , and will be presented later in this report.

Species	**Life stage**	**Adult Feeding Behavior**	**Larval Habitat(s)**
▪ *Aedes albopictus*	▪ A, L	▪ Avian, mammal, reptile	▪ Standing water
▪ *Aedes triseriatus*	▪ A	▪ Avian, mammal	▪ Tires, treeholes, some artificial containers
▪ *Aedes vexans*	▪ A	▪ Large mammals	▪ Grassy Ditches, rain pools, tire ruts
▪ *Anopheles punctipennis*	▪ A	▪ Mammal	▪ Ditches, slow streams, swamps, tire ruts
▪ *Culex pipiens* complex	▪ A	▪ Avian, mammal	▪ Foul water in ditches, large containers, pools
▪ *Culex restuans*	▪ A, L	▪ Mammal	▪ Standing water
▪ *Culex territans*	▪ A	▪ Amphibian, reptile	▪ Grassy margins of clean water
▪ *Ochlerotatus canadensis*	▪ L	▪ Amphibian, avian, mammal, reptile	▪ Shaded woodland pools
▪ *Orthopodomyia signifera*	▪ A	▪ Avian	▪ Treeholes, artificial containers

Table One. Species of mosquito collected in 2009 at the Riverbanks Zoo, with life stages represented in collections (A = adult, L = larvae), the previously recorded host preferences of adult females, and the habitats where larvae are found.

Site number	Location description
16	Behind machine shop, across from vernal pool
17	Side of gorilla viewing bldg. near front door
18	Against fence behind gorilla bldg.
19	Behind gorilla goodies stand
20	Side of grizzly bear exhibit, by bamboo stumps
21	Backside of Starbucks
~~22~~	Side of aviary, across from baboons – INACCESSIBLE
23	Front of 3D Adventure theater
24	Behind merry-go-round wall
25	Front of raptor clinic, facing parking lot
26	Behind fence, by dumpsters near Safari station
27	Behind pony ring
28	Side of reptile house across from cafeteria
29	Side of tortoise shelter, against bldg
30	Behind cafeteria
31	Corridor in front of vet clinic
32	Back corner of storage shed by landscaping, in ivy
33	Between back of Ndoki lodge and largest vernal pool
34	Front corner of reptile house
35	Corridor between ostrich cage and safari station

Table Two. Sites for placement of adult traps during 2009 and 2010

Results of 2008 – 2009 study on habitats with mosquito larvae

Objectives:

5. Survey the mosquito species present as larvae in the Riverbanks Zoo
6. Determine environmental factors associated with larval presence
7. Determine seasonality of species
8. Provide control and monitoring recommendations to the zoo

Results:

9. 977 larvae collected representing 16 species
10. 4 species comprised 88% of collections
    a. *Aedes albopictus*: 28%
    b. *Aedes triseriatus*: 32%
    c. *Culex pipiens* complex: 10%
    d. *Culex restuans*: 18%

11. Mosquito larvae were most abundant in spring (59.6% of sites sampled had larvae) and summer (59.3% of sites sampled had larvae), followed by fall (33.3% of sites had larvae), then winter (20% of sites sampled had larvae)
12. Mosquito larvae were found in artificial and natural containers, and artificial and natural pools.
13. The most abundant species, *Aedes triseriatus*, was 3.6 times more likely to be found in natural habitats, and 3.5 times more likely to be found when the shade source for the habitat is less than or equal to 2 meters (e.g., low bushes or shrubs).
14. The second most abundant species, *Aedes albopictus*, was 6.3 times more likely to be found in container habitats than in other habitat types.
15. Mosquito larvae, regardless of species, were 1.4 times more likely to be found in natural habitats, and 1.7 times more likely when aquatic vegetation was absent from the habitat.
16. Presence of mosquito larvae was positively correlated with temperature and precipitation.

Recommendations:

6. Empty or eliminate container and natural habitats or remediate (e.g., fill with sand).
7. Regularly clean or flush all container and pool habitats that are integral to animal exhibits.
8. Eliminate shade sources less than 2 meters above aquatic habitats.
9. Use mosquito larvicides for habitats that cannot be eliminated, remediated, or regularly flushed.
10. Provide a yearly training seminar or educational video for zoo employees on habitat and larval recognition, and control strategies, before peak mosquito abundance.

<u>Control of mosquito breeding on zoo property</u>

Although many breeding sites cannot be controlled reliably (e.g., treeholes, ephemeral puddles) many others can be (e.g., pumphouse, permanent puddles, buckets, tarps). To foster effective control on zoo grounds all employees should look for potential breeding sites and eliminate them when they can. When sites cannot be eliminated *Bti* treatments should be used in a regulated manner. Dr. Tiffany Moore suggested that *Bti* pellets could be dispensed to different departments in the zoo in the manner of a prescription. There would be a point person in each department responsible for picking up *Bti* pellets from the animal hospital each month. This person would also be tasked with treating sites identified in their respective area. In this way a monthly check would show which departments have picked up *Bti* pellets each month. Additionally, these point people could receive instruction in how to recognize potential breeding sites and how to monitor those sites for mosquito activity. The ideal situation would be for one person to have a monthly maintenance day where they walk around the zoo and treat sites with *Bti*, tip over buckets, pick up trash, and identify potential mosquito pest problems.

Specific mosquito-breeding sites of concern at the Riverbanks Zoo

Data collected over the six sampling visits indicate that some sites, or site types, have consistently high numbers of immature mosquitoes but can be controlled or altogether eliminated. The most notable of these are:

1. In the area behind Kenya café at least one container every visit had hundreds of immature mosquitoes (these containers are probably sources of biting adults in the pony ring). Containers included drink carts, large cooking pots with old food scraps, cigarette buckets, and various kitchen containers. Prevention of mosquitoes breeding in these containers requires that they be overturned, periodically tipped, or drained. Some of the worst nuisance species breeding at these sites are *Aedes albopictus*, *Culex restuans*, and *Culex pipiens*.

2. The frame for the marshmallow roaster stored on perimeter road. During the months when this is not being used it is covered with a tarp. The tarp develops many rain-filled pockets ranging from 1L to 5L in volume. Hundreds of adult mosquitoes were emerging from this site every month. At one point during the study the tarp was treated with *Bti* granules but did not receive a thorough application. Because of this, pockets nearest perimeter road did not have immature moquitoes but those furthest away were still producing the same number of adults as before the application.

3. The vernal ponds located between the back of the horticulture house and Ndoki lodge. These pools were dry from June to October but thousands of adult mosquitoes emerged from March to May. Mosquito species collected from these sites during the active months were *Culex restuans*, *Culex pipiens*, *Culex salinarius*, and *Culex territans*. Personnel in the reptile department told me that they deliberately flood these habitats during winter months (to provide habitat for salamanders).

4. Although not on the zoo grounds, there is an extensive bog adjacent to the perimeter road fence. This is probably a significant source of adults during the months that it is flooded. During this study it was flooded in March, May, and October.

5. During the course of the study thousands of immature mosquitoes of multiple species were observed in puddles and tire tracks along the length of perimeter road. Although some puddles are ephemeral (and therefore hard to treat) others held water during all but one month (July) of the study. The most notable of these are beside the dumpsters and the horticulture building. Additionally, garbage located along perimeter road provided productive breeding habitats (e.g., old lamp posts, drink bottles, broken plastic containers).

6. The pump housing located on the side of the machine shop consistently had high numbers of multiple species. I was told by an employee that it cannot be drained and is not being treated with *Bti* pellets.

Conclusions

Currently, at the zoo, the best method for reducing the number of mosquito bites received by employees and visitors is to reduce the number of mosquito breeding habitats. The best remedy is for employees to know what breeding habitats look like and how to eliminate or treat them when habitats are recognized.

Although I did not sample every potential breeding site at the zoo, the survey conducted was comprehensive enough to provide a basis for determining which sites should be managed and which are not problems. The bias inherent in my sampling scheme (e.g., limited access to animal enclosures) calls for vigilance on the part of zoo employees regarding identification of mosquito breeding sites. Additionally, I spoke with many employees who work near breeding sites but do not know what immature mosquitoes look like. To ameliorate this, **I would like to give a 30-minute seminar at the zoo in April 2010 to educate employees about mosquito breeding habitat recognition and control**. This seminar could be repeated three times over the course of three days to include as many employees as possible.

