FACTORS CONTRIBUTING TO THE HOST SPECIFICITY OF THE FROG-FEEDING MOSQUITO *CULEX TERRITANS* WALKER (DIPTERA: CULICIDAE)

by

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A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of Doctor of Philosophy

Graduate Program in Entomology

Written under the direction of

Dr. Randy Gaugler

And approved by

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New Brunswick, New Jersey

[January, 2009]

ABSTRACT OF DISSERTATION

Factors contributing to the host specificity of the frog-feeding mosquito *Culex territans* Walker (Diptera: Culicidae)

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We tested the hypothesis that *Culex territans* uses amphibian vocalizations as a long distance attractant. Two thirds of females oriented toward sound across all experiments. Females exhibited positive phonotaxis to frog calls, including those of *P. crucifer*, *Hyla versicolor* (northern gray tree frog), *Bufo americanus* (American toad), and *R. clamitans* (green frog). Multiple regression analysis showed that call frequency is the best predictor for phonotaxis, with pulse duration and call amplitude increasing the attractiveness of the source. Females oriented to calls in the range of 50 to 75 dB, with particle velocities of 0.02 to 0.3 mm/s, indicating that phonotaxis occurs at distances greater than 5 m from the source.

To examine synchrony of *Cx. territans* with amphibian species, ten larval habitat sites were sampled weekly from March to November of 2004. *Culex territans* larvae were temporally and spatially associated with the green frog, *Rana clamitans* Latrielle. Using the thermal heat summation model, 192.3 days above 3.9°C were required to complete the gonotrophic cycle. This is the lowest thermal minimum reported for a

Nearctic species of mosquito. Using this model, we calculated that the first larvae of *Cx. territans* field- collected on 6 May 2004 were the progeny of females which bloodfed during the last week of March or first week of April.

The bloodmeals of field-collected female *Culex territans* (Diptera: Culicidae) were concurrently assayed for the presence of trypanosomes and for vertebrate host identification. We amplified vertebrate DNA in 42 of 119 females, and made positive identification to the host species level in 29 of those samples. Of the 119 field-collected *Cx. territans* females, 24 were infected with trypanosomes. Phylogenetic analysis placed the trypanosomes in the amphibian portion of the aquatic clade of the Trypanosomatidae. These trypanosomes were isolated from *Cx. territans* females that had fed on the frog species, *Rana clamitans, R. catesbeiana, R. virgatipes,* and *R.* spp. Results support an unknown lineage of dipteran transmitted amphibian trypanosomes occur within the aquatic clade.

Acknowledgements

I would like to thank my committee members, Randy Gaugler, Wayne Crans, Frank Carle, and Joan Ehrenfeld for their guidance and support. I would especially like to thank Wayne Crans for teaching me about mosquitoes, trees, catching and bleeding frogs, and how to peep like a spring peeper.

I would like to thank my fellow graduate students and friends in various departments that I have met along the way, Priscilla Collins, Ary Farajollahi, Jennifer Gruener, Ayelet Klartag, Tadhgh Rainey, Scott Crans, Dana Price, Eric Williges, Jeremy Feinberg, Elena Tartaglia, Alicia Buchanan, Holly Vuong, and Diana Carle.

I would like to thank the 21 county mosquito agencies throughout New Jersey, and the New Jersey Mosquito Control Association and Northeastern Mosquito Control Association for financial support. I would especially like to thank Warren Staudenger of Bergen County Mosquito, Jennifer Gruener, Jeff Riker, Don Gunther, and John Necina of Sussex County Mosquito, Sara May of Warren County Mosquito, Sean Healy of Monmouth County Mosquito, and Ary Farajollahi of Mercer County Mosquito for going above and beyond to help in the collection of *Culex territans.*

I would like to thank Linda McCuiston for always sharing her knowledge on mosquito rearing techniques, mosquito identification, forced mating, and other areas of mosquito biology.

I would like to thank Lisa Reed for statistical guidance, as well as being an eager frog catcher. I would also like to thank Scott Crans, Sean Healy and Wayne Crans for their assistance with catching and bleeding frogs. I would like to thank my dog Bucky,

for being a willing field assistant for the price of a hamburger. I would like to thank Rose Puelle and Vivian Roegner for expanding my knowledge of molecular techniques.

I would like to thank several Post Docs in the department, Banu Kesavaraju, Mark Nelder, and Emily Cameron for their willingness to edit manuscripts, provide statistical guidance, and exchange ideas. I would also like to thank Henry Rupp for providing his expertise in grammar and editing. I promise to never say “Mosquito Breeding”.

I would like to thank the Entomology support staff, Carol Terry, Nancy Lyon, Diane Nale, Susan Puckett, and Marie Helmond for making my life easier. I would also like to thank the General Biology staff, Diana Martin, Chris Brey, Bruce Mohn, and Jennifer Dan for providing me with an enjoyable opportunity.

I would like to thank my mom for teaching me independence and strength, especially during hard times. I would like to thank my sister for being a great friend and driving me to be better. I would also like to thank those that I have loved that have passed on, especially my brother and father. It is because of my brother’s strength and desire to be more, when times were the hardest, that I will never give up in life.

Lastly, I would like to thank my husband Sean for teaching me to believe in myself. You make me want to reach for the stars. I am truly blessed to have you and all these wonderful people in my life.

Dedication

To my best friend and love of my life, Sean

To my mother

and

To my sister Kim

Thank you for showing me that anything is possible

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Rationale

Over the past three decades, amphibian decline has become a serious global issue. Potentially, 122 amphibian species have become extinct, and 32.5% of the 5743 described species are currently threatened (Mendelson et al. 2006). Factors such as disease, climate change, habitat loss, environmental factors, and invasive species have contributed to the decrease in amphibian species (Mendelson et al. 2006). Emerging infectious diseases in amphibians, such as ranaviruses and chytridiomycosis, are partly responsible for amphibian population declines and extinctions on a global scale (Mazzoni et al. 2003). These drastic declines of amphibian species and diversity underscore a need for a better understanding of amphibian stressors.

Culex territans Walker (Diptera: Culicidae) ingests bloodmeals from amphibian hosts (Crans 1970), and transmits parasites and pathogens to amphibians (Benach and Crans 1973). Several of these parasites and pathogens include *Foleyella flexicauda* Schacher and Crans (Benach and Crans 1973), frog erythrocytic ranavirus (Gruia-Gray and Desser 1992), and *Hepatozoon clamatae* Stebbins (Kim et al. 1998). *Culex territans* has also tested positive for West Nile virus in the United States (CDC 2005). High parasitic burdens not only cause disease in amphibians, but also stress animal populations making them more susceptible to other afflictions (Bustnes et al. 2006). The importance of disease in the decline of amphibian populations stresses a need for understanding potential vectors to amphibians.

Culex territans may threaten declining amphibian populations by competing for available food and habitats. Mosquitoes and amphibians are dependant on water for their development, and share common habitats, such as vernal pools, permanent swamps, and

temporary flooded habitats. Within these habitats, both mosquito larvae and tadpoles are using the same food sources (Mokany and Shine 2003). These populations often overlap due to suitable temperature and precipitation. In situations where both mosquitoes and tadpoles are living within a temporary habitat, there may be tremendous amount of pressure to develop quickly before the habitat dries down. In addition, competition for resources may slow the developmental rate for many species of tadpoles, affecting the ability to mature before the habitat becomes dry. Loss of valuable habitats emphasizes a need to understand the interactions between *Cx. territans* and amphibian populations.

The purpose of my study is to examine the relationship between amphibians and mosquitoes by examining the factors contributing towards the host specificity of *Cx. territans*. This research encompasses a wide range of associations, including temporal and spatial synchrony, host location, host feeding preference, and disease prevalence. By understanding the relationships between amphibians and mosquitoes, we might be able to alleviate potential stressors to threatened amphibians, develop better mosquito control strategies that minimize the impacts on amphibian populations, and understand the role of *Culex territans* in transmission of mosquito-borne diseases to amphibians. This information is particularly useful in conservation of threatened and endangered amphibians in New Jersey.

Objectives

Culex territans has often been called the frog-feeding mosquito. The objective of my research is to determine the factors contributing to host specificity of *Culex territans*. This objective includes the following hypotheses.

1. *Culex territans* temporally and spatially synchronizes its lifecycle to match the lifecycle of its amphibian hosts.

2. *Culex territans* prefers amphibian bloodmeals to other classes of vertebrates.

3. *Culex territans* utilizes host-specific cues to locate amphibian blood-meals.

4. *Culex territans* is capable of digesting bloodmeals at temperatures close to freezing, corresponding to when amphibians in New Jersey are exiting diapause.

5. *Culex territans* readily acquire amphibian trypanosomes during bloodfeeding.

Introduction

Biology of *Culex territans*

Culex territans Walker (Diptera: Culicidae) has often been considered a scientific curiosity, because it has never been implicated as a vector to humans. As a result, few studies have been conducted to examine the biology of this species. *Culex territans* belongs to the subgenus, *Neoculex*, which includes 26 of the 796 described species of *Culex* worldwide (Walter Reed Biosystematics Unit 2001). Within the subgenus, *Cx. territans* is the most widely distributed, being found throughout the northern hemisphere, in North America, Europe, and Asia (Walter Reed Biosystematics Unit 2001). There are 6 species of *Neoculex* that occur within North and Central America (*Cx. territans, Cx. boharti* Brookman and Reeves, *Cx. reevesi* Wirth, *Cx. apicalis* Adams, *Cx. arizonensis* Bohart and *Cx. derivator* Dyar and Knab), all of which prefer amphibian and reptilian bloodmeals (Linam and Nielson 1970). Of the 6 species that occur in the Nearctic and Neotropical regions, *Cx. territans* has the widest altitudinal and longitudinal distribution, and occurs in the greatest variety of habitats (Linam and Nielson 1970). *Culex territans* has also been reported to withstand temperatures as low as -27 °C in nature (Berg and Lang 1948). The ability of *Cx. territans* to survive in a variety of habitats and environmental conditions may explain its wide geographic distribution in the northern hemisphere.

The subgenus *Neoculex* is believed to have evolved in the Old World (Linam and Nielson 1970), where the majority of species are known to occur. The New World species of *Neoculex*, except *Cx. territans*, are believed to have evolved from an ancestor that arrived from Asia from across the Bering Sea (Ross 1964). *Culex territans* likely

evolved in Eurasia (Ross 1964), and arrived in the New World from the North during a later period, where it spread across Canada and into the United States (Linam and Nielson 1970).

There are currently seven described species of *Culex* that occur in New Jersey, including *Culex (Culex) pipiens pipiens* Linnaeus, *Cx. (Cx.) pipiens molestus* Forskal, *Cx. (Cx.) restuans* Theobald, *Cx. (Cx.) salinarius* Coquillett, *Cx. (Cx.) tarsalis* Coquillett, *Cx. (Melanoconion) erraticus* (Dyar and Knab), *and Cx. (Neoculex) territans* Walker (Crans 2004). *Culex territans* is easily identifiable as an adult by having apical abdominal bands, as opposed to basal bands that occur in the remaining *Culex* (Andreadis et al. 2005).

Culex territans is often called the frog-feeding mosquito, due to the preference for amphibian bloodmeals. Using group antisera tests, Crans (1970) showed that *Cx. territans* preferred amphibians to other groups of vertebrates. Savage et al. (2007) found *Cx. territans* feeding primarily on amphibians, and occasionally reptiles, aves, and mammals. *Culex territans* are primarily crepuscular (McIver 1969) and nocturnal bloodfeeders (Bosak 1998), but may be active during daylight hours (Benach 1970). Frohne and Frohne (1954) found diurnal swarms of *Culex territans* occurred primarily during late afternoon.

Culex territans overwinter as adult females, and exit diapause in the early spring. *Culex territans* adult females have been collected overwintering in New York (Nasci et al. 2001) and New Jersey (Farajollahi 2005). Collections of larvae in the spring (Smith 1903) are consistent with the report that females exit diapause in New Jersey in March. Many amphibian species in New Jersey frequently exit diapause throughout March

(Gessner and Stiles 2001). At this time, female *Cx. territans* will seek a bloodmeal, and oviposit their first batch of eggs. *Culex territans* eggs are generally laid above the water line on emergent vegetation (McIver 1969). Subsequent populations will continue to increase in abundance throughout the season, with the greatest numbers occurring before the first frost in the fall.