Mosquito collections at the Greenville and Riverbanks Zoos March 2010 to October 2010: Annual Report

Holly Tuten
Department of Entomology, Soils & Plant Sciences
Clemson University

Overview and Results

From April to September 2010, adult mosquitoes were collected 1-2x per month by hand aspiration from resting habitats at the Greenville and Riverbanks Zoos. The zoos were inspected for potential adult aspiration sites on an ongoing basis (Table one). Molecular analyses were used on mosquitoes collected in 2009 and 2010 to determine the identity of mosquito hosts (Figure one, Table two). The following species were never found bloodfed (total number of individuals collected in parentheses):

- *Aedes*
  - *canadensis* (1), *japonicus* (7), *vexans* (6),
- *Culex*
  - *pipiens/restuans* (116), spp. (6)
- *Orthopodomyia signifera* (1)
- *Psorophora ferox* (1)
- *Uranotaenia sapphirina* (3)

In total 2,522 mosquitoes were collected from the two zoos. Ninety-five of the collected mosquitoes were bloodfed and vertebrate hosts were successfully identified from fifty-three of those bloodmeals. Additionally, bloodfed mosquitoes were tested for the causative agent of dog heartworm, *Dirofilaria immitis*. No mosquitoes tested positive for *D. immitis*. For the three species most commonly bloodfed species there were avian, human, and mammalian hosts, and there were notable differences in the ratio of avian to mammalian hosts depending on whether the animals were captive or wild (Figure two).

These results were presented at the annual meeting of the Entomological Society of America in December 2010. Data from both zoos are presented together to convey the total breadth and patterns of vertebrate hosts used.

Conclusions

- At least eight mosquito species are feeding on captive and wild animals, and humans, in SC zoos.
- *Cx. erraticus* and *Cx. pipiens* complex foraged more often on birds, and *An. punctipennis* foraged more often on mammals.
- Mosquitoes appear to have a broader host range when feeding on captive as opposed to wild animals.
- There were several novel host records from mosquitoes feeding on captive animals.
- No bloodfed mosquitoes were positive for *D. immitis*.

Table One. Sites for adult aspirations during 2010. GZ = Greenville Zoo, RZ = Riverbanks Zoo.

Zoo		Description
	GZ	Tree stump in woods behind duck pond
	GZ	Trees and fence behind alligator
	GZ	Ivy area between elephants and education
	GZ	Ivy covered wall behind reptile
	GZ	Ivy area behind reptile
	GZ	Pumphouse behind South America
	GZ	Bushes and ivy behind South America
	GZ	Trail behind South America
	GZ	Ivy across from reptile/snack area
	GZ	Underside of playground porch
	GZ	Ivy and shed behind duck pond
	GZ	Underside of duck pond porch
	GZ	Gutters behind reptile house
RZ		Ivy in front of education building
RZ		Pool with larvae behind reptile house
RZ		Bridge beside train (small access bridge)
RZ		Under bridge by old hippo pool
RZ		Front wall of education bldg behind garden
RZ		Bunker inside landscaping shed
RZ		Wall of aviary across from train
RZ		Sewer grates behind storage sheds by clinic
RZ		Outside bathroom by merry-go-round
RZ		Large fan unit at entrance to ape island
RZ		Pumphouse behind bears
RZ		Area underneath cafeteria
RZ		Underground pumphouse near Gorilla Goodies
RZ		Pumphouse area at machine shop
RZ		Under ape island
RZ		Interior RCO
RZ		Groundcover and bushes on back of aviary

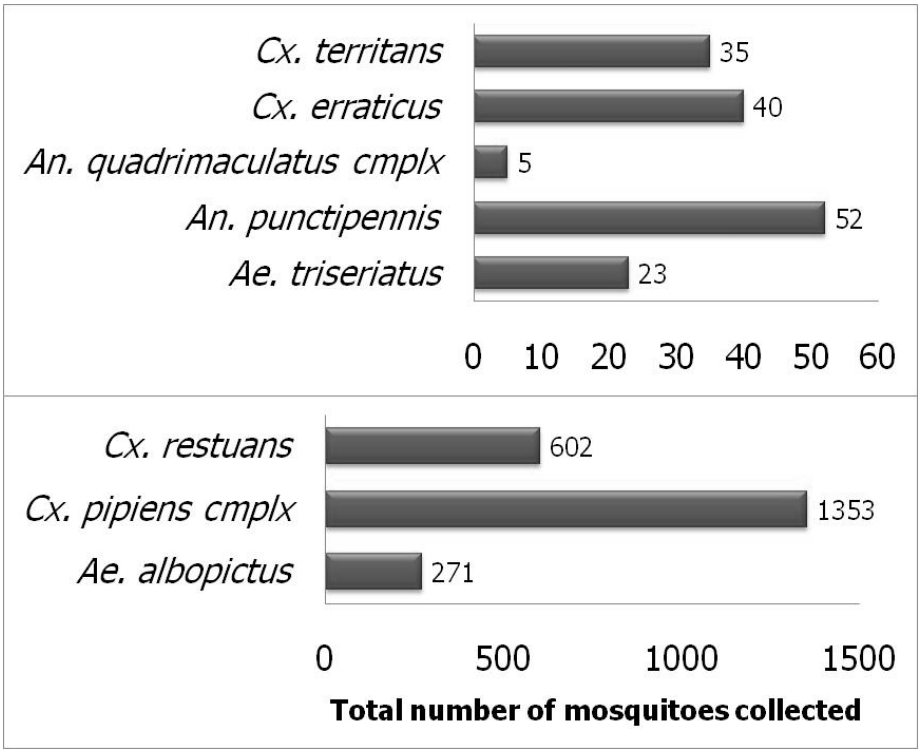


Figure One. The total number of mosquitoes collected of species that were found bloodfed at least once. "*Ae*." = *Aedes*, "*An*." = *Anopheles*, and "*Cx*." = *Culex*

Table Two. Species of mosquito collected in 2010 at the Riverbanks and Greenville Zoos with identity of hosts. Captive species in bold.

Zoo	Site Description
Ae. albopictus	Human, Opossum
Ae. triseriatus	**Brown bear**
An. punctipennis	**Cow, Horse, Ostrich, Spotted hyena, Summer tanager**
An. quadrimaculatus cmplx	**Brown bear, Ostrich**
Cx. erraticus	**American flamingo, Cow, Grey-crowned crane, Horse,** Human, **Indefatigable island tortoise, Keel-billed toucan,** Mourning dove, Northern cardinal, **Ostrich,** Raccoon, **Thick-billed parrot, Toco toucan, Turkey vulture**

Cx. pipiens complex	**American flamingo,** Carolina chickadee, Carolina wren, **Cow, Eastern box turtle,** Human, Mourning dove, Northern cardinal, **Ostrich, Red-billed hornbill, Ring-tailed lemur, Siamang, Spotted hyena, Toco toucan, Wreathed hornbill**

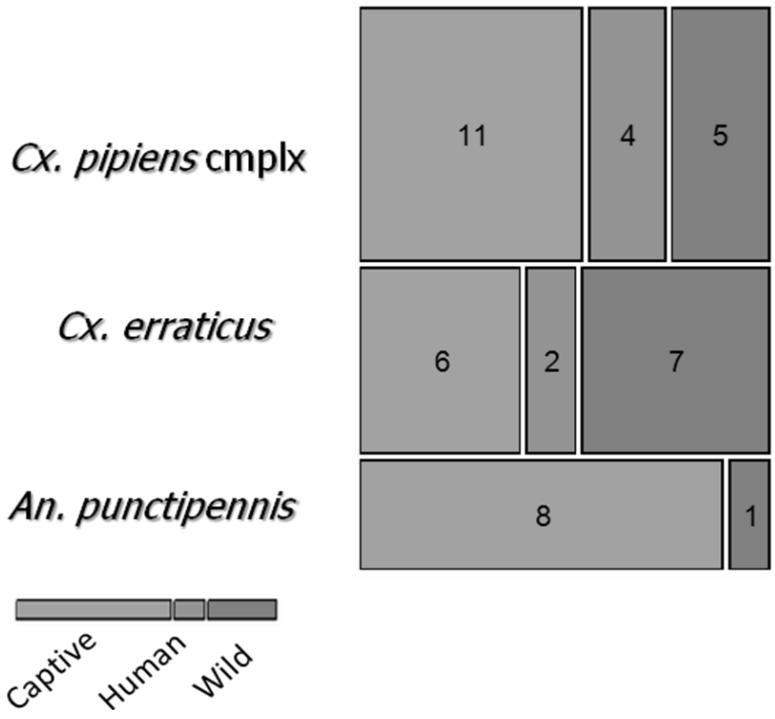


Figure One. The ratio of avian to human to mammal hosts used by three species of mosquitoes across both zoos. The total number of hosts indicated by numbers in middle of cells. Percentage bar across bottom of figure.

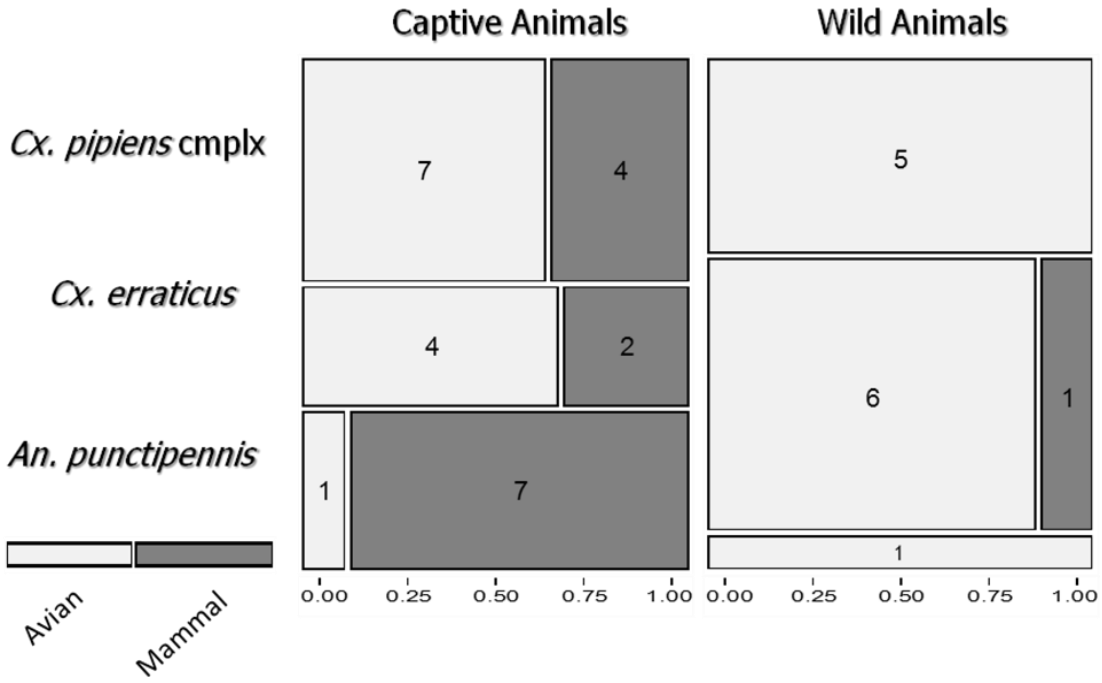


Figure Two. The ratio of avian to mammal hosts used by three species of mosquitoes, grouped by captive versus wild status. The total number of hosts indicated by numbers in middle of cells. Percentage bars across bottom of figure.

REFERENCES

Abraham, D. 1988. Biology of *Dirofilaria immitis*, pp. 29-46. In P. F. L. Boreham and R. B. Atwell (eds.), Dirofilariasis. CRC Press, Boca Raton, FL, USA.

Abràmoff, M. D., P. J. Magalhães, and S. J. Ram. 2004. Image processing with ImageJ. BioPhotonics International 11: 36-42.

Adler, P. H., H. C. Tuten, and M. P. Nelder. 2011. Arthropods of medicoveterinary importance in zoos. Ann. Rev. Entomol. 56: 123-142.

Allan, S. A., J. F. Day and J. D. Edman. 1987. Visual ecology of biting flies. Ann. Rev. Entomol. 32: 297-316.

Alley, M. R., R. A. Fairley, D. G. Martin, L. Howe, and T. Atkinson. 2008. An outbreak of avian malaria in captive yellowheads/mohua (*Mohoua ochrocephala*). New Zeal. Vet. J. 56: 247-251.

Alto, B.W., and S.A. Juliano. 2001a. Temperature effects on the dynamics of *Aedes albopictus* (Diptera: Culicidae) populations in the laboratory. J.Med. Entomol. 38: 548-556.

Alto, B.W., and S.A. Juliano. 2001b. Precipitation and temperature effects on populations of *Aedes albopictus* (Diptera: Culicidae): Implications for range expansion. J. Med. Entomol. 38: 646-656.

Arav, D., and L. Blaustein. 2006. Effects of pool depth and risk of predation on oviposition habitat selection by temporary pool dipterans. J. Med. Entomol. 43: 493-497.

Barrera, R., M. Amador, and G. G. Clark. 2006. Ecological factors influencing *Aedes aegypti* (Diptera: Culicidae) productivity in artificial containers in Salinas, Puerto Rico. J. Med. Entomol. 43: 484-492.

Bartlett-Healy, K., W. Crans, and R. Gaugler. 2009. Vertebrate hosts and phylogenetic relationshiops of amphibian trypanosomes from a potential invertebrate vector, *Culex territans* Walker (Diptera: Culicidae). J. Parasitol. 95: 381-387.

Bataille, A., A. A. Cunningham, W. Cedeño, L. Patiño, A. Constantinou, L. D. Kramer, and S. J. Goodman. 2009. Natural colonization and adaptation of a mosquito species in Galápagos and its implications for disease threats to endemic wildlife. PROC. NATL. ACAD. SCI. USA 106:10230-10235.

Beehler, J. W. 1991. The oviposition behavior of *Aedes triseriatus*. Proc. Calif. Mosq. Vector Control Assoc. 59: 108-113.

Beehler, J. W., and M. S. Mulla. 1995. Effects of organic enrichment on temporal distribution and abundance of Culicine egg rafts. J. Am. Mosq. Control Assoc. 11: 167-171.

Beier, J. C., and M. K. Stoskopf. 1980. The epidemiology of avian malaria in Black-footed penguins (*Spheniscus demersus*). J. Zoo. Animal Med. 11: 99-105.

Beier, J. C., and M. Trpis. 1981a. Incrimination of natural culicine vectors which transmit Plasmodium elongatum to penguins at the Baltimore Zoo. Can. J. Zool. 59: 470-475.

Beier, J.C., and M. Trpis. 1981b. Local distribution of *Aedes triseriatus* (Diptera, Culicidae) at the Baltimore, Maryland (USA) Zoo. Mosq. News 41: 447-455.

Beier, J. C., M. Travis, C. Patricoski, and J. Kranzfelder. 1983. Habitat segregation among larval mosquitoes (Diptera: Culicidae) in tire yards in Indiana, USA. J. Med. Entomol. 20: 76-80.

Beketov, M. A., Y. A. Yurchenko, O. E. Belevich, and M. Liess. 2010. What environmental factors are important determinants of structure, species richness, and abundance of mosquito assemblages. J. Med. Entomol. 47:129-139.

Bentley, M. D., and J.F. Day. 1989. Chemical ecology and behavioral aspects of mosquito oviposition. Ann. Rev. Entomol. 34: 401-421.

Berner, R., W. Rudin, and H. Hecker. 1983. Peritrophic matrixes and protease activity in the midgut of the malaria mosquito, *Anopheles stephensi* (Liston) (Insecta: Diptera) under normal and experimental conditions. J. Ultrastructure Res. 83: 195-204.

Blackwell, A., and S. N. Johnson. 2000. Electrophysiological investigation of larval water and potential oviposition chemo-attractants for *Anopheles gambiae* s.s. Ann. Trop. Med. Parasitol. 94: 389-398.

Blitvich, B.J. 2008. Transmission dynamics and changing epidemiology of West Nile virus. Anim. Health Res. Rev. 9: 71-86.

de Boissezon, P. 1930. Contribution à l'étude de la biologie et de l'histophysiologie de *Culex pipiens* L. Arch. Zool. Exp. Gen. 70: 281-431.

Boreham, P. F. L., and R. B. Atwell. 1988. Preface. In P. F. L. Boreham and R. B. Atwell (eds.), Dirofilariasis. CRC Press, Boca Raton, FL, USA.

Borkent, A. and P. Belton. 2006. Attraction of female *Uranotaenia lowii* (Diptera: Culicidae) to frog calls in Costa Rica. Can. Entomol. 138: 91-94.

Bradley, C. A., and S. Altizer. 2007. Urbanization and the ecology of wildlife diseases. Trends Ecol. Evol. 22: 95-102.