Amphibians are frequently used as indicators of water quality (Boyer and Grue 1995), because they are sensitive to toxicants and acidic conditions (Freda and Taylor 1992). Therefore amphibians are restricted to habitats that are non-polluted. *Culex territans* are often collected in non-polluted water including permanent swamps, marshes, ponds, streams, containers, and large wetland pools (Joy and Clay 2002), and share habitats with amphibian species (Benach 1971). Mosquito larvae that share habitats with amphibian tadpoles often compete for limited resources (Mokany and Shine 2003).

Gjullin et al. (1961) found *Cx. territans* larvae commonly occurred in a variety of habitats containing amphibians, and were abundant in habitats regardless of insect predators. The ability of *Cx. territans* larvae to utilize a variety of habitats increases the likelihood they will interact with amphibians. Several researchers have examined relationships between insects and amphibians (Morin et al. 1988; Morin 1983; Blaustein and Margalit 1994; Blaustein and Margalit 1996; McLachlan 1985; Mokany and Shine 2003; and Mokany and Shine 2002). Morin et al. (1988) examined competition between aquatic insects and tadpoles of *Hyla andersoni* and *Bufo woodhousi fowleri.* The authors showed that insects and tadpoles would decrease the total biomass of *H. andersoni*, due to competition for important resources, such as periphyton (Morin et al. 1988).

It is likely that mosquitoes and tadpoles compete for resources, because they share a similar habitat, and feed on a wide range of organic matter, including detritus, algae, bacteria, and protozoans (Mokany and Shine 2003). Mokany and Shine (2003) performed gut content analysis on tadpoles and mosquito larvae in Australia and found a high dietary overlap, with both feeding predominantly on algae and bacteria. The authors examined competition between mosquito larvae of *Culex quinquefasciatus* and the striped marsh frog, *Limnodynastes peronii;* and between the mosquito larvae *Oc. australis* and the common Eastern toadlet *Crinia signifera*. The authors found that tadpoles in both systems grew more rapidly when given more food, and less rapidly when combined with mosquito larvae (Mokany and Shine 2003).

Blaustein and Margalit (1994 and 1996) examined the relationship between the mosquito *Culiseta longiareolata* and the green toad *Bufo viridis,* which co-exist within temporary pools in Israel. Blaustein and Margalit (1994) found that both species were competing for resources, and later instars of *Cs. longiareolata* were preying on *B. viridis* tadpoles (Blaustein and Margalit 1994). The authors also found a direct correlation between tadpole size and development time with density of organisms within the temporary pool (Blaustein and Margalit 1996). These density-dependant effects may be a result of an abundance of tadpoles, or an abundance of other organisms with the tadpoles (Blaustein and Margalit 1996). This data suggests that mosquitoes and amphibians are more likely to be competing for resources in areas that are smaller in size, such as a temporary pool, or where there are limited resources. Because the woodfrog, *Rana sylvatica*, depends on vernal pools, it is most likely competing with mosquito larvae for important food resources.

Mosquito biologists have long been concerned about safely controlling mosquitoes, while minimizing impacts on humans or the environment. One concern is whether or not water management projects have eliminated habitats for amphibians. One of the greatest impacts on amphibian declines is loss of habitat (Mendelson et al. 2006). Loss of suitable amphibian habitat may be the result of urbanization, clearcutting forests, logging, decreasing habitat complexity, changing vegetation, draining wetlands, and habitat modification (Alford and Richards 1999). Water management practices in the early 1900's to control mosquitoes did not take into consideration loss of amphibian habitat, and were often deleterious to wildlife (Mitsch and Gosselink 2000). However, as understanding of the values of wetlands to the environment and water quality increases, the impacts to the flora and fauna are taken into consideration in the management of wetlands to reduce mosquitoes (Knight et al. 2003). Current wetland management strategies for reducing mosquito larvae are designed to enhance wetlands in order to increase animal diversity, by attracting wildlife (Meredith and Lesser 2007).

Mosquito fish, *Gambusia holbrooki*, are commonly used to biologically control mosquito larvae, but often decimate amphibian populations by feeding on the tadpoles (Hamer et al. 2002). Several papers have examined the effects of mosquito fish on amphibian populations (Hamer et al. 2002; Goodsell and Kats 1999; and Lawler et al. 1999). Goodsell and Kats (1999) examined the effect of mosquito fish on tree frogs in California, and found the mosquito fish preyed heavily on treefrog tadpoles, even when mosquito larvae were abundant (Goodsell and Kats 1999). Studies by Lawler et al. (1999) showed mosquito fish prefer prey other than tadpoles, but readily feed on tadpoles of the red-legged frog, *Rana aurora*. Lawler suggested that predation on the frogs may

be reduced if additional prey is added to ponds along with the mosquito fish (Lawler et al. 1999).

Mosquito fish have reduced the suitability of permanent water habitats as breeding sites for many amphibian species (Hamer et al. 2000). Hamer et al. (2002) examined the effect of mosquito fish on the endangered golden bell frog, *Liroria aurea*, which inhabits permanent ponds, swamps, and Lagoons in Australia. The mosquito fish are widespread in permanent habitats in Australia, and cannot live in temporary habitats (Hamer et al. 2000). Hamer et al. (2000) noted that *L. aurea* have switched from a permanent water habitat to a temporary water habitat in response to the mosquito fish.

The physiology of mosquitoes often corresponds to their biology (Lehane 1991). McIver (1970) examined the antennal receptors of *Cx. restuans, Cx. pipiens, Cx. tarsalis, Cx. fatigans,* and *Cx. territans,* and found that *Cx. territans* had the fewest number of antennal olfactory receptors. The reduced number of receptors may correlate with the biology of this species. *Culex territans* females lay eggs in habitats that may contain amphibians, and do not travel far in search of a blood meal. McIver and Charlton (1970) compared the receptors on the maxillary palps of several *Culex* species and found that *Cx. territans* had fewer CO_2 receptors than the other C*ulex*. McIver and Charlton (1970) attributed these differences to *Cx. territans* preference for poikilothermic hosts. Carbon dioxide receptors would be limited as amphibians emit CO_2 mostly through their skin and into the surrounding water (Jorgensen 2000). The use of CO_2 receptors would potentially lead *Cx. territans* females to an undesirable avian or mammalian host.

McKeever (1986) examined the maxillary palps of *Corethrella*, which is a sister group to Culicidae (McKeever and French 2000). The *Corethrella* had an average of 32

CO_2 receptors on the maxillary palps, similar in number to those for *Cx. territans.* *Corethrella* also acquire blood meals from amphibians (McKeever 1977). The similarities between *Cx. territans* and *Corethrella* support Chapman's hypothesis that host-feeding behavior may influence receptor numbers.

Mosquito hearing and phonotaxis

In 1980, McKeever and French examined the relationship between a chaoborid midge (*Corethrella)*, and several species of tree frogs. They found that Chaoborid midges were attracted to the calls of certain species of tree frogs. The authors examined this by placing tape recorders of frog calls around CDC light traps, and found *Corethrella* readily went to the traps (McKeever and French 1980). Johnson et al. (1993) examined the infection levels of a frog trypanosome in male versus female frogs, because only the male frogs make their distinctive calls. They found that 72% of the males he examined were infected with trypanosomes; where as 0% of the females were infected. Johnson et al. (1993) attributed this significant difference to the fact that *Corethrella* are attracted to the calls of the male frogs, and use this to find a source of a blood meal.

Several arthropods have been shown to exhibit phonotaxis in the field and in the laboratory. The argasid tick, *Ornithodorus concanensis*, is attracted to the calls of its host, the cliff swallow, *Petrochelidon pyrrohonoto* (Webb et al. 1977). Gopfert et al. (1999) examined mosquito hearing in *Aedes aegypti,* and found the resonant frequency (sensitivity) of the male *Ae. aegypti* corresponded to the wing beat frequency of the female (Gopfert et al. 1999). This supports prior studies (Tischner and Schieff 1955; Gould 1975; Heran 1959; Bennet-Clark 1967). Gopfert et al. (1999) found that the resonant frequency of the female did not correspond to any sounds or wing beat

frequencies of the male, and were unable to make any speculations as to why she is tuned to these frequencies. Male *Aedes aegypti* were sensitive to around 650 to 680 Hz, and female mosquitoes were sensitive to frequencies around 445 to 475 Hz (Gopfert et al. 1999). Although there is a tremendous amount of variation in regards to frequencies of frog calls (under 50 Hz to over 4000 Hz), the male green frog, *Rana clamitans* calls with a frequency around 400 to 450 Hz (Bee et al. 2000).

Auditory behavior in insects requires an accurate ability to utilize mechanoreception to detect specific sound cues (Robert and Gopfert 2002). This requires behavioral, biological, neurological, and physiological mechanisms. These mechanisms are so precise, they can discriminate frequencies that differ by just 1% (Robert and Gopfert 2002). Mosquitoes hear with their antennae, which act as a sound receiver to antennal vibrations (Gopfert and Robert 2001). Although the male mosquitoes generally have more advanced hearing, both the male and females can respond to antennal deflections as low as 0.0001° and 0.0005° respectively (Gopfert and Robert 2001). Therefore, acoustic behavior in mosquitoes must serve several important functions.

Insects use a variety of techniques in order to hear, such as tympanal ears, subgenual organs, and antennal or leg hairs. In each type, the hearing organs are called chordotonal organs, which are made up of mechanical receptors called scolopidia (Clements 1999). These scolopidia are clustered together and connected to a moveable part of the insect cuticle or tracheal system (Clements 1999). The chordotonal organ forms a bridge between the cuticle (not bathed in hemolymph), and the trachea (bathed in hemolymph), and responds to stretching and relaxing (Bailey 1991).

Mosquitoes use structures on their antennae for detecting sound. The antennae in insects are made up of the scape, pedicel, and flagellum (Snodgrass 1935). In mosquitoes, the scape provides a site for muscle attachment for the antenna (Clements 1999). The scape probably does not function in insect hearing. In mosquitoes, the length of the antennae is mostly flagellum, which is made up of 13 flagellomeres varying in size and structure (Clements 1999). The flagellum is different in males and females. In males, the flagellum is plumose, and contains long, grooved and thin flagellar sensory hairs called fibrils (Gopfert et al. 1999). In contrast, the females have short ungrooved setae on the antennae (Clements 1999). In some species, *Culiseta inornata, Uranotaenia lowii,* and *Deinocerites cancer,* both the male and female antennae may lack the long fibrils, making them more similar in appearance (Clements 1963).

Within the pedicel, many insects have a hearing organ called the Johnston's organ (Snodgrass 1935). The Johnston's organ is a chordotonal organ that is present in the pedicel of all adult insects (Clements 1999). In male mosquitoes, this organ almost completely fills the pedicel (Clements 1999). The pedicel is deeply invaginated in the mosquito, which forms a deep depression (Clements 1963). A thickened area, called the sclerotized ring occurs halfway down the pedicel, and the cuticle becomes thin below this ring (Clements 1963). At the bottom of the pit, there is a basal plate, which is not sclerotized (Clements 1963). The flagellum is inserted within the basal plate, and acoustically, they become one functional unit (Clements 1999). Radiating from the basal plate are the prongs and septa, both of which have connections to the scolopidia (Clements 1999). The scolopidia are the mechanical receptors that are used for hearing in insects. They consist of a scolopale cell, an envelope cell, a long cap, and a sheath

(Boo and Richards 1974). In male mosquitoes, there are four distinct types of scolopidia, which are termed A, B, C, and D (Clements 1999). Female mosquitoes lack the type D scolopidia (Boo and Richards 1974). These types of scolopidia differ in their location within the Johnston's organ. The type A are radially arranged and are inserted into the inner surface of many prongs (Clements 1999). The type B are distributed around the inner wall of the pedicel, and have both ends attaching to the same prong (Clements 1999). Type C insert at the basal plate of the Johnston's organ, and type D insert into the flagellum (Clements 1999).

The hearing structures differ slightly in male and female mosquitoes. In females, the pedicel is slightly smaller and the apical pit is shallower than in the male (Clements 1999). In females, the basal plate is thinner and smaller than the males, but is similar in appearance to the male structure (Clements 1999). Males and females have a similar amount of prongs in the Johnston's organ, however, the male prongs are thinner and almost four times as long (Clements 1999). The males and females both have a similar amount of type B and C scolopidia, however, the female has half the amount of type A, and completely lacks the type D (Clements 1999). In addition, the antennae of males are plumose, and contain short setae in females (Gopfert et al. 1999).