Braks, M. A. H., R. A. Anderson, and B. G. J. Knols. 1999. Infochemicals in mosquito host selection: human skin microflora and *Plasmodium* parasites. Parasitol. Today 15: 409-413.

Britch, S. C., K. J. Linthicum, A. Anyamba, C. J. Tucker, E. W. Pak and The Mosquito Surveillance Team. 2008. Long-term surveillance data and patterns of invasion by *Aedes albopictus* in Florida. J. Am. Mosq. Control Assoc. 24: 115-120.

Brossy, J. J., A. L. Plos, J. M. Blackbeard, and A. Kline. 1999. Diseases acquired by captive penguins: what happens when they are released into the wild? Mar. Ornithol. 27: 185-186.

Buse, E., and F. Kuhlow. 1979. Rasterelektronenoptische studien zur morphologie und bedeutung der vorderdarmbewehrung verschiedener stechmückenarten für die aufnahme von mikrofilarien. Tropenmed. Parasit. 30: 446-454.

Byers, J. R., and E. F. Bond. 1971. Surface specializations of the hindgut cuticle of lepidopterous larvae. Can. J. Zool. 49:867-76.

Chadee, D. D., J. C. Beier, and R. Martinez. 1996. The effect of the cibarial armature on blood meal haemolysis of four anopheline mosquitoes. Bull. Entomol. Res. 86:351-354.

Chan, K. L., B. C. Ho and Y. C. Chan. 1971. *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) in Singapore City: 2. Larval habitats. Bull. Wld. Hlth. Org. 44: 629-633.

Chatterjee, S., and A. S. Hadi. 2006. Regression Analysis by Example. Wiley, Hoboken, N.J.

Chaves, L. F., L. C. Harrington, C. L. Keogh, A. M. Nguyen, and U. D. Kitron. Blood feeding patterns of mosquitoes: random or structured? Front. Zool. 7: 3.

Christensen, H. A., A. Herrer, and G. B. Fairchild. 1971. Pyloric armature of new world phlebotomine sandflies (Diptera, Psychodidae). J. Med. Entomol. 8: 116-119.

Christophers, S. 1960. *Aëdes aegypti* (L.) the yellow fever mosquito: its life history, bionomics and structure. The Syndics of the Cambridge University Press, London, UK.

Chwatt, L. J. 1945. The morphology of the pharyngeal armature in *Anopheles gambiae* and *Anopheles gambiae* var. *melas* from southern Nigeria. Ann. Trop. Med. Parasitol. 39: 124-128.

Ciborowski, J. J. H., and P. H. Adler. 1990. Ecological segregation of larval black flies (Diptera: Simuliidae) in northern Saskatchewan, Canada. Can. J. Zool. 68: 2113-2122.

Ciferri, F. 1982. Human pulmonary dirofilariasis in the United States: a critical review. Am. J. Trop. Med. Hyg. 31: 302-308.

Clements, A. N. 1963. The physiology of mosquitoes. Oxford: Pergamon, London, UK.

Coluzzi, M., A. Concetti, and F. Ascoli. 1982. Effect of cibarial armature of mosquitoes (Diptera, Culicidae) on blood-meal haemolysis. J. Ins. Physiology. 28: 885-888.

Costanzo, K. S., K. Mormann, and S. A. Juliano. 2005. Asymmetrical competition and patterns of abundance of *Aedes albopictus* and *Culex pipiens* (Diptera: Culicidae). J. Med. Entomol. 42: 559-570.

Courtney, C. H. 1988. Chemotherapy and chemoprophylaxis, pp. 151-162. In P. F. L. Boreham and R. B. Atwell (eds.), Dirofilariasis. CRC Press, Boca Raton, FL, USA.

Dapples, C. C., and A. O. Lea. 1974. Inner surface morphology of the alimentary canal in *Aedes aegypti* (L.) (Diptera: Culicidae). Int. J. Insect Morphol. Embryol. 3: 433-442.

Darsie, R. F., and R. A. Ward. 2004. Identification and geographical distribution of the mosquitoes of North America, north of Mexico. University press of Florida, FL, USA.

Deem, S. L., D. J. Heard, and R. LaRock. 1998. Heartworm (*Dirofilaria immitis*) disease and glomerulonephritis in a black-footed cat (*Felis nigripes*). J. Zoo Wildlife Med. 29: 199-202.

Dennett, J. A., A. Bala, T. Wuithiranyagool, Y. Randle, C. B. Sargent, H. Guzman, M. Siirin, H. K. Hassan, M. Reyna-Nava, and T. R. Unnasch. 2007. Associations between two mosquito populations and West Nile virus in Harris County, Texas, 2003–06. J. Am. Mosq. Control Assoc. 23: 264-275.

Derraik, J. B. 2004. A survey of the mosquito (Diptera: Culicidae) fauna of Auckland Zoological Park. N. Z. Entomol. 27: 51–55.

Derraik, J. G. B. 2005. Recommendations for mosquito control in zoological parks to reduce disease transmission risk. The Weta 29: 16-20.

Derraik, J.G.B. and D. Slaney. 2005. Container aperture size and nutrient preferences of mosquitoes (Diptera: Culicidae) in the Auckland region, New Zealand. J. Vector Ecol. 30: 73-82.

Derraik, J. B., D. Slaney, and P. Weinstein. 2008. Effect of height on the use of artificial larval container habitats by *Aedes notoscriptus* in native forest and zoo in Wellington, New Zealand. N. Z. Entomol. 31: 31–34.

Dhileepan, K. 1997. Physical factors and chemical cues in the oviposition behaviour of arboviral vectors *Culex annulirostris* and *Culex molestus* (Diptera: Culicidae). Environ. Entomol. 26: 318-326.

Edman, J. D., L. A. Webber, and H. W. Kale II. 1972. Host-feeding patterns of Florida mosquitoes. J. Med. Entomol. 9: 429-434.

Ejiri, H., Y. Sato, R. Sawai, E. Sasaki, R. Matsumoto, M. Ueda, Y. Higa, Y. Tsuda, S. Omori, S., K. Murata, and M. Yukawa. 2009. Prevalence of avian malaria parasite in mosquitoes collected at a zoological garden in Japan. Parasitol. Res. 105: 629-633.

Ejiri, H., Y. Sato, K. S. Kim, T. Hara, Y. Tsuda, T. Imura, K. Murata, and M. Yukawa. 2011. Entomological study on transmission of avian malaria parasites in a zoological garden in Japan: bloodmeal identification and detection of avian malaria parasite DNA from blood-fed mosquitoes. J. Med. Ent. 48: 600-607.

Elzinga, R. J. 1998. Microspines in the alimentary canal of arthropoda, onychophora, annelida. Int. J. Insect Morphol. Embryol. 27: 341-349.

Eysell, A. 1905. In Mense, C. (ed.). Handbuch der Tropenkrankheiten.Vol. II. J. A. Barth, Leipzig, Germany.

Ferguson, H. M., A. Dornhaus, A. Beeche, C. Borgemeister, M. Gottlieb, M. S. Mulla, J. E. Gimnig, D. Fish, and G.F. Killeen. 2010. Ecology: a prerequisite for malaria elimination and reduction. PLoS Med . 7:e1000303.

Fix, A. S., C. Waterhouse, E. C. Greiner and M. K. Stoskopf. 1988. *Plasmodium relictum* as a cause of avian malaria in wild-caught Magellanic penguins (*Spheniscus magellanicus*). J. Wildlife Dis. 24: 610-619.

Foley, D. H., E. P. Torres and I. Mueller. 2002. Stream-bank shade and larval distribution of the Philippine malaria vector *Anopheles flavirostris*. Med. Vet. Entomol. 16: 347-355.

Fonseca, D. M., N. Keyghobadi, C. A. Malcolm, C. Mehrnet, F. Schaffner, M. Mogi, R. C. Fleischer, and R. C. Wilkerson. 2004. Emerging vectors in the *Culex pipiens* complex. Science 303: 1535-1538.

Forattini, O. P. and M. A. M. Sallum. 1992. Cibarial armature as taxonomic characters for the Spissipes Section of *Culex* (Melanoconion) (Diptera: Culicidae). Mosq. Systematics 24:70-84.

Foster, W. A., and E. D. Walker. 2009. Mosquitoes (Culicidae), pp. 207-260. In G. R. Mullen and L. A. Durden (eds.), Medical and Veterinary Entomology, 2nd ed. Elsevier, Burlington, MA, USA.

Frank, J. H. and E. D. McCoy. 1990. Attack and Defense - behavioral ecology of predators and their prey - endemics and epidemics of shibboleths and other things causing chaos - introduction. Fla. Entomol. 73: 1-9.

Frimeth, J. P. and H. P. Arai. 1984. A survey for the microfilariae of canine heartworm, *Dirofilaria immitis*, in the Calgary region of southern Alberta. Can. Vet. J. 25: 14-16.

Fyodorova, M. V., H. M. Savage, J. V. Lopatina, T. A. Bulgakova, A. V. Ivanitsky, O. V. Platonova, and A. V. Platonova. 2006. Evaluation of potential West Nile virus vectors in Volgograd region, Russia, 2003 (Diptera: Culicidae): species composition, bloodmeal host utilization, and virus infection rates of mosquitoes. J. Med. Entomol. 43: 552-63.

Gamble, K. C., J. J. Fried, and G. J. Rubin. 1998. Presumptive dirofilariasis in a pale-headed saki monkey (*Pithecia pithecia*). J. Zoo Wild. Med. 29: 50-54.

Geong, H. 2001. Carving a niche for medical entomology: a quest for professional identity in American entomology. Am. Entomol. 47: 236-243.

Gibson, G., and S. J. Torr. 1999. Visual and olfactory responses of haematophagous Diptera to host stimuli. Med. Vet. Entomol. 13: 2-23.

Gimnig, J. E., M. Ombok, L. Kamau, and W. A. Hawley. 2001. Characteristics of larval anopheline (Diptera: Culicidae) habitats in western Kenya. J. Med. Entomol. 38: 282-288.

Gottfried, K. L., R. R. Gerhardt, R. S. Nasci, M. B. Crabtree, N. Karabatsos, K. L. Burkhalter, B. S. Davis, N. A. Panella, and D .J. Paulson. 2002. Temporal abundance, parity, survival rates, and arbovirus isolation of field-collected container inhabiting mosquitoes in eastern Tennessee. J. Am. Mosq. Control Assoc. 18: 164-172.

Graham, S. P., H. K. Hassan, N. D. Burkett-Cadena, C. Guyer, and T. R. Unnasch. 2009. Nestedness of ectoparasite-vertebrate host networks. PLoS One 4: e7873.

Gratz, N. G. 2004. Critical review of the vector status of *Aedes albopictus*. Med.Vet.Entomol. 18: 215-227.

Grieve, R. B., J. B. Lok, and L. T. Glickman. 1983. Epidemiology of canine heartworm infection. Epidem. Rev. 5: 220-246.

Grim, C. K., T. McCutchan, J. Li, M. Sullivan, T. K. Graczyk, G. McConkey and M. Cranfield. 2004. Preliminary results of an anticircumsporozoite DNA vaccine trial for protection against avian malaria in captive African black-footed penguins (*Spheniscus demersus*). J. Zoo Wildlife Med. 35: 154-161.

Griner, L.A. 1974. Some diseases of zoo animals. *Adv. Vet. Sci. Comp. Med.* 18: 251–71.

Gu, W. D., J. Utzinger and R. J. Novak. 2008. Habitat-based larval interventions: a new perspective for malaria control. Am. J. Trop. Med. Hyg. 78: 2-6.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41: 95-98.

Hamada, N., J. W. McCreadie, and P. H. Adler. 2002. Species richness and spatial distribution of blackflies (Diptera: Simuliidae) in streams of Central Amazonia, Brazil. Freshwater Biol. 47: 31-40.

Hamer, G. L., U. D. Kitron, T. L. Goldberg, J. D. Brawn, S. R. Loss, M. O. Ruiz, D. B. Hayes and E. D. Walker. 2009. Host selection by *Culex pipiens* mosquitoes and West Nile virus amplification. Am. J. Trop. Med. Hyg. 80: 268-278.

Haramis, L. D. 1984. *Aedes triseriatus* - a comparison of density in tree holes vs discarded tires. Mosq. News 44: 485-489.

Harbach, R. E. 2007. The Culicidae (Diptera): a review of taxonomy, classification, and phylogeny. Zootaxa 1668: 591-638.

Harrington, L. C., J. D. Edman, and T. W. Scott. 2001. Why do female *Aedes aegypti* (Diptera: Culicidae) feed preferentially and frequently on human blood? J. Med. Entomol. 38: 411-422.

Hassan, H. K., E. W. Cupp, G. E. Hill, C. R. Katholi, K. Klingler and T. R. Unnasch. 2003. Avian host preference by vectors of Eastern Equine Encephalomyelitis virus. Am. J. Trop. Med. Hyg. 69: 641-647.

Hess, A. D., R. O. Hayes, and C. H. Tempelis. 1968. The use of the forage ratio technique in mosquito host preference studies. Mosq. News 28: 386-389.

Hocking, B. 1971. Blood-sucking behavior of terrestrial arthropods. Ann. Rev. Entomol. 16: 1-28.

Hosmer, D. W., and S. Lemeshow. 2000. Applied Logistic Regression. Wiley, Hoboken, N.J., USA.

Horn, B. W. 2001. Physiological adaptations of Trichomycetes to the insect gut: *Smittium culisetae* and its mosquito host, p 99–116. In J.W. Misra and B. W. Horn (eds.), Trichomycetes and other fungal groups. Science Publishers, Enfield, NH, USA.

Hribar, L. J., J. M. Smith, J. J. Vlach, and T. N. Verna. 2001. Survey of container-breeding mosquitoes from the Florida Keys, Monroe County, Florida. J. Am. Mosq. Control Assoc. 17: 245-248.

Huang, J., E. D. Walker, J. Vulule and J. R. Miller. 2007. The influence of darkness and visual contrast on oviposition by *Anopheles gambiae* in moist and dry substrates. Physiol. Entomol. 32: 34-40.

Huijben, S., W. Schaftenaar, A. Wijsman, K. Paaijmans, and W. Takken. 2007. Avian malaria in Europe: an emerging infectious disease?, pp. 59-74. In W. Takken and B. G. Knols (eds.), Emerging pests and vector-borne diseases in Europe. Wageningen Academic Publishers, Wageningen, The Netherlands.

Hurd H., J.C. Hogg, and M. Renshaw. 1995. Interactions between blood feeding, fecundity, and infection in mosquitoes. Parasitol. Today. 11: 411–416.

Hurlbert S.H. 1969. A coefficient of interspecific association. Ecology 50:1–9.

Jackson, B. T., S. L. Paulson, R. R. Youngman, S. L. Scheffel, and B. Hawkins. 2005. Oviposition preferences of *Culex restuans* and *Culex pipiens* (Diptera: Culicidae) for selected infusions in oviposition traps and gravid traps. J. Am. Mosq. Control Assoc. 21: 360-365.

Juliano, S. A., and L. P. Lounibos. 2005. Ecology of invasive mosquitoes: effects on resident species and on human health. Ecol. Lett. 8: 558-574.

Kaufman, M. G., and E. D. Walker. 2006. Indirect effects of soluble nitrogen on growth of *Ochlerotatus triseriatus* larvae in container habitats. J. Med. Entomol. 43: 677-688.

Kennedy, S., and S. Patton. 1981. Heartworms in a Bengal tiger. J. Zoo Anim. Med. 12: 20-22.

Kent, R. J. 2009. Molecular methods for arthropod bloodmeal identification and applications to ecological and vector-borne disease studies. Mol. Ecol. Resources 9: 4-18.

Kent, R., L. Juliusson, M. Weissmann, S. Evans, and N. Komar. 2009. Seasonal blood feeding behavior of *Culex tarsalis* (Diptera: Culicidae) in Weld County, Colorado, 2007. J. Med. Entomol. 46: 380-390.

Kershaw, W. E., M. M. Lavoipierre, and T. A. Chalmers. 1953. Studies on the intake of microfilariae by their insect vectors, their survival, and their effect on the survival of their vectors. I. *Dirofilaria immitis* and *Aëdes aegypti*. Ann. Trop. Med. Parasitol. 47: 207-224.

Kesavaraju, B., D. A. Yee, and S.A. Juliano. 2007. Interspecific and intraspecific differences in foraging preferences of container-dwelling mosquitoes. J. Med. Entomol. 44: 215-221.

Kilpatrick, A. M., P. Daszak, M. J. Jones, P. P. Marra and L. D. Kramer. 2006a. Host heterogeneity dominates West Nile virus transmission. Proc. Royal Soc. Biol. 273: 2327-2333.