The mosquitoes use their antennae in flight in response to drag and gravitational forces (Clements 1999). It has long been recognized that male mosquitoes are capable of using mechanoreceptors and chordotonal organs on their antennae for receiving sound signals (Clements 1999). Hearing in female mosquitoes has only recently been studied. Hearing in mosquitoes is a three-step process that involves coupling, transduction, and encoding (Clements 1999).

During coupling, the sensillum on the antennae absorbs energy from physical stimuli and converts them into mechanical energy transmitted to the sensory neurons (Clements 1999). While a sound is heard, the antennae rocks like a stiff rod at the pedicel-flagellum joint (Gopfert and Robert 2000). This mechanism functions like a harmonic oscillator in both male and female mosquitoes (Gopfert and Robert 2000). This step involves the multiple structures of the antennae and Johnston's organ. It was long debated whether or not the flagellum played any role in sound reception. In one experiment by Roth (1948), the male mosquitoes were stripped of their fibrillae. This resulted in male response to tuning forks, but not to female flying mosquitoes. They deduced that this indicated that the antennae were the organs of hearing, and sound reception depended on movement of the flagella (Clements 1999). It was also suggested that the presence of the long fibrils increases the surface area of the male's flagellum, which increases the force imposed on the multiple scolopidia (McVean 1991). During certain frequencies, the flagellum vibrates. These vibrations give rise to receptor potentials in the Johnston's organ, which is the process of transduction (Clements 1999). The male flagellum general vibrates at frequencies between 100 and 600 Hz, and the maximum amplitude of oscillation is called the resonant frequency (Clements 1999). These resonant frequencies differ between species and temperature (Tischner and Schieff 1955). As the flagellum vibrates, the sound energy is absorbed and conducted through the basal plate and scolopidia (Clements 1999).

Once the sound is absorbed, the process of transduction occurs. During this process the flagellar vibrations are transduced by around 15,000 mechanosensory neurons from the Johnston's organ (Robert and Gopfert 2002). During transduction, the dendritic

membrane is deformed and causes receptor potentials (Clements 1999). These receptor potentials have no threshold or refractory period, and reproduce the waveform of the stimulus (Clements 1999). This waveform is sinusoidal and occurs at twice the frequency of the acoustic stimulus (Clements 1999). In addition, the amount of depolarization increases with the intensity of the stimulus (Clements 1999). This depolarization is then changed into action potentials. During encoding, the receptor potentials are converted into action potentials (Clements 1999). There is little information regarding encoding in mosquitoes. During this process mechanosensory receptor cells encode for the acoustic energy from an action potential (Robert, 2002).

It is believed that male mosquitoes utilize their hearing capabilities to locate female mosquitoes. In general they do this by responding to the wing beat frequencies of the female mosquito. Each species of mosquito responds to a range of frequencies, the maximum being the resonant frequency. These frequencies can be as low as 50 Hz, and as high as several thousand Hz. Research on this subject has led to the knowledge of male mosquitoes cueing in to the wing beat frequencies of females of the same species.

The male mosquito flagellum bears a large number of long hairs (Gopfert and Robert 2001). These long hairs increase the hearing capabilities of male mosquitoes. Some of the earliest studies by Roth (1948) showed that by cutting off the flagellum or coating the pedicel with shellac, the males no longer responded to female mosquitoes. When just the tips of the antennae were cut, the males had a normal response to females (Roth 1948). Lastly, males would not respond to females if the fibrils of the antennae were removed, although they were responding to tuning forks. This indicated that males were utilizing their antennae to detect specific frequencies of females (Roth 1948).

The male and female flagellums resonate at different frequencies. Gopfert et al. (1999) provided the first experimental evidence of sound induced antennal vibrations in mosquitoes, using a laser vibrometer and loudspeaker. The mosquitoes were attached to the vibrometer, which measured antennal vibrations to various sound frequencies (Gopfert et al. 1999). The authors showed the frequencies at which the male and female antennae vibrate. Gopfert and Robert (2000), further expanded the work by showing the resonant frequency of the male flagellum coincided with the fundamental frequency of the female flight sounds. This supported prior studies that demonstrated that males cue into female flight sounds. They also found that their hearing was so acute, they could respond to antennal deflections as low as 0.0001° (Gopfert and Robert 2000). This indicates a high sensitivity to sound from females (Gopfert and Robert 2000). They found that males were just as sensitive to acoustic stimulation when they were under hypoxic conditions (Gopfert and Robert 2001).

Clements (1999) suggests a hypothetical model of using sound to locate the female using the angle the sound is coming from. He postulates that if a sound is coming from in front of the mosquito, it will activate a response in both antennae more strongly than if it were coming from a side direction (Clements 1999). He further states that they can detect a sound source both vertically and horizontally (Clements 1999). Bailey (1991) suggested that the antennae may be too close together to orient direction, and flying insects might utilize the mechanoreceptors for sound, similar to a pair of headphones. Direction may be determined by time differences, pressure differences or phase differences, which cause receptors in one antenna to respond quicker than the other (Bailey 1991).

Research has shown that the female Johnston's organ is less sensitive to acoustic vibrations than male mosquitoes (Gopfert and Robert 2000), but females are capable of responding to a range of sound frequencies. Unlike male mosquitoes, females do not cue into the flight sounds of males. In a study by Gopfert and Robert (2000), the males produced flight sounds that were much higher than the resonance frequency of the male and female flagellum.

Culex territans and vector-borne parasites and pathogens

Frogs are host to a large number of parasites and pathogens. Often times, it is uncertain why some species of frogs may be infected with a pathogen, where other species within the same habitat remain uninfected. In addition, there may be differences in infection levels between the sexes of frogs. *Culex territans* is a competent vector for several parasite species, including *Foleyella flexicauda* (Benach and Crans 1975), frog erythrocytic virus (Gruia-Gray and Desser 1992) and *Hepatozoon catesbeianae* (Desser et al. 1995) to amphibians. *Culex territans* has also tested positive for West Nile virus in the United States (CDC 2005). In New Jersey, bullfrogs show high levels of co-infection with the nematode *F. flexicauda* and trypanosomes (Benach 1971). Amphibian trypanosomes infect most anuran species (Bardsley and Harmsen, 1973). Barta and Desser (1984) found trypanosomes are prevalent parasites infecting amphibians in Ontario, an area where *Cx. territans* is common (Desser et al. 1973).

Culex territans is considered a potential vector of trypanosomes to amphibians, such as *Trypanosoma rotatorium*, a flagellated protozoan that affects amphibians (Desser et al. 1973). Within *Cx. territans,* this trypanosome undergoes transformation and multiplication in the midgut and hindgut (Desser et al. 1973) to develop into the

epimastigote stage. Experiments have shown that *T. rotatorium* may also be transmitted to frogs by *Cx. pipiens, Rhodnius prolixus,* and *Ae. aegypti* (Ramos and Urdaneta-Morales 1977).

Anuran trypanosomes are cosmopolitan in distribution and have been found infecting most anuran species (Bardsley and Harmsen 1973). Barta and Desser (1984) found that trypanosomes were one of the most prevalent parasites infecting amphibians in Ontario. Amphibians typically survive these infections, but several species may cause fatal infections (Marcus 1981). High parasitic burdens can stress animal populations making them more susceptible to other stressors (Bustnes et al. 2006).

West Nile virus was recently discovered in the United States in 1999. Since then, researchers have been looking into possible reservoirs, vectors, disbursement, and overwintering mechanisms of the virus. In Russia, amphibians have been shown to be possible reservoirs of West Nile Virus (Kostyukov et al. 1985). In this study, a lake frog *(Rana ridibunda*), was experimentally infected with WNV, and showed very high levels of viremia (Kostyukov et al. 1985). In addition, *Culex pipiens,* in the laboratory, was shown to pick up WNV from amphibians, and transmit it to other animals (Kostyukov et al. 1986). In New Jersey, *Cx. pipiens* and *Cx. restuans* account for the majority of positively infected mosquitoes. However, little is known about their host preference, especially at different times in the year. It is hypothesized that these species may switch their host preference at different times of the year, based on host availability. Because they are some of the earliest adults to appear in the spring, it is likely that these two species could readily find amphibians close by, and utilize them as a blood meal source. A review of the literature showed that many important vectors have been shown to feed

on amphibians. These include, but are not limited to *Aedes albopictus* (Miyagi 1972), *Aedes vexans* (Miyagi 1972), and *Culex pipiens* (Hayes 1961; Miyagi 1972; Wright and DeFoliart 1970). In addition, many of our New Jersey species of mosquitoes have been found to feed on turtles. These include *Ae. canadensis* (Crans and Rockel 1968; DeFoliart 1967, Nolan et al. 1965; and Hayes 1961), *Cx. pipiens* (Hayes 1961; Miyagi 1972), and *Cx. restuans* (Hayes 1961). What is interesting is that these three species have been found positive for West Nile, as early as April and May.

Klenk and Komar (2003) investigated viral titers and antibody levels of WNV in experimentally infected amphibians and reptiles. They found that some species, including bullfrogs (*Rana catesbeiana*), could develop viral titers high enough to infect some species of mosquitoes, yet the role of amphibians and reptiles in WNV is still not determined (Klenk and Komar 2003). Reptiles and amphibians are often infected with EEE, WEE, and SLE (Hoff and Trainer 1973). Serological and experimental evidence suggests that amphibians and reptiles are capable of being hosts for a variety of arboviruses (Hoff et al. 1984). Recent infections of WNV in alligators showed that alligators might obtain the virus by ingesting infected horse meat (Miller et al. 2003).

Bullfrogs (*Rana catesbeiana)* are commonly infected with a nematode parasite *Foleyella sp.* (Filarioidea*:Nematoda).* This nematode is transmitted to frogs from the bite of an infected mosquito, *Culex territans*. *Foleyella flexicauda* is a species that was first described by Crans (1969), from New Jersey bullfrogs. Further investigation by Benach and Crans (1973) showed that *Cx. territans* is capable of transmitting this parasite to several species of frogs. Several species of mosquitoes have been shown to die from parasitic burden when experimentally infected with this nematode. Experiments with

Aedes triseriatus, showed the microfilarial parasites caused severe damage to the gut, killing the mosquito (Benach and Crans 1975). Terwedow and Craig (1977b) examined infection levels of *Foleyella flexicauda* in A*edes aegypti,* and found that two different strains of *Ae. aegypti* responded differently to *F. flexicauda* infections. One was refractory to infection; the other was susceptible to infection (Terwedow and Craig 1977b). After inoculating four strains of *Ae. aegypti,* they deduced that susceptibility to infection may be based on a single genetic factor (Terwedow and Craig 1977b).

Iridoviruses, such as Ranaviruses, may contribute to the worldwide decline of amphibians (Gantress et al. 2003). Mazzoni et al. (2003) considers Ranaviruses to be a main concern in the decline and extinction of amphibian species. It has been suggested that an iridovirus may be the primary cause of periodic population crashes of an endangered tiger salamander, *Ambystoma tigrinum* stebbinsi (Jancovich et al. 1997). However, more information is needed to understand how these viruses infect amphibians.

The two major types of iridoviruses include those occurring in invertebrates, and those occurring in amphibians (Ranaviruses). Ranaviruses are usually highly virulent in amphibians, often causing systemic infections (Daszak et al. 1999). These viruses occur throughout the United States in many important species of frogs, salamanders, and turtles, yet the epizootiology of the disease is not very well understood. The iridovirus is also common in invertebrates, including mosquitoes (Mosquito iridescent virus). In invertebrates, Iridoviruses normally affect soil dwelling and aquatic insects (Marina et al. 2003). However, little is known about invertebrate transmission mechanisms. Iridoviruses have been found in mosquito larvae of *Cs. annulata* and *Cx. territans* (Buchatsky 1977). Interestingly, *Cx. territans* is a frog feeding species of mosquito,

which is found in New Jersey. The larvae of *Cx. territans* share habitat with many species of amphibians. However, the role of *Cx. territans,* or other possible vectors, in transmitting Iridoviruses are completely unknown.

Frog erythrocytic virus is a type of Ranavirus that is transmitted from *Culex territans* to amphibians (Gruia-Gray and Desser 1992). The virus is a large double stranded DNA iridovirus that is contained within an envelope (Gruia-Gray and Desser 1992). When the virus is transmitted to amphibians, the blood cells change shape from oval to spheroid. As a result, amphibians become anemic (Gruia-Gray and Desser 1992). Frog erythrocytic virus has been detected in various *Rana* species. Although ranaviruses can be host specific, many occur within a wide variety of amphibians, reptiles, and fish (Jancovich et al. 1997).