Kilpatrick, A. M., L. D. Kramer, M. J. Jones, P. P. Marra, and P. Daszak. 2006b. West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior. PLoS Biol. 4: e82

Kilpatrick, A. M., S. L. LaDeau, and P. P. Marra. 2007. Ecology of West Nile virus transmission and its impact on birds in the western hemisphere. The Auk 124: 1121-1136.

Kim, S. K., and P. H. Adler. 2009. Digestive system of larval black flies (Diptera: Simuliidae): structure and phylogeny. Invertebr. Biol. 128: 182-204.

Kitron, U. D., D. W. Webb and R. J. Novak. 1989. Oviposition behavior of *Aedes triseriatus* (Diptera Culicidae) prevalence intensity and aggregation of Eggs in oviposition Traps. J. Med. Entomol. 26: 462-467.

Klowden, M. J., and A. O. Lea. 1979. Effect of defensive host behavior on the blood meal size and feeding success of natural populations of mosquitoes (Diptera: Culicidae). J. Med. Entomol. 15: 514-517.

Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Paabo, F. X. Villablanca, and A. C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86: 6196-6200.

Kreeger, T. J., U. S. Seal, M. Callahan, and M. Beckel. 1990. Treatment and prevention with ivermectin of dirofilariasis and ancyclostomiasis in captive gray wolves (*Canis lupus*). J. Zoo Wild. Med. 21: 310-317.

Lampman, R. L. and R. J. Novak. 1996. Oviposition preferences of *Culex pipiens* and *Culex restuans* for infusion-baited traps. J. Am. Mosq. Control Assoc. 12: 23-32.

Lanciotti, R.S., J. T. Roehrig, V. Deubel, J. Smith, M. Parker, K. Steele, B. Crise, K. E. Volpe, M. B. Crabtree, J. H. Scherret, R. A. Hall, J. S. MacKenzie, C. B. Cropp, B. Panigrahy, E. Ostlund, B. Schmitt, M. Malkinson, C. Banet, J. Weissman, N. Komar, H. M. Savage, W. Stone, T. McNamara, and D. J. Gubler. 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 286: 2333-2337.

Lardeux, F., R. Tejerina, C. Aliaga, R. Ursic-Bedoya, C. Lowenberger, and T. Chavez. 2008. Optimization of a semi-nested multiplex PCR to identify *Plasmodium* parasites in wild-caught *Anopheles* in Bolivia, and its application to field epidemiological studies. Trans. R. Soc. Trop. Med. Hyg. 102: 485-492.

Lee, R., and D. A. Craig. 1983. Maxillary mandibulary and hypopharyngeal stylets of female mosquitoes (Diptera: Culicidae) a scanning electron microscope study. Can. Entomol. 115: 1503-1512.

Le Menach, A, F. E. McKenzie, A. Flahault and D. L. Smith. 2005. The unexpected importance of mosquito oviposition behaviour for malaria: non-productive larval habitats can be sources for malaria transmission. Malaria Journal 4: 23

Lester, P. J., and A. J. Pike. 2003. Container surface area and water depth influence the population dynamics of the mosquito *Culex pervigilans* (Diptera: Culicidae) and its associated predators in New Zealand. J. Vector Ecol. 28: 267-274.

Licitra, B., E. W. Chambers, R. Kelly and T. R. Burkot. 2010. Detection of *Dirofilaria immitis* (Nematoda: Filarioidea) by polymerase chain reaction in *Aedes albopictus*, *Anopheles punctipennis*, and *Anopheles crucians* (Diptera: Culicidae) from Georgia, USA. J. Med. Entomol. 47: 634-638.

Lok, J. B. 1988. Dirofilaria sp.: taxonomy and distribution, pp. 1-28. In P. F. L. Boreham and R. B. Atwell (eds.), Dirofilariasis. CRC Press, Boca Raton, FL, USA.

Ludwig, G. V., P. P. Calle, J. A. Mangiafico, B. L. Raphael, D. K. Danner, J. A. Hile, T. L. Clippinger, J. F. Smith, R. A. Cook, and T. McNamara. 2002. An outbreak of West Nile virus in a New York City captive wildlife population. Am. J. Trop. Med, Hyg. 67: 67-75.

Lyimo, I. N., and H. M. Ferguson. 2009. Ecological and evolutionary determinants of host species choice in mosquito vectors. Trends in Parasit. 25: 189-196.

Macdonald, W. W., and C. P. Ramachandran. 1965. The influence of the gene fm (filarial susceptibility, *Brugia malayi*) on the susceptibility of *Aedes aegypti* to seven strains of *Brugia*, *Wuchereria* and *Dirofilaria*. Ann. Trop. Med. Parasitol. 59: 64-73.

Mackay, A. J. 2007. Detection of West Nile virus activity in male and female mosquitoes, and evaluation of host-utilization patterns of mosquitoes, in east Baton Rouge parish, Louisiana. Ph.D. Dissertation. Louisiana State University, Baton Rouge, LA, USA. 185 pp.

Malmqvist, B., D. Strasevicius, O. Hellgren, P. H. Adler, and S. Bensch. 2003. Vertebrate host specificity of wild-caught blackflies revealed by mitochondrial DNA in blood. Proc. R. Soc. B (Suppl.) 271: 152-155.

Matsuda, K., B. Baek, C. Lim, and B. Back. 2003. Eurasian otter (*Lutra lutra*), a definitive host for *Dirofilaria immitis*. J. Zoo Wild. Med. 34: 200-201.

McNamara ,T. 2007. The role of zoos in biosurveillance. *Int. Zoo Yearb.* 41:12-15.

Mighthew, P. J. 2001. Herbivore host choice and optimal bad motherhood. Trends Ecol. Evol. 16: 165-167.

McConkey, G. A., J. Li, M. J. Rogers, D. C. Seeley, T. K. Graczyk, M. R. Cranfield, and T. F. McCutchan. 1996. Parasite diversity in an endemic region for avian malaria and identification of a parasite causing penguin mortality. J. Eukaryot. Microbiol. 43: 393-399.

McCreadie, J. W., and P. H. Adler. 1999. Parasites of larval black flies (Diptera: Simuliidae and environmental factors associated with their distributions. Invert. Biol. 118: 310-318.

McCreadie, J. W., and C. E. Beard. 2003. The microdistribution of the trichomycete *Smittium culisetae* in the hindgut of the black fly host *Simulium vittatum*. Mycologia 95: 998-1003.

McGreevy, P. B., J. H. Bryan, P. Oothuman, N. Kolstrup. 1978. The lethal effects of the cibarial and pharyngeal armature of mosquitoes on microfilariae. Trans. R. Soc. Trop. Med. Hyg. 72: 361-368.

McNamara, T. 2007. The role of zoos in biosurveillance. Int. Zoo Yearb. 41: 12-15.

Means, R.G. 1968. Host Preferences of Mosquitoes (Diptera - Culicidae) in Suffolk County New York. Ann. Entomol. Soc. Am. 61: 116-120.

Mercer, D. R., S. L. Sheeley, and E. J. Brown. 2005. Mosquito (Diptera: Culicidae) development within microhabitats of an Iowa wetland. J. Med. Entomol. 42: 685-693.

Merritt, R. W., R. H. Dadd, and E. D. Walker. 1992. Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. Ann. Rev. Entomol. 37: 349-374.

Minakawa, N., P. Seda and G. Y. Yan. 2002. Influence of host and larval habitat distribution on the abundance of African malaria vectors in western Kenya. Am. J. Trop. Med. Hyg. 67: 32-38.

Moore, C. G. 1999. *Aedes albopictus* in the United States: current status and prospects for further spread. J. Am. Mosq. Cont. Assoc. 15: 221-227.

Molaei, G., T. G. Andreadis, P. M. Armstrong, J. F. Anderson, and C. R. Vossbrinck. 2006. Host feeding patterns of *Culex* mosquitoes and West Nile virus transmission, northeastern United States. Emerg. Infect. Dis. 12: 468-474.

Molaei, G., T. G. Andreadis, P. M. Armstrong, and M. Diuk-Wasser. 2008. Host-feedng patterns of potential mosquito vectors in Connecticut, USA: Molecular analysis of bloodmeals from 23 species of *Aedes, Anopheles, Culex, Coquilletidia, Psorophora,* and *Uranotaenia*. J. Med. Entomol. 45: 1143-1151.

Molaei, G., A. Farajallohi, P. M. Armstrong, J. Oliver, J. J. Howard, and T. G. Andreadis. 2009. Identification of bloodmeals in *An. quadrimaculatus* and *An. punctipennis* from Eastern Equine Encephalitis virus foci in northeastern U.S.A. Med. Vet. Entomol. 23: 350-356.

Moore, C. G., D. B. Francy, D. A. Eliason, and T. P. Monath. 1988. *Aedes albopictus* in the United States: rapid spread of a potential disease vector. J. Am. Mosq. Cont. Assoc. 4: 356-361.

Mukabana, W. R., W. Takken and B. G. J. Knols. 2002. Analysis of arthropod bloodmeals using molecular genetic markers. Trends Parasitol. 18: 505-509.

Munga, S., N. Minakawa, G. Zhou, E. Mushinzimana, O. J. Barrack, A. K. Githeko, and G. Yan. 2006. Association between land cover and habitat productivity of malaria vectors in western Kenyan highlands. Am. J. Trop. Med. Hyg. 74: 69-75.

Murata, K., T. Yanai, T. Agatsuma, and S. Uni. 2003. *Dirofilaria immitis* infection of a Snow leopard (*Uncia uncia*) in a Japanese zoo with mitochondrial DNA analysis. J. Vet. Med. Sci. 65: 945-947.

Muturi, E. J., J. Mwangangi, J. Shililu, S. Muriu, B. Jacob, E. Kabiru, W. Gu, C. Mbogo, J. Githure, and R. Novak. 2007. Mosquito species succession and physicochemical

factors affecting their abundance in rice fields in Mwea, Kenya. J. Med. Entomol. 44: 336-344.

Neary, J. M., A. J. Trees, D. D. Ekale, V. N. Tanya, U. Hetzel, and B. L. Makepeace. *Onchocerca armillata* contains the endosymbiotic bacterium *Wolbachia* and elicits a limited inflammatory response. Vet. Parasitol. 174: 267-276.

Neiffer, D. L., E. C. Klein, P. P. Calle, M. Linn, S. P. Terrell, R. L. Walker, D. Todd, C. C. Vice, and S. K. Marks. 2002. Mortality associated with melarsomine dihydrochloride administration in two North American river otters (*Lontra canadensis*) and a red panda (*Ailurus fulgens fulgens*). J. Zoo Wild. Med. 33: 242-248.

Nelder, M. 2007. Arthropods at the interface of exotic and native wildlife: A multifaceted approach to medical and veterinary entomology in the zoos of South Carolina. Ph. D Dissertation. Clemson University, Clemson, SC, USA. 264 pp.

Ngo K. A., Kramer L. D. (2003) Identification of mosquito bloodmeals using polymerase chain reaction (PCR) with order-specific primers. J. Med. Entomol. 40: 215-222.

Novak, R. J., and J. J. Peloquin. 1981. Substrate modification for the oviposition trap used for detecting the presence of *Aedes triseriatus*. Mosq. News 41: 180-181.

O'Neill, S. L., R. Giordano, A. M. E. Colbert, T. L. Karr, and H. M. Robertson. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmatic incompatibility in insects. Proc.Natl. Acad. Sci. USA 89: 2699-2702.

Paradise, C. J., and W. A. Dunson. 1997. Effects of water cations on tree hole insect communities. Ann.Entomol. Soc. Am. 90: 798-805.

Paradise, C. J. 2000 Effects of pH and resources on a processing chain interaction in simulated tree holes. J. Anim. Ecol. 69: 651-658.
Perrone, J. B., and A. Spielman. 1986. Microfilarial perforation of the midgut of a mosquito. J. Parasitol. 72: 723-727.

Pombi, M., C. Costantini, and A. della Torre. 2003. *Aedes albopictus* (Diptera: Culicidae) in Rome: experimental analysis of the relevant parameters in control strategies. Parassitologia (Rome) 45: 97-102.

Ratliff R.D. 1982. A correction of Cole's C(7) and Hurlbert's C(8) coefficients of interspecific association. Ecology 63: 1605–1606.

Reddy, M. R., T. J. Lepore, R. J. Pollack, A. E. Kiszewski, A. Spielman, and P. Reiter. 2007. Early evening questing and oviposition activity by the *Culex* (Diptera : Culicidae)

vectors of West Nile virus in northeastern North America. J. Med. Entomol. 44: 211-214.

Reisen, W. K. 2009. Epidemiology of vector-borne diseases, pp. 19-35. In G. R. Mullen and L. A. Durden (eds.), Medical and Veterinary Entomology, 2nd ed. Elsevier, Burlington, MA, USA.

Reiskind, M. H. and M. L. Wilson. 2004. *Culex restuans* (Diptera: Culicidae) oviposition behavior determined by larval habitat quality and quantity in southeastern Michigan. J. Med. Entomol. 41: 179-186.
Reiter. P. 2007. Oviposition, dispersal, and survival in *Aedes aegypti*: implications for the efficacy of control strategies. Vector Borne Zoonotic Dis. 7: 261–273.

Reiter, P. 2008. Climate change and mosquito-borne disease. Env. Health Persp. 109: 141-161. Suppl.1.

Rejmankova, E., H. M. Savage, M. Rejmanek, J. I. Arrendondo-Jiminez, and D. R. Roberts. 1991. Multivariate analysis of relationships between habitats, environmental factors and occurrence of anopheline mosquito larvae *Anopheles albimanus* and *A.pseudopunctipennis* in southern Chiapas, Mexico. J. Appl. Ecol. 28: 827-841.

Rejmankova, E., R. Higashi, J. Grieco, N. Achee, and D. Roberts. 2005. Volatile substances from larval habitats mediate species-specific oviposition in *Anopheles* mosquitoes. J. Med. Entomol. 42: 95-103.

Rey, J. R., N. Nishimura, B. Wagner, M. A. H. Braks, S. M. O'Connell and L. P. Lounibos. 2006. Habitat segregation of mosquito arbovirus vectors in south Florida. J. Med. Entomol. 43: 1134-1141.

Richins, C. A. 1938. The metamorphosis of the digestive tract of Aedes dorsalis Meigen. Ann. Entomol. Soc. Am. 31: 69-73.

Rishniw M., S. C. Barr, K. W. Simpson, M. F. Frongillo, M. Franz, and J. L. Dominguez-Alpizar. 2006. Discrimination between six species of canine microfilariae by a single polymerase chain reaction. Vet. Parasitol. 135: 303–314.

Roberts, L. S., and J. Janovy Jr. 2005. Foundations of Parasitology 7th ed. McGraw-Hill, New York, NY, USA.

Rodriguez-Prieto, I., E. Fernandez-Juricic, and J. Martin. 2006. Anti-predator behavioral responses of mosquito pupae to aerial predation risk. J. Insect Behav. 19: 373-381.

Romoser, W. S., and E. Cody. 1975. The formation and fate of the peritrophic matrix in adult *Culex nigripalpus* (Diptera: Culicidae). J. Med. Entomol. 12: 371-378.

Romoser, W. S., R. M. Moll, A. C. Moncayo, and K. Lerdthusnee. 2000. The occurrence and fate of the meconium and meconial peritrophic matrixes in pupal and adult mosquitoes (Diptera: Culicidae). J. Med. Entomol. 37: 893-896.

Rossignol, P. A., J. M. C. Ribeiro, M. Jungery, M. J. Turell, A. Spielman, and C. L. Bailey. 1985. Enhanced mosquito blood-finding success on parasitemic hosts: evidence for vector-parasite mutualism. Proc. Natl. Acad. Sci. USA 82: 7725-7727.

Russell, R. C. and M. J. Geary. 1992. The susceptibility of the mosquitoes *Aedes notoscriptus* and *Culex annulirostris* to infection with dog heartworm *Dirofilaria immitis* and their vector efficiency. Med. Vet. Entomol. 6: 154-158.

Sall, J., L. Creighton, and A. Lehman. 2007. JMP start statistics: a guide to statistics and data analysis using JMP. 4th ed. SAS Institute Inc., Cary, NC, USA.

Sano, Y., M. Aoki, H. Takahashi, M. Miura, M. Komatsu, Y. Abe, J. Kakino, and T. Itagaki. 2005. The first record of *Dirofilaria immitis* infection in a Humboldt penguin, *Spheniscus humboldti*. J. Parasitol. 91: 1235-1237.

Schneider, J. R., A. C. Morrison, H. Astete, T. W. Scott, and M. L. Wilson. 2004. Adult size and distribution of *Aedes aegypti* (Diptera: Culicidae) associated with larval habitats in Iquitos, Peru. J. Med. Entomol. 41: 634-642.