Culex territans is involved in transmission of other pathogens, including Hepatozoons (Desser et al. 1995; and Smith et al. 2000), and microsporidia (White et al. 1994; and Andreadis 1989). Hepatozoons are very similar to Malaria parasites, having sexual stages of development within the mosquito, and asexual stages within their vertebrate hosts (Marcus 1981). Two species of Hepatozoon, *H. catesbianae* and *H. clamatae,* were found to be transmitted by *Cx. territans* (Desser et al. 1995). In the mosquito, sporogonic development occurs in the Malphighian tubules (Desser et al. 1995). Development in the frog is similar to *Plasmodium* in humans, with asexual development starting in the liver, and then invading the erythrocytes, where they become gametes (Desser et al. 1995).

Amblyospora opacita is a microsporidia that was originally described from *Cx. territans* (White et al. 1994). Further examination showed that spores of this

microsporidia develop in the fat body of *Cx. territans* larvae, and were infectious to the copepod, *Paracyclops fimbriatus chiltoni* (White et al. 1999). Transmission between the copepod and the mosquito occurs horizontally (White et al. 1994). Andreadis (1989) examined horizontal transmission between *Amblyospora connecticus* and *Aedes cantator*. *Amblyospora* species were found to be extremely host specific, and only completed development within a few species of mosquitoes (Andreadis 1989). Therefore, *Cx. territans,* is probably necessary for maintaining this cycle in nature.

Chapter 1:

Phonotaxis of *Culex territans* Walker (Diptera: Culicidae) to Amphibian Vocalizations

Introduction

Culex territans Walker (Diptera: Culicidae), a widely distributed species of mosquito in the United States, takes bloodmeals from amphibians (Crans 1970). Crans (1970) frequently observed female *C. territans* blood feeding on the spring peeper, *Pseudacris crucifer* (Wied-Neuwied), in nature. *Culex territans* overwinters as an adult inseminated female in New Jersey, and exit from diapause in the early spring correlates with the mating calls *P. crucifer* (unpublished data). Therefore, early season host-seeking behavior occurs at a time when preferred hosts are vocalizing.

Use of auditory cues in host seeking behavior has been documented in the Diptera for Corethrellidae (McKeever and French 1991), Tachinidae (Muller and Robert 2001) and Sarcophagidae (Kohler and Lakes-Harlan 2001). McKeever and French (1991) found blood-feeding flies in the genus *Corethrella* were attracted to the calls of several species of tree frogs from which they acquire bloodmeals. Kohler and Lakes-Harlan (2001) reported that the parasitic fly *Emblemasoma auditrix* Shewell locates cicada hosts acoustically. Muller and Robert (2001) found parasitic flies, *Ormia ochracea* (Bigot), were attracted to the calls of the male cricket hosts. These authors reported *O. ochracea* were accurate in locating hosts in complete darkness and could gauge the direction and distance in three dimensions.

Sound attraction has been examined in several mosquito species. Gibson and Russell (2006) found female *Toxorhynchites brevipalpis* Theobald altered wing beat frequency in response to the male flight tones. Gopfert et al. (1999) found female *Aedes aegypti* L. were sensitive to specific frequencies that did not correspond to the wing beat of males. Thongrungkiat (1990) examined sound trapping of *Culex tritaeniorhynchus*

Giles at 530 Hz, collecting 70% more females with sound. Leemingsawat et al. (1988) showed similar results, with an increase in females at higher frequencies between 800 and 1000 Hz. Borkent and Belton (2006) recently reported collecting *Uranotaenia lowii* Theobald females in traps baited with frog calls. Toma et al. (2005) had similar results, with 863 female mosquitoes collected in traps baited with frog calls, as opposed to 5 females without.

Mosquitoes use olfactory, visual, humidity, temperature, and tactile cues in host-finding and recognition (McIver 1982). The use of these cues depends on the distance from a host (Lehane 1991). Dekker et al. (2005) categorized stimuli as a long-range attractant if sensed at more than 5 m, and short range at less than 1 m. At long distances, host odor and CO_2 guide mosquitoes to the bloodmeal source (Gillies and Wilkes 1969, Lehane 1991). As mosquitoes approach their host, short-distance cues including vision, temperature, and odor become increasingly important (Lehane 1991). Mosquitoes can detect sounds in near-field ranges of less than 30 cm (Clements 1999). Observations by Smith and Gadawski (1994) indicated that male *Aedes provocans* (Walker) could detect females at greater distances. Recent reports of female mosquitoes collected in traps baited with frog calls (Toma et al. 2005, Borkent and Belton 2006) suggest that mosquitoes can detect sounds further than one meter, corresponding to the far-field range.

Auditory behavior in insects requires an accurate ability to use mechanoreception to detect and recognize specific sound cues (Robert and Gopfert 2002). Mosquitoes sense vibrations in the air using their antennae (Gopfert and Robert 2001), which in turn are transmitted to the Johnston's organ. The Johnston's organ is a chordotonal organ located at the base of the antennae (Yack 2004), which detects near-field sounds within one or

two wavelengths of the source. It is comprised of four types of scolopidia that differ in number and structure in male and female mosquitoes (Clements 1999). There is also variation in scolopidia type, structure and number between different species of mosquitoes. Both near-field and far-field (sound pressure) receptors are chordotonal organs. Far-field receptors have independently evolved in several species of Diptera where there is a selective pressure to locate a vocalizing host (Lakes-Harlan et al. 1999, Kohler and Lakes-Harlan 2001, Yack 2004), suggesting a far-field receptor could exist in Nematoceran Diptera.

If the antennal resonance frequency of a given species of mosquito is determined, then sound trapping could potentially target that species for surveillance. Sound waves travel in a predictable pattern from a point source and are less limited by wind direction than odors (Fishbane et al. 1996). Sound decreases by 6 dB as source distance doubles (Marten and Marler 1977, Bailey 1991). Gerhardt (1975) examined the distance attenuation of calls for 20 species of frogs and found the expected 6 dB was consistent for most species in field conditions. Depending on the initial volume, type of sound, and background noise, there is variation in how far a sound can be detected. However, Brenowitcz et al. (1984) found that a *P. crucifer* chorus could be detected within 8 acres around the source. Therefore, sound could potentially attract mosquitoes from greater distances than most conventional trapping methods.

We tested the hypothesis that *C. territans* females use amphibian vocalizations as a long distance attractant that can be detected at over 5 m. We propose that positive phonotaxis toward amphibian vocalizations may allow *C. territans* to locate an amphibian bloodmeal.

Materials and Methods

Laboratory colony maintenance

C. territans were colonized from larvae and adults field-collected throughout New Jersey, based on techniques described by Benach (1970). All stages of development were maintained at 24^{o}C at a 16:8 light:dark cycle. Larvae were reared in shallow pans and daily fed ground rat chow (Purina Mills, St. Louis, MO.). Water was changed daily. Pupae were transferred to glass bowls, and placed in 0.23 m^3 emergence cages. Adults were provided a continuous supply of 10% sucrose, which was removed 24 h before experimentation to increase host-seeking behavior.

Experimental Arena

The test arena consisted of two 0.029 m^3 cages separated by a 30 x 12 cm tube. A 2.5-cm hole in the tube center provided a port to release mosquitoes. In the distance attenuation study, a longer tube was used (76 x 8 cm), with a wider release port (8 cm). The frog calls and bird songs were played from compact discs (CD). Mating calls from New Jersey frogs and toads (Golden and Bunnell 2002) were used. Common bird songs (sparrows, finches, grosbeaks, and buntings) from Eastern and Central North America (Peterson 1990) were played on an Audiovox® CDA1361 (60 Hz, 14 W, 120 V) portable compact disc player (Audiovox Corp., Hauppauge, NY.), at an amplitude of 80 $\pm$ 2 dB. A mature male bullfrog (*Rana catesbeiana* Shaw) was used for the live frog. In order to determine the effect of background noise and attraction of CD player emissions, a control consisted of a plugged in CD-player that was not playing a compact disc. For consistency, the compact disc player, live frog, and control were placed 30.5 cm away from the experimental cage, and under the same lighting and temperature conditions

(24.7 $\pm$ 0.7˚C). Experiments were conducted in low light conditions, with a single overhead light 2.5 m away from the test arena. Background noise levels were recorded using a digital sound level meter (Fisher Scientific Co, Pittsburgh, PA.). During each trial, only one cage contained either the treatment or the control. The opposite cage was empty. Seven-day-old *C. territans* were introduced into the tube, and subjected to either the treatment or control for 15 min before the number of mosquitoes in each cage was counted. Those mosquitoes moving into the cage containing the treatment or control were counted as toward, and those moving into the empty cage were counted as away. Females not moving into either cage were recorded as no response.

Comparison of different treatments

If amphibian vocalizations are utilized as a long-distance attractant, then significantly more females would orient in the direction of the vocalizations when compared to the control. To remove direction as a confounding variable, treatments (frog calls, bird songs, live frog, and control) were replicated 5 times each in the left and right directions. Twenty females were used for each of the ten replications (200 females for each treatment or control), resulting in 800 total females examined in this experiment.

Comparison of different frog calls

We compared the responses of mosquitoes orienting to calls of six frog species. If vocalizations of particular frog species are used as an attractant, the attractiveness of calling individuals to host-seeking mosquitoes is predicted to differ among the frog species. We predicted that if *C. territans* orients toward sound, then there are components of sound that increase the attractiveness. The frog calls tested included *R. sylvatica* LeConte (wood frog), *R. catesbeiana* (bullfrog), *R. clamitans* Latreille (green frog), *Bufo*

americanus Holbrook (American toad), *Hyla versicolor* LeConte (northern gray tree frog), *P. crucifer* (spring peeper), and a control. Twenty-five females were used for each of the 5 replications (125 females), resulting in 875 females examined in this experiment.

We performed a comparison study to examine the responses of *C. pipiens pipiens* L. orienting toward *P. crucifer* calls and a control. Twenty-five females were used for each of the 5 replications (125 females), resulting in 250 females examined.

Distance attenuation and attraction of sound specific cues

We examined the response of *C. territans* to decreasing intensity of *P. crucifer* calls. If vocalizations are used as a long distance attractant, then an optimum distance away from the source at which vocalizations are most attractive could be predicted. We examined the response of females at increasing dB (46.8, 50, 54.8, 60.7, 63, 69, 73.5, and 76.7 dB). Sound intensity was recorded with a digital sound level meter (Fisher Scientific Co, Pittsburgh, PA.), using the reference pressure and frequency for human hearing (20 μPA, 1 KHz). Each sound intensity level was replicated 3 times with 50 females per dB level (150 females), resulting in 1200 females examined in this experiment.

Using the inverse square law, distance attenuation was extrapolated from the dB data. Field recorded dB levels were made for *P. crucifer* at 50 and 100 cm away. We examined the literature for measurements on *P. crucifer* dB levels (Gerhardt 1975, Brenowitz et al. 1984, and Wilczynski et al. 1984). We chose the lower mean of 86.6 dB at 50 cm, even though values above 95 dB at 50 cm are not that uncommon (Gerhardt 1975). Based on the literature, there are two dominant frequencies in *P. crucifer* recordings; 2800 Hz is the dominant frequency of the vocalization, and 500 Hz is the

dominant frequency of wind and white noise detected in the recordings. We defined near-field as the distance in which the sound pressure of a particular frequency (*f*) travels through one wavelength (λ), where $\lambda = \upsilon_{sound}/f$ (Fishbane et al. 1996). The speed of sound through air was calculated as $\upsilon_{sound} = 331.5 + 0.6\,T$ (˚C), where $T = 24.3$˚C during our experiments. From our calculations $\upsilon_{sound} = 346.1$, resulting in a near-field distance of 12 cm for 2800 Hz, and 69 cm for 500 Hz. Using these values we were able to determine sound pressure levels (SPL) and particle velocity levels (PVL) for each frequency at increasing distances (Fig. 1.1). The SPL decreased 12 dB within near-field and 6 dB in far-field, and PVL decreased 18 dB within near-field and 6 dB in far-field for every doubling of the distance (Clements 1999). Particle velocity (*u*) was converted to m/s using the formula $u = I/p$, where *I* is the sound intensity (W/m^2), and *p* is the sound pressure (N/m^2).

Field trials

If vocalizations are utilized as a long-range attractant, then more females would be predicted to be collected in mosquito traps baited with sound than in comparable traps without sound. Trap types examined included CDC miniature light traps, pickle jar traps, and resting boxes. Three traps of each type contained an Audiovox® portable CD player. The players were set to continuously play *P. crucifer* calls at 92 dB. The pickle jar traps were tested in Warren County New Jersey. The CDC light traps and resting boxes were examined in the Pine Barrens region of New Jersey. Five field trials were conducted. Because we were unsuccessful using CDC light traps and pickle jar traps to collect *C. territans*, only one trial was conducted for each of these two trap types.