Sella M. 1920. Parte II, p. 188. In B. Grassi (ed.), Lotta antimalarica a Fiumicino (Roma): Seconda relazione. Ministero Dell'interno, Rome, Italy.

Senior-White, R. 1926. Physical factors in mosquito ecology. Bull. Entomol. Res. 16: 187-248.

Service, M. W. 1978. A short history of early medical entomology. J. Med. Entomol. 14: 603-626.

Service, M. W. 1997. Mosquito (Diptera: Culicidae) Dispersal — The long and short of it. J. Med. Entomol. 34: 579-588.

Shaman, J., M. Stieglitz, C. Stark, S. Le Blancq, and M. Cane. 2002. Using a dynamic hydrology model to predict mosquito abundances in flood and swamp water. Emerg. Infect. Dis. 8: 6-13.

Shimonski, J. 2009. An experiment in arboriculture and mosquito control. City Trees 45: 6-10.

Shimonski J. 2010. Integrated pest management in a zoological theme park. Wing Beats (Spring): 37–41.

Shoukry, A., and B. A. Soliman. 1995. The ultrastructure of the foregut and its influence on bancroftian microfilariae ingestion in three Egyptian mosquito species. J. Egypt Soc. Parasitol. 25: 367-375.

Silver, J. B. 2008. Mosquito ecology - Field Sampling Methods. Springer, The Netherlands.

Smith, D. L., J. Dushoff, and F. E. McKenzie. 2004. The risk of a mosquito-borne infection in a heterogeneous environment. PLoS Biol. 2: 1957-1964.
Snodgrass, R. E. 1959. The anatomical life of the mosquito. Smithsonian Institution, Washington D.C., USA.

Snyder, G. K., and B. A. Sheafor. 1999. Red blood cells: centerpiece in the evolution of the vertebrate circulatory system. Amer. Zool. 39: 189-198.

Somboon, P., K. Yamniam, and C. Walton. 2009. Scanning electron microscopy of the cibarial armature of species in the Anopheles dirus complex (Diptera: Culicidae). Southeast Asian J. Trop. Med. Public Health. 40: 937-941.

Sota, T., M. Mogi, and E. Hayamizu. 1992. Seasonal Distribution and Habitat Selection by *Aedes albopictus* and *Aedes riversi* (Diptera, Culicidae) in Northern Kyushu, Japan. J. Med. Entomol. 29: 296-304.

Spencer, M., L. Blaustein, and J. E. Cohen. 2002. Oviposition habitat selection by mosquitoes (*Culiseta longiareolata*) and consequences for population size. Ecology 83: 669-679.

Strand, M., D. A. Herms, M. P. Ayres, M. E. Kubiske, M. G. Kaufman, E. D. Walker, K. S. Pregitzer, and R. W. Merritt. 1999. Effects of atmospheric CO_2, light availability and tree species on the quality of leaf detritus as a resource for treehole mosquitoes. Oikos 84: 277-283.

Sulaiman, I., and H. Townson. 1980. The genetic basis of susceptibility to infection with *Dirofilaria immitis* in *Aedes aegypti*. Ann. Trop. Med. Parasitol. 74: 635-646.

Sunahara, T., K. Ishizaka, and M. Mogi. 2002. Habitat size: a factor determining the opportunity for encounters between mosquito larvae and aquatic predators. J. Vector Ecol. 27: 8-20.

Talluri, M. V., E. Bigliardi, and G. Cancrini. 1993. Comparative ultrastructural study of *Dirofilaria repens* (Nematoda: Filarioidea) development in susceptible and refractory strains of *Aedes aegypti*. Ital. J. Zool. 60: 377-383.

Thompson, M. T. 1905. Alimentary canal of the mosquito. Proc. Boston Soc. Nat. Hist. 32: 145-202.

Torres-Estrada, J. J., R. A. Mesa-Alvarez, J. Cibrian-Tovar, M. H. Rodriguez, J. I. Arredondo-Jimenez, L. Cruz-Lopez, and J. C Rojas-Leon. 2005. Vegetation-derived cues for the selection of oviposition substrates by *Anopheles albimanus* under laboratory conditions. J. Am. Mosq. Control Assoc. 21: 344-349.

Trembley, H. L. 1951. Pyloric spines in mosquitoes. J. Natl. Mal. Soc. 10: 213-215.

Trexler, J. D., C. S. Apperson, and C. Schal. 1998. Laboratory and field evaluations of oviposition responses of *Aedes albopictus* and *Aedes triseriatus* (Diptera: Culicidae) to oak leaf infusions. J. Med. Entomol. 35: 967-976.

Tuten, H.C. 2011. Habitat characteristics of larval mosquitoes in zoos of South Carolina, USA. J. Am. Mosq. Control Assoc. 27: 111-119.

Udevitz, M. S., P. Bloomfield, and C. S. Apperson. 1987. Prediction of the occurrence of 4 species of mosquito larvae with logistic regression on water chemistry variables. Environ. Entomol. 16: 281-285.

Unnasch, R.S., E. W. Cupp, and Unnasch T. R. 2006. Host selection and its role in transmission of arboviral encephalitides, pp. 73-89. In S.K. Collinge and C. Ray (eds.), Disease Ecology. Oxford University Press, New York, USA.

Vanwambeke, S. O., P. Somboon, R. E. Harbach, M. Isenstadt, E. F. Lambin, C. Walton, and R. K. Butlin. 2007. Landscape and land cover factors influence the presence of *Aedes* and *Anopheles* larvae. J. Med. Entomol. 44: 133-144.

Vaughan, J. A., B. H. Noden, and J. C. Beier. 1991. Concentration of human erythrocytes by anopheline mosquitoes (Diptera: Culicidae) during feeding. J. Med. Entomol. 28: 780-786.

WHO. World Health Organization. 1975. Manual on practical entomology in malaria. Part II. Methods and techniques. World Health Organization, Geneva, Switzerland.

Walters, L. L., G. B. Modi, R. B. Tesh, and T. Burrage. 1987. Host-parasite relationship of *Leishmania mexicana mexicana* and *Lutzomyia abonnenci* (Diptera: Psychodidae). Am. J. Trop. Med. Hyg. 36: 294-314.

Warburg, A. 2008. The structure of the female sand fly (*Phlebotomus papatasi*) alimentary canal. Trans. R. Soc. Trop. Med. Hyg. 102: 161-166.

Washino, R. K., and C. H. Tempelis. 1983. Mosquito host bloodmeal identification: methodology and data analysis. Ann. Rev. Entomol. 28: 179-201.

Watts, S. L., D. M. Fitzpatrick, and J. E. Maruniak. 2009. Blood Meal Identification from Florida Mosquitoes (Diptera: Culicidae). Fla. Entomol. 92: 619-622.

White, G. L. 1975. *Dirofilaria immitis* and heartworm disease in the California sea lion. J. Zoo Anim. Med. 6: 23-24.

Wigglesworth, V. B. 1950. The principles of insect physiology. 4th ed. Methuen & Co. Ltd., London, UK.

Williams, J. F., and A. W. Dade. 1976. *Dirofilaria immitis* infection in a wolverine. J. Parasitol. 62: 174-175.

Williams, D. D., S. E. MacKay, R. C. M. Verdonschot, and P. J. P. Tacchino. 2007. Natural and manipulated populations of the treehole mosquito, *Ochlerotatus triseriatus*, at its northernmost range limit in southern Ontario, Canada. J. Vector Ecol. 32: 328-335.

Wynn, G., and C.J. Paradise. 2001. Effects of microcosm scaling and food resources on growth and survival of larval *Culex pipiens*. BMC Ecology 1: 3.

Wozniak, A., H. E. Dowda, M. W. Tolson, N. Karabatsos, D. R. Vaughan, P. E. Turner, D. I. Ortiz, and W. Wills. 2001. Arbovirus surveillance in South Carolina, 1996 – 1998. J. Am. Mosq. Cont. Assoc. 17: 73-78.

Yee, D. A., B. Kesavaraju, and S. A. Juliano. 2007. Direct and indirect effects of animal detritus on growth, survival, and mass of invasive container mosquito *Aedes albopictus* (Diptera: Culicidae). J. Med. Entomol. 44: 580-588.

Yee, D. A., and S. H. Yee. 2007. Nestedness patterns of container-dwelling mosquitoes: effects of larval habitat within variable terrestrial matrices. Hydrobiologia 592: 373-385.