Statistics

Data were analyzed using SPSS software (SPSS Inc. 2005). To determine if a particular sound was attractive to mosquitoes, we used a *t*-test to compare the number of females orienting toward and away from the sound source for all experiments. In the first experiment, we were interested in determining which treatments were most attractive, and compared treatments to each other and to the control using a two-way Analysis of Variance (ANOVA). Only the females entering the cage containing the treatment or control were used in the statistical analysis. In addition, we compared the number of females orienting towards treatments in the left direction versus the right direction. For all analysis, a Tukey's test was used to compare means.

In the second experiment, we were interested in determining which frog calls were most attractive. We compared the number of females orienting towards each treatment to each other and the control using a one-way ANOVA. A Tukey's test was used to compare means. A stepwise multiple regression analysis was performed to determine which characteristics of sound (frequency, pulse duration, call duration, call amplitude, and call rate) could predict phonotaxis in *C. territans* females.

For the distance attenuation study, curvilinear regression analysis was performed for both near-field and far-field data to determine a trend. The best-fit line was determined using the p-values and R^2 values obtained from the curve estimation regression analysis. Those traps baited with sound were compared to those without sound using a *t*-test. A chi-square test was performed to determine if the week of sound trapping influenced the number of mosquitoes collected, by comparing the number of *C. territans* females collected in resting boxes at three other sites not baited with sound. All resting box sites were located within the New Jersey Pine Barrens.

Results

We examined a total of 2875 *C. territans* females for phonotaxis in the laboratory. When provided with a sound source, 70.3% moved in the direction of the sound, as opposed to 29.6% that moved away from the sound ($P < 0.001$). Orientation occurred regardless of the source of the sound. Although birds are not a preferred host for *C. territans*, a large proportion (60%) of females oriented toward the bird songs. While *C. territans* readily feed on *R. catesbeiana* in the laboratory, only 58% of the females oriented toward the live frog in the 15-min period. The number of females orienting toward the control averaged 51% across all experiments.

Significantly more *C. territans* females oriented to frog calls ($F = 5.07$; df = 3, 36; $P = 0.005$), when compared to bird songs, live frog, or the control. There was a 30% increase in the number of females orienting to the frog calls when compared to the other treatments. The original design compared responses in both left and right directions. Neither direction ($F = 1.54$; df = 7, 32; $P = 0.223$) nor the interaction ($F = 1.33$; df = 7, 32; $P = 0.28$) were significant, so the data were collapsed to include 10 replicates each. In both the two-way ($F = 5.29$; df = 7, 32; $P = 0.004$) and one-way designs ($F = 5.07$; df = 3, 36; $P = 0.005$), the effects of call type were significant.

Females ($F = 15.04$; df = 6, 28; $P < 0.001$) were attracted to the calls of *P. crucifer*, *H. versicolor*, *B. americanus*, and *R. clamitans* when compared to the control (Fig. 1.2). Females were not attracted to the calls of *R. sylvatica* or *R. catesbeiana*. *Pseudacris crucifer* attracted more mosquitoes (16.8 females) than the other treatments. *Hyla versicolor* (12.0 females) and *B. americanus* (12.0 females) attracted the second highest number of mosquitoes. These frogs have high-frequency vocalizations compared

to the other species examined (Table 1.1). A comparison study was conducted with *C. pipiens,* and there was no significant difference between those orienting toward *P. crucifer* (5.88 $\pm$ 0.88 SE) when compared to the control (6.3 $\pm$ 1.4 SE).

The multiple regression analysis showed that orientation was positively correlated with frequency ($P < 0.001$) (Table 1.2). There was a slight correlation with call rate, calling period, and call amplitude. Call duration and pulse duration did not correlate with the number of females orienting. We developed a model based on these data (Table 1.3). Although frequency was sufficient to predict orientation ($R^2 = 0.7$, $P < 0.001$), the best fit model included frequency, pulse duration, amplitude, and error ($R^2 = 0.85$, $P < 0.001$). This model best explains the data, suggesting that frequency is the best predictor for orientation, with pulse duration and amplitude increasing the attractiveness of the source.

The call of *P. crucifer* was analyzed as a potential long-distance attractant. The highest (76 dB PVL, 1.8 m, $u = 0.4$ mm/s) and lowest dB levels (46 dB PVL, 40 m, $u = 0.01$ mm/s) attracted fewer mosquitoes ($F = 3.91$; df = 7, 16; $P = 0.05$) than those in the middle range (50 to 75 dB PVL, 2 to 28 m, $u = 0.02$ to 0.3 mm/s). The number of *C. territans* females orienting (y) to *P. crucifer* call sound intensity (x) was described by a quadratic trend ($y = -0.033 (\pm 0.01)x^2 + 4.3 (\pm 1.27)x - 105.49 (\pm 38.4)$; $R^2 = 0.456$, $P = 0.002$; Fig. 1.3).

The number of mosquito females orienting to spring peeper calls decreased with distance away from the source (Fig. 1.4). A cubic trend described the number of *C. territans* females orienting (y) orienting to frog calls as a function of distance from the source ($y = 0.001 (\pm 0.001)x^3 - 0.053 (\pm 0.045)x^2 + 0.772 (\pm 0.738)x + 29.6 (\pm 2.75)$; $R^2 = 0.422$, $P = 0.011$). The optimum distance at which *C. territans* responded to vocalizations

was between 2 and 32 m from the source. As particle velocities reach below 0.05 mm/s, the number of females orienting toward frog calls rapidly decreases (Fig 1.5).

Neither the CDC light trap nor the pickle jar trap collected *C. territans* in any of the trials. Placing a compact disc player of *P. crucifer* calls next to resting boxes resulted in an increase in the number of *C. territans* females with sound (69%) compared to those without sound (31%). Note that even with small collection sizes (25 females) our data were significant at $P = 0.07$. A chi-square test showed sound trapping increased the observed number of *C. territans* collected ($\chi^2 = 59.84$, $P < 0.001$). Our highest collection of *C. territans* (15 females) occurred in resting boxes baited with sound, compared to the mean of 2.8 females in resting boxes without sound.

Discussion

Our laboratory study shows that *C. territans* females are attracted to sound. The proportion of female mosquitoes orienting to sound, regardless of the source, was greater than that moving away from the sound source. Both male and female mosquitoes use a Johnston's organ to sense air-borne vibrations (Clements 1999). Gopfert and Robert (2000) found that female mosquitoes are almost as sensitive to sounds as males. Fewer females oriented toward the live frog in the 15-min period compared to the frog calls and bird songs. *Culex territans* readily blood-feed on *R. catesbeiana* in the lab; however, females do not attempt blood-feeding immediately and will continually land on *R. catesbeiana* throughout a 3-h period. Because *C. territans* have fewer chemoreceptors (McIver 1970) than other species of *Culex*, females might not orient toward most odors. Experiments were conducted in an open environment in the laboratory, as opposed to a wind tunnel. Therefore, mosquitoes did not fly upwind to the amphibian odor.

Females were attracted to the calls of *P. crucifer*, *H. versicolor*, and *B. americanus*, three species commonly heard calling in New Jersey. *Pseudacris crucifer* are among the earliest amphibians to call in the spring, beginning to call in New Jersey from March to early April (Gessner and Stiles 2001), corresponding to when *C. territans* exit hibernation and seek their first bloodmeal. Resting boxes reveal the first collections of adult female *C. territans* coincide with the calling of *P. crucifer.*

Phonotaxis was correlated with higher frequency vocalizations. A curve estimation test indicated that the data showed a linear trend. As the frequency increases, then the number of females orienting increases. *Pseudacris crucifer* calls had the highest frequency examined (2.6 to 3.5 kHz), and were also the most attractive to *C. territans*. Fewer females oriented toward the lower frequency calls of *R. catesbeiana* (200 to 400 Hz) and *R. clamitans* (350 to 450 Hz). Most frogs and toads produce calls in frequencies ranging from 100 Hz to 5–6 kHz (Capranica 1965). Bird songs are produced in frequencies ranging from 80 Hz to 10.7 kHz (Narins et al. 2004). This overlap in frequencies may explain why *C. territans* females were orienting toward bird songs in the laboratory. Since birds sing during the day and amphibians call during the evening, the nocturnal feeding behavior of *C. territans* would reinforce orienting only toward amphibians.

Prior studies show female mosquitoes respond to specific sound frequencies. Gopfert et al. (1999) found that the antennae of female *A. aegypti* respond to frequencies around 230 Hz, with a second peak around 1500 Hz; the lower frequency being the resonant frequency of the antennae, and the higher being the resonant frequency of the antennal hairs (Gopfert et al. 1999). Gibson and Russell (2006) found *T. brevipalpis*

females respond to frequencies around 400 Hz. Resonance frequency can be influenced by age, temperature, size of antennae, and stiffness of the cuticle (Gopfert and Robert 2001). The antennal hairs do not independently receive sounds (Belton 1994), and it is the resonant frequency of the antennae that stimulates the Johnston's organ, with the antennal hairs increasing surface area for sound reception (McVean 1991). If these resonance frequencies can be determined for a target species of mosquito, then sound can be added to traps to improve surveillance techniques. Although we were unsuccessful in sound trapping for *C. territans*, Borkent and Belton (2005) collected large numbers of *U. lowii* by adding frog calls to CDC miniature light traps.

Although *C. territans* females were attracted most to the vocalizations of *P. crucifer*, it is unclear what frequency in the call was the most attractive to host-seeking individuals. The *P. crucifer* vocalization is made up of a range of frequencies from 0 to 4 KHz (Wilcynski et al. 1984), with a dominant peak around 2.5 to 3.5 KHz. We used the dominant frequency in the statistical analysis, although the full range was present in our treatment. For *P. crucifer,* Wilcynski et al. (1984) and Schwartz and Gerhardt (1998) showed a second dominant peak at 500 Hz, which both authors attributed to wind and white noise. Because we were using a CD of field-recorded amphibians, it is likely we had the same noise conditions in our recordings. This white noise might correspond to the resonant frequency of the antennae. Therefore, if the white noise stimulated the Johnston's organ, the higher frequency vocalizations might have increased the attractiveness of the recorded calls by stimulating the antennal hairs. The CD player might emit low frequency components that stimulate the Johnston's organ; however, female *C. territans* did not orient towards all vocalizations. If female mosquitoes

responded to sounds produced by the CD player, then higher frequency vocalizations increased the attractiveness of the source.

Near-field receptors detect air-borne vibrations; typically lower frequencies within one wavelength of the source (Yack 2004). The two peak frequencies in the *P. crucifer* vocalization were 500 Hz (λ = 69.2 cm), and 2800 Hz (λ = 12.3 cm). If females were using only near-field receptors, they would not be able to detect calls at > 69 cm for the low frequency, and > 12 cm for the high frequency component of the *P. crucifer* call. In our experiments, the CD player was placed 30.5 cm from the cage. Females were never within one wavelength of the high frequency band. Given the cage and tube sizes in our arena, the females were released 99 cm away from the CD player. This would be within two wavelengths of the low frequency band, but not the high frequency band. If females were only responding to the low frequency band and white noise, then there would be no significant difference between treatments, suggesting females can detect particle velocities at distances greater than two wavelengths. This hypothesis seems plausible considering the high sound intensity levels of amphibian vocalizations compared to female wing beats. It would be worthwhile to repeat experiments with the CD player greater than 70 cm away from the release port.

Call and pulse durations did not influence the number of female mosquitoes orienting toward calls. *P. crucifer* has a short continuous peep, lasting 0.3 sec (Gerhardt 1975). *Rana clamitans* calls, which were not attractive to *C. territans* females, also have a short continuous peep lasting 0.16 sec (Bee and Perrill 1996). *Bufo americanus* calls, which were attractive to *C. territans* females, has a long series of trills lasting 6 to 10 sec (Gerhardt et al. 2000). However, multiple regression analysis showed pulse duration may

explain the data if coupled with other characteristics. When combining frequency, pulse duration, and call amplitude into a model, 84.5 % of the variation in the mosquito responses was explained. This finding indicates that *C. territans* may orient toward multiple parameters of a host's call and supports other studies of phonotaxis in Diptera. DeVries and Lakes-Harlan (2005) found that flies were attracted to several features of the host's calling song, the most important of which was frequency. However, pulse duration and pulse repetition rate may also allow further discrimination of hosts (DeVries and Lakes-Harlan 2005). *Culex territans* will obtain bloodmeals from a variety of amphibian species, indicating females do not need accurate discrimination of host species.
Call amplitude did not show a strong correlation to the number of females orienting, but increased the attractiveness of the call based on our model. Our data suggest that amplitude can influence the distance from which the sound can be detected.

Vocalizations by particular frog species may be a mid to long-range attractant, with higher numbers of host-seeking mosquito females orienting toward vocalizations between 50 to 75 dB PVL. This amplitude corresponds to a mosquito female at 2 to 28 m away from the point source. The degree of attraction may also increase with higher call amplitudes ranging from 90 to 100 dB (2 to 5 cm), the call amplitude reflecting the distance-attenuation of the particular frequency (Gerhardt 1975). The distance at which a sound can be detected depends on the frequency, intensity, and attenuation of sound (Webster et al. 1992). A sound output of 90 dB SPL at a distance of 1 m, which has a typical 6 dB decrease for every doubling of the distance, has the potential to be detected at 1000 m from the source (Webster et al. 1992) by a sound pressure detector. During calling periods, *P. crucifer* space themselves apart from other calling males based on

their call amplitude (Brenowitz et al. 1984). This behavior maximizes their active calling space and maximizes the advertisement call of the entire chorus. Call amplitudes of a *P. crucifer* chorus can potentially reach 100 dB on a humid spring night, with a potential to be detected with a sound pressure receptor from a kilometer away.

The Johnston's organ is a near-field receptor that detects particle velocities and not sound pressures (Tischner and Schief 1955). Even though the tympanic membrane and vertebrate ears can detect far-field sound pressures, the mosquito antenna detects near-field particle velocities (Clements 1999). Our data show that as particle velocity approaches 0.05 mm/s, the attractiveness of the vocalization rapidly decreases. In addition, there was little difference between those female mosquitoes responding to particle velocities above 0.05 mm/s, corresponding to 60 dB. These values seem reasonable considering male mosquitoes respond to female wing beats at 45 dB (Charlwood and Jones 1979). Typical background noise, which can affect sound detection, may be around 40 to 60 dB (Mankin 1994) or 30 to 50 dB (Brenowitz et al. 1984), depending on location. Amplitudes of the female wing beats might be similar to typical background noise.

The near-field sounds are most-likely detected by *C. territans*; however, far-field receptors also are present in dipterans. The chordotonal organs for near-field and far-field hearing are structurally similar, and differ by connectivity, attachment, cap structure, and type of scolopidia (Yack 2004). The mosquito Johnston's organ is made up of 4 different types of scolopidia, the most of which are type A and B scolopidia (amphinematic), which are used for detecting particle velocities. Mosquitoes have few Type C scolopidia (mononematic), which are similar in structure to far-field receptors.

Far-field receptors have independently evolved in several species of Diptera where there has been selective pressure to locate a vocalizing host (Lakes-Harlan et al. 1999, Kohler and Lakes-Harlan 2001, Yack 2004). Because several Nematoceran Diptera, including *Corethrella, C. territans* and *Ur. lowii* have vocalizing hosts, and have been collected in traps baited with frog calls (McKeever and French 1991, Borkent and Belton 2006), it would be worthwhile to further study the ultrastructure and function of the chordotonal organs in these species.

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Table 1.1. Characteristics of select amphibian vocalizations.

Species	Dominant call frequency (HZ)	Call amplitude (db)	Call duration (sec/call)	Pulse duration (pulses/sec)	Call rate (calls/hr)	Calling period in NJ	References
Pseudacris crucifer	2600 to 3500	80.2 to 97	0.10 to 0.30	continuous	600 to 990	March to May	Brenowitz et al. 1984, Wilczynski et al. 1984 Gerhardt 1975 Marshall et al. 2003 Gessner and Stiles 2001
Hyla versicolor	1800 to 2200	86 to 100.5	0.475 to 0.875	16 to 25	600 to 1400	May to July	Gerhardt 1975 Fellers 1979 McLister 2001 Gerhardt 2000 Taigen and Wells 1985 Gessner and Stiles 2001
Bufo americanus	1300 to 2000	90 to 115	6.0 to 10.8	30 to 40	60 to 100	March to June	Howard and Young 1998 Gerhardt 1975 Moffat and Capranica 1976 Blair 1958 Gessner and Stiles 2001
Rana clamitans	350 to 450	84	0.16	continuous	300 to 1800	April to August	Bee et al. 2000 Bee and Perrill 1996 Gessner and Stiles 2001
Rana catesbeiana	200 to 400	64 to 76	0.37 to 0.97	continuous	600 to 2000	April to July	Capranica and Moffat 1977 Capranica 1965 Simmons 2004 Gessner and Stiles 2001
Rana sylvatica	not available	not available	not available	not available	not available	March to May	Gessner and Stiles 2001

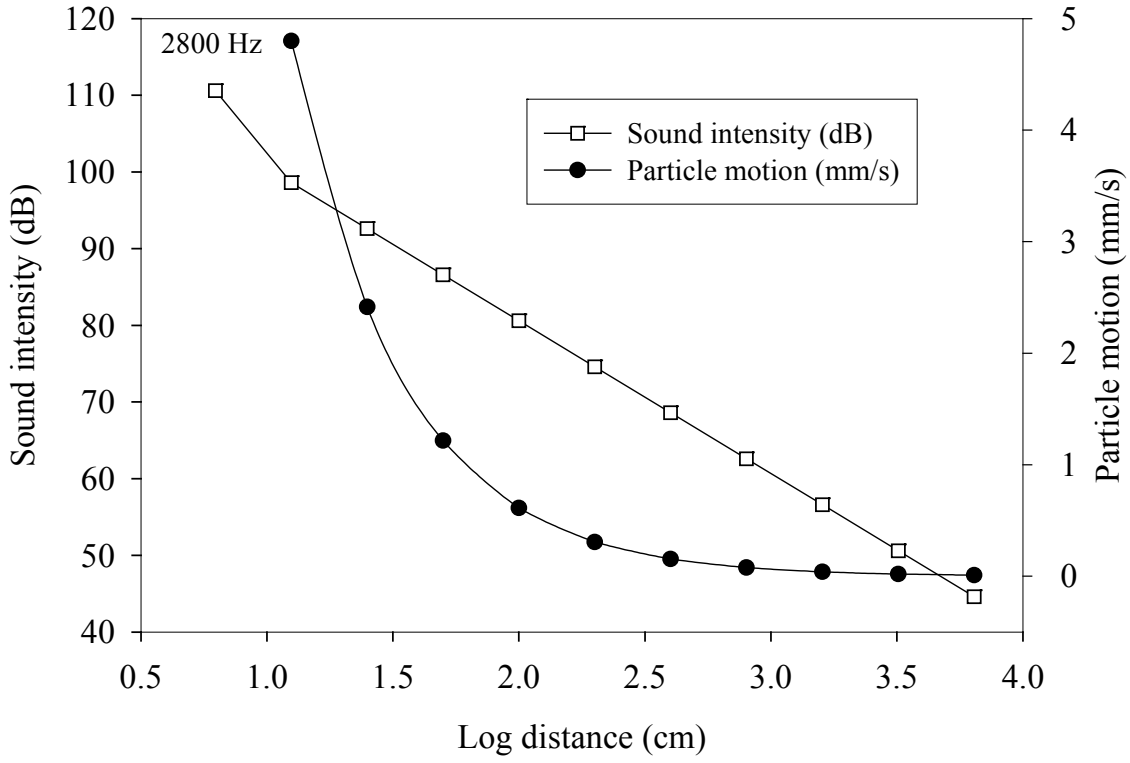
Table 1.2. Linear associations between number of females orienting and call characteristics of amphibians using stepwise multiple regression

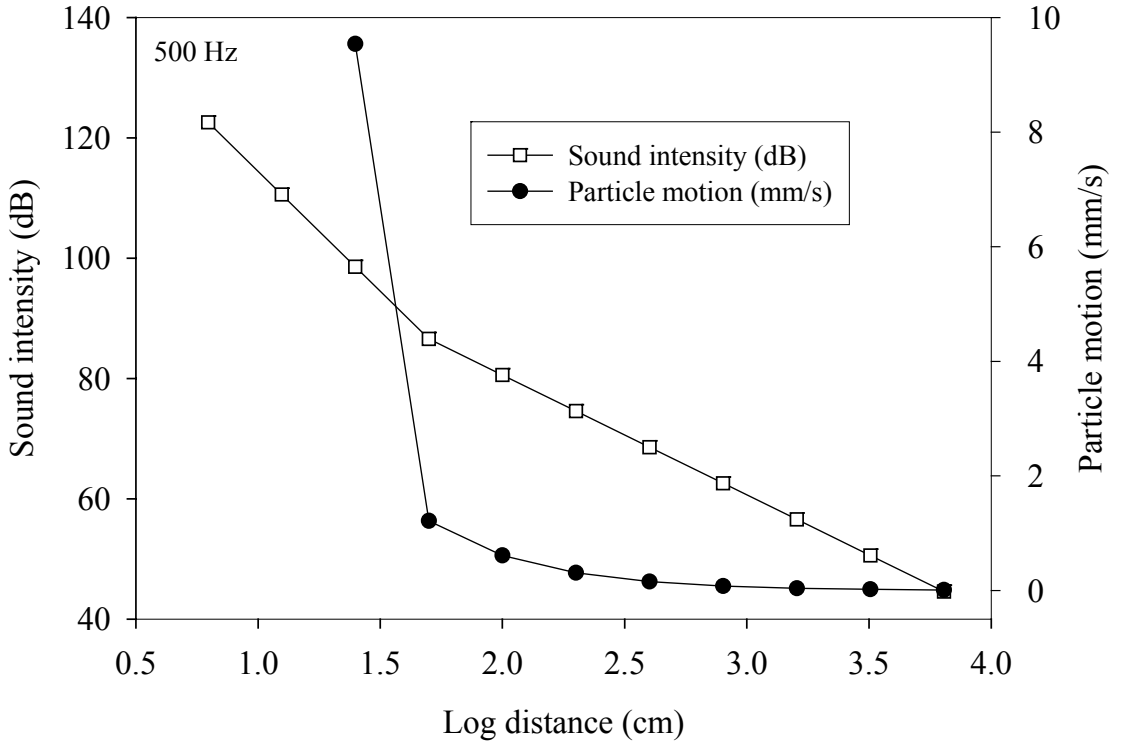
Characteristic	Pearson correlation	P-value
Frequency	+0.836	<0.001
Call duration	-0.082	0.349
Pulse duration	-0.069	0.372
Call rate	-0.414	0.020
Calling period	-0.527	0.003
Call amplitude	-0.393	0.026

Table 1.3. Comparison of models predicting the number of females orienting toward frog calls

Model	Model Predictors	R-squared	F-value	P-value
1	Frequency	0.698	53.175	<0.001
2	Frequency Pulse duration	0.777	38.428	<0.001
3	Frequency Pulse duration Call amplitude	0.845	38.183	<0.001

Fig. 1.1. Sound attenuation for the two dominant frequencies of *Pseudacris crucifer* recordings. Measurements of sound pressure levels (SPL) and particle velocity levels (PVL) are shown for 2800 Hz (Fig 1a) and 500 Hz (Fig 1b) at increasing distances. Note that the distance on the x-axis has been logarithmically transformed. Far-field decibel values are from Brenowitz et al. (1984), Wilczynski et al. (1984) and Gerhardt (1975). The transition from near-field to far-field (one wavelength) at 2800 Hz and 500 Hz occurs at 12 cm ($10^{1.1}$), and 69 cm ($10^{1.84}$) respectively.


2800 Hz
Sound intensity (dB)
Particle motion (mm/s)
Sound intensity (dB)
Particle motion (mm/s)
Log distance (cm)
120
110
100
90
80
70
60
50
40
5
4
3
2
1
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0


500 Hz
Sound intensity (dB)
Particle motion (mm/s)
Sound intensity (dB)
Particle motion (mm/s)
Log distance (cm)
140
120
100
80
60
40
10
8
6
4
2
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0

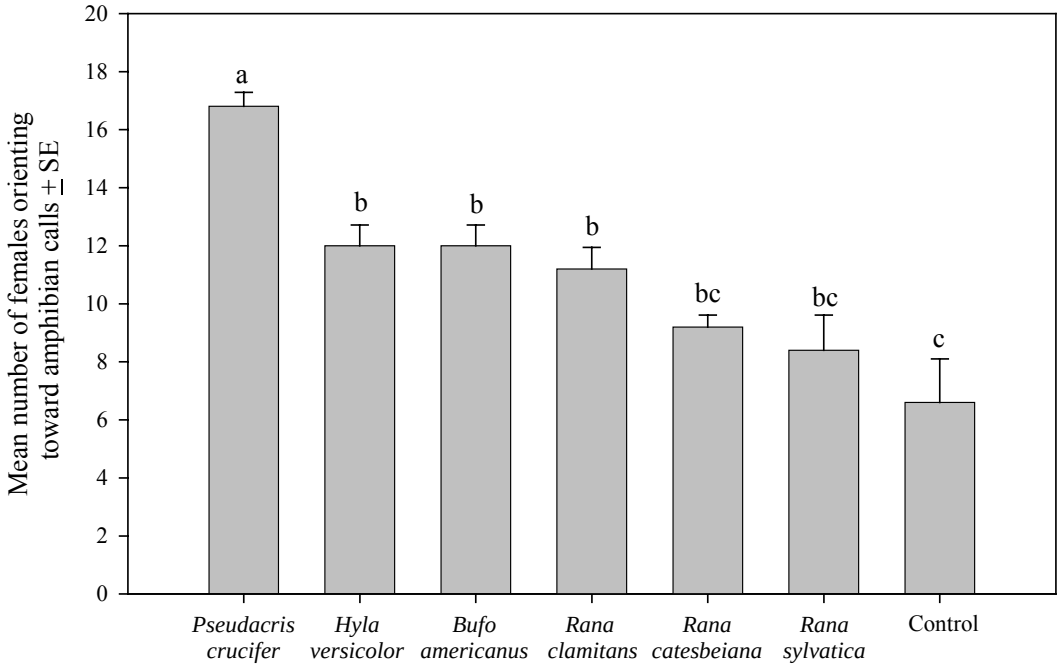


Fig. 1.2. *Culex territans* orientation to different frog calls. Responses were analyzed using Analysis of Variance. Numbers followed by same letter are not significantly different.

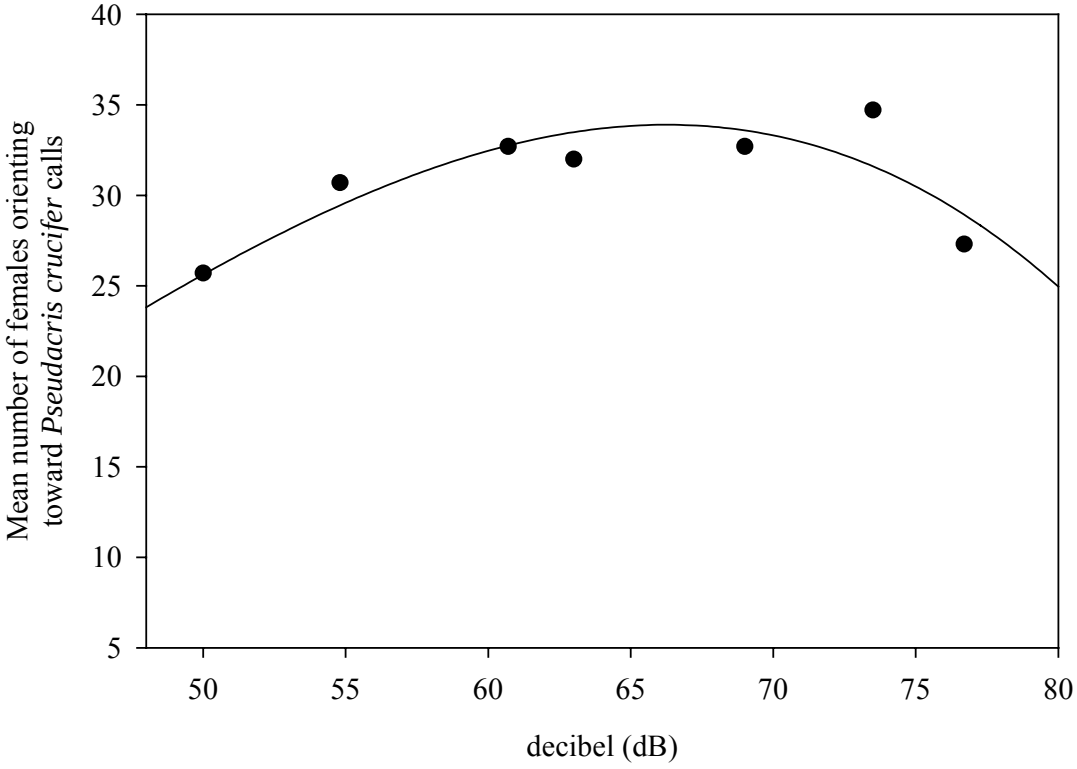


Fig. 1.3. Influence of frog call intensity of orientation of *Culex territans.*

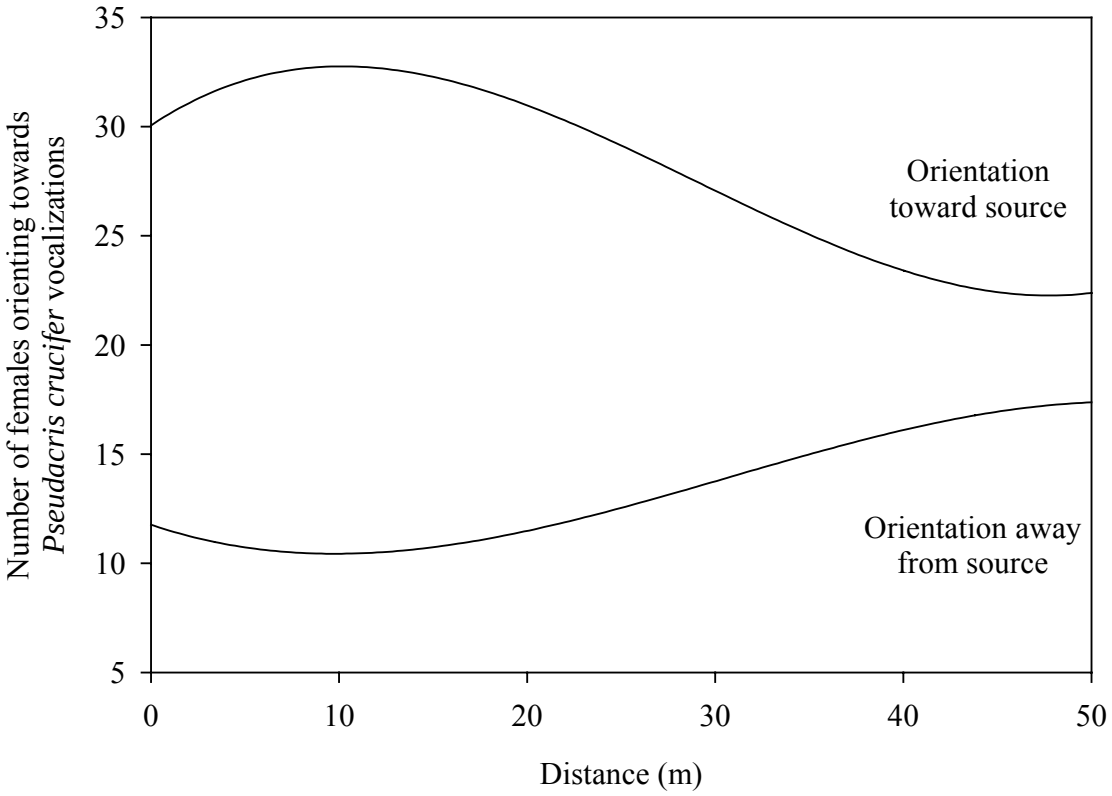


Fig. 1.4. *Culex territans* orientation to *P. crucifer* calls at increasing distances from source.

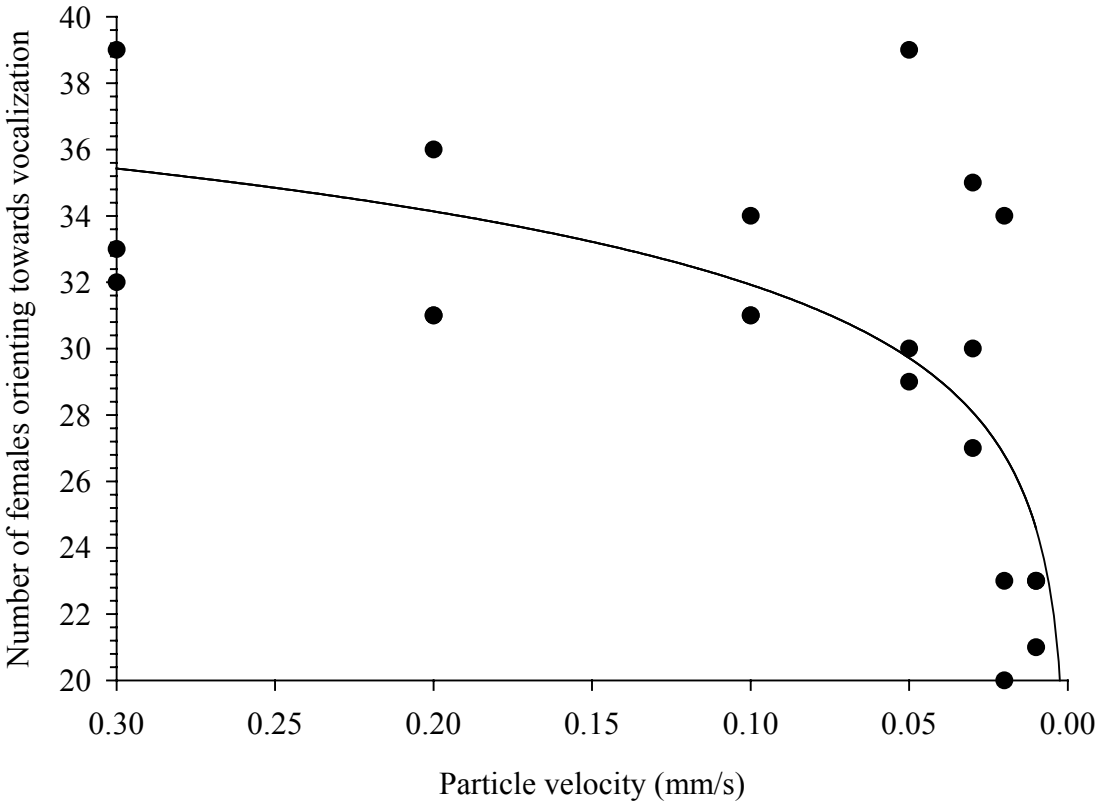


Fig 1.5. Effect of particle velocity on the orientation of female *Culex territans.*

Chapter 2:

Temporal and spatial synchrony of *Culex territans* Walker (Diptera: Culicidae) with their amphibian hosts

Introduction

Culex territans Walker (Diptera: Culicidae*),* a widely distributed mosquito species, is found throughout most of the Northern hemisphere (Knight and Stone 1977). *Culex territans* females in Tennessee have a preference for amphibian bloodmeals (63%), and also feed on Reptilia, Aves, and Mammalia (Savage et al. 2007). In New Jersey, *Cx. territans* occasionally feed on reptile and avian sources, but prefer amphibians (88.5%) (Crans 1970). *Culex territans* females harbor several parasites and pathogens which may be transmitted to amphibians, including *Foleyella flexicauda* Schacher and Crans (Benach and Crans 1973), frog erythrocytic virus (Gruia-Gray and Desser 1992), and *Hepatozoon clamatae* Stebbins (Kim et al. 1998). *Culex territans* has also tested positive for West Nile virus in the United States (CDC 2005).

Culex territans adult females have been collected overwintering in New York (Nasci et al. 2001) and New Jersey (Farajollahi 2005). Collections of larvae in the spring (Smith 1903) are consistent with the report that females exit diapause in New Jersey in March (Crans, personal communication). At this time, females will seek a bloodmeal, and oviposit their first batch of eggs. Eggs are generally laid above the water line on emergent vegetation (McIver 1969). Reproduction continues throughout the growing seasons, with the greatest numbers of this species occurring before the first frost in the fall.

Culex territans are often collected in non-polluted water including permanent swamps, marshes, ponds, streams, containers, and large wetland pools (Joy and Clay 2002), and share habitats with amphibian species (Benach 1971). Amphibians are good indicators of water quality (Boyer and Grue 1995), because they are sensitive to toxicants

and acidic conditions (Freda and Taylor 1992). Therefore amphibians are restricted to habitats that are non-polluted. Some species of mosquito that share larval habitats with amphibians may compete with tadpoles for limited resources (Mokany and Shine 2003).

Many amphibian species in New Jersey exit diapause throughout March (Gessner and Stiles 2001). At this time, adult female *Cx. territans* have been collected in traps as part of the NJ State Surveillance and Vector Surveillance projects (Reed et al. 2008). Monthly mean temperatures in northern New Jersey in March are approximately 3˚C (NJ Weather and Climate Network, Office of the NJ State Climatologist). Rates of bloodmeal digestion and ovarian development are temperature dependant (Vinogradova 2000). *Culiseta melanura* Coquillett is a common mosquito species in New Jersey, which can only complete a gonotrophic cycle at temperatures above 6.4˚C (Mahmood and Crans 1997). *Culex pipiens pipiens* L., which overwinters as an adult female, requires temperatures above 9.6˚C (Madder et al. 1983) or 10˚C (Vinogradova 2000) to complete a cycle. If *Cx. territans* coordinates its life cycle to that of its amphibian host, then it might be able to complete a gonotrophic cycle at temperatures around 3˚C, when its hosts are exiting diapause.

Our goal was to determine which factors contribute to the temporal and spatial synchrony of *Cx. territans* larvae with their amphibian hosts, and to use these factors to predict the presence of *Cx. territans* larvae. Our hypothesis was that *Cx. territans* synchronize their life cycle to match their amphibian hosts. Because amphibian hosts exist in aquatic habitats, we predicted that the presence of amphibians might coincide with the temporal and spatial distribution of *Cx. territans* larvae. We also predicted that

if *Cx. territans* females co-occur temporally with their amphibian hosts, then females might be capable of digesting bloodmeals at those temperatures.

Materials and Methods

Field study sites

Field study sites (N=11) were located in a 47-km^2 area of Stokes Forest and High Point State Park in northwestern New Jersey. Sites were 1 to 2 km apart, and were examined the summer before field experiments, to identify the presence or absence of mosquito larvae and amphibians, vegetation type, and flooding regime. The eleven sites were classified according to the Cowardin classification system (Cowardin et al. 1979), and consisted of five seasonally flooded forested wetlands, and five permanently flooded habitats with emergent vegetation. One permanent pond acted as a comparison site, whose depth and lack of vegetation was suitable for amphibians, but not for *Cx. territans* larvae. Global positioning system (GPS) coordinates were recorded for each site and analyzed using ArcView 3.1 software (ESRI, Redlands, CA). GPS site locations were overlaid onto Geographic information system (GIS) maps to determine soil type, soil acidity, percent organic matter, slope, drainage, elevation, wetland type, and wetland size. Additional information was collected weekly at each site, including current weather conditions (raining, overcast, sunny, snowing, and foggy), absence of water, and habitat disturbance. Weekly temperature, precipitation, barometric pressure, water table depth, and wind speed records were downloaded from the High Point and Sussex County weather stations (NJ Weather and Climate Network, Office of the NJ State Climatologist). The High Point station was chosen because it is located within our 47-

km^2 study area. The Sussex County station is located 11.8 km away from the High Point station, and was used to fill in any gaps in the weather data.

Field collections

Collections were made weekly from 14 March to 10 November 2004. Road closings during the first week limited access to four sites within hiking distance. Sampling was conducted in 20-min periods, to allow equal access to all 11 sites during daylight hours. During the first 15 min, all aquatic organisms were sampled using a standard mosquito dipper at different depths in the water column. Each site was sampled by skimming the surface, scanning below the surface, dipping into aquatic vegetation, and dipping into small areas and cavities. Mosquito larvae were placed in two-liter coolers, and transported to the lab for species and instar identification. When other invertebrates were collected while sampling, they were identified to family, and immediately released.

Amphibians were also collected with the mosquito dipper, or by hand. The final five minutes of each sampling period was used to sample additional amphibians and organisms using an aquatic net. All amphibians were identified on site by stage and species, and released.

Laboratory colony maintenance

Culex territans were colonized from field-collected larvae and adults from Sussex County based on techniques described by Benach (1970). Cultures were maintained at $23.8 \pm 0.3^{\circ}$ C and a 16:8 (L: D) cycle. Field collected larvae were held in shallow pans of tap water and fed ground rat chow daily (Purina Mills, St. Louis, MO). Water was

changed daily, and pupae were removed and placed in glass bowls, which were transferred to 61-cm^3 cages for emergence. Adults were provided a continuous supply of 10% sucrose, on cotton wicks, which was removed 24 h before experiments to increase blood-feeding frequency.

Laboratory studies

The effect of temperature on the length of the gonotrophic cycle was examined in nulliparous females (F1 and F2 generations), six to nine days old. During each experiment, an unrestrained bullfrog was placed into a cage containing approximately 100 female *Cx. territans*. Mosquitoes were given 4 hr to blood feed, and a fully engorged female was removed and placed in a separate 30.5-cm^3 cage, with a continuous supply of 10% sucrose. Cages were immediately placed in an incubator with a 16:8 (L:D) cycle. Blood feeding was repeated until 10 females were available for each temperature. Ten individual females were held at each of 11 temperatures: 3.5, 6.7, 9.0, 13.7, 16.8, 23.8, 24.5, 25.6, 26.0, 27.3, and 28^oC, for a total of 110 females. Each cage contained one fully engorged female, a white enamel pan with an octagonal brick on which females oviposited, and 10% sucrose. Cages were covered with wet towels and placed under plastic to increase humidity. Females were examined daily to record egg rafts, and mortality. The mean duration from blood feeding to oviposition (g_c) was determined for each temperature.

The number of days from egg to adult at temperatures 23.8, 26.0, and 27.3^oC were recorded for 15 egg rafts (5 per temperature), for a total of 1662 mosquitoes examined. Each egg raft was placed in a separate white enamel pan with ground rat chow. Water was changed daily. Pupae were placed in glass bowls, and emerging adults were added to

an existing colony. The three temperatures were chosen to simulate the peak monthly temperatures for High Point weather station (NJ Weather and Climate Network, Office of the NJ State Climatologist) for June (23.5^{o}C), July (26.1^{o}C), and August (25.2^{o}C).

Statistical analysis

Data were analyzed using SPSS software (SPSS Inc. 2005). The database consisted of 10 sample sites examined weekly over a 35 week period. To determine which factors correlated spatially with *Cx. territans* larvae, a single week was chosen from the dataset in order to compare the 10 sites. A Pearson correlation analysis was performed, where the dependent variable, presence of *Cx. territans* larvae at a given site, was correlated to soil pH, drainage, organic matter, elevation, slope, and the presence/absence of amphibians within the same sites. The analysis was repeated three times with randomly selected weeks. Variables with correlations greater than 0.5 for two or more of the weeks were added to a logistics regression model to predict the presence of *Cx. territans* larvae. For the logistics regression model, a single week was randomly selected for the months of June, July, August, and September. Variables not significant at 0.05 were removed from the model, until an appropriate model was determined. A model was determined satisfactory when all predictors in the model were significant, and the accuracy of predicting *Cx. territans* larvae was greater than 80%.

To analyze the temporal distribution of *Cx. territans* larvae an Autoregressive integrated moving average (ARIMA) time series analysis was performed using density (number of sites containing *Cx. territans* larvae) as the dependent variable. This was analyzed against water table depth, day length, temperature, precipitation, relative humidity, wind, barometric pressure, *R. catesbeiana*, *R. clamitans, Pseudacris crucifer*

Wied-Neuwied (spring peeper), invertebrate predators, salamanders, water presence, and other mosquito species. Normality of the data was assessed using Q-Q plots, and data was transformed according to results. The best ARIMA model was chosen based on stationary R-squared values. Variables that were determined to be predictors of *Cx. territans* larvae using the ARIMA model were further analyzed using a cross correlation analysis to find significant relationships between the data and weekly lags. Significant lags were determined by Box-Ljung Q-statistic less than 0.05.

Thermal heat summation model

The thermal heat summation model (Mahmood and Reisen 1981) was used to predict the temporal distribution of *Cx. territans* larvae based on temperature and degree-days. This model ($V = (t-t_0)/k$) was used to determine the thermal minimum (t_o) in which females are capable of digesting bloodmeals, and the number of degree-days above this thermal minimum (k) to complete one gonotrophic cycle. The time in days to complete one gonotrophic cycle (g_c) for *Cx. territans* was calculated for increasing temperatures, where g_c = mean number of days from eggs in stage I (Christophers 1911) to oviposition. The rate of ovarian development (V), was calculated as $V = 1/g_c$. By plotting rate of ovarian development (V) over increasing temperatures, a linear regression ($V = a + bt$) was created. The thermal minimum (t_o) was calculated using the regression as (–a/b). The number of degree-days above thermal minimum (k) was calculated as (1/b) using this regression.

To estimate the days since blood feeding (D) for field-collected larvae, a new model was created using the thermal minimum t_0 and the thermal constant k. In this model $D = k - \Sigma\, n_i(t_i - t_0)$, where n_i = the number of days occurring at the mean

temperature t_i. This model was used to determine the date when *Cx. territans* females took their first bloodmeal based on the first field collections of larvae. To test our model, we used the formula $g_c=k/\sum(t-t_0)$, to predict the first occurrence of *Cx. territans* larvae based on mean daily temperatures at the Sussex County weather station. We calculated the total degree-days for temperatures above the thermal minimum (t_0), starting with the first day in which the mean was above t_0. From these data, we could estimate the date on which degree-days approached the thermal constant (k). If our model is accurate, then this date should accurately predict the date of our first collection of *Cx. territans* larvae.

Results

Field studies

Culex territans larvae were present in 71 of the 350 collection attempts and were present at least once in 7 of the 10 collection sites. Two of the sites that did not hold larvae were small temporary pools that briefly held water. The third site that did not contain larvae was the lake that served as our control. Out of the total collections, 58% of amphibians were collected in permanent water, and 42% in seasonally flooded habitats. *Culex territans* larvae showed a similar trend towards permanent water (77%), as opposed to seasonally flooded habitats (23%).

Culex territans larvae were present earlier than other adult overwintering species. In 2004, *Cx. territans* larvae in northern New Jersey were first detected on 6 May. By contrast, *Anopheles quadrimaculatus* Say larvae first appeared on 28 May and *Uraenotania sapphirina* (Osten Sacken) on 7 June.

Culex territans were collected with other species of mosquito larvae, including *Ur. sapphirina, An. quadrimaculatus, Aedes canadensis canadensis* (Theobald),

Psorophora sp. and *Ae. trivittatus* (Coquillett). Sampling sites were rich in invertebrate fauna, including Dysticidae, Notonectidae, Chaoboridae, Lestidae, Aeshnidae and Amphipoda. *Culex territans* larvae were collected (89%) in habitats with invertebrate predators.

Of the 71 collections of *Culex territans* larvae, the following amphibians occurred concurrently: *R. clamitans* (56), *R. catesbeiana* (20) *P. crucifer* (10), *Ambystoma sp.* (9), *R. palustris* (5), *R. sylvatica* (3), and *H. versicolor* (2). Pearson correlations were conducted for week 23 of sampling, and although they were not significant, the presence of *Cx. territans* larvae showed the strongest correlations with the presence of *Rana clamitans* (r = 0.56, *P*=0.074) and number of weeks with continuous water in habitat (r = 0.56, *P*=0.074). The presence of *Cx. territans* larvae did not correlate with soil pH, drainage, elevation, slope, other amphibian species, or wetland size. Results did not differ when we re-ran the analysis using weeks 21 or 27. Both presence of *R. clamitans* and water were added to the logistics regression analysis to predict *Cx. territans* larvae. In this analysis, the presence of water was not significant, and could not predict the presence of mosquito larvae. Water was removed from the logistics regression analysis, and the presence of *R. clamitans* was significant (*P* = 0.05), and alone could predict *Cx. territans* larvae (88.9%) using this model.

Time series analysis produced two separate models to predict the temporal distribution of *Cx. territans* larvae. The first model predicted *Cx. territans* larvae based on its own temporal distribution (R^2 = 0.495). The second model showed the presence of *R. clamitans* was the best fitting model to temporally predict the presence of *Cx. territans* larvae (R^2=0.794). Cross correlation analysis (Fig. 2.1) showed *Cx. territans* larvae

correlated temporally with the presence of *R. clamitans* during the same week (R^2 = 0.672, P = 0.004) and when *R. clamitans* occurred two weeks prior (R^2 = 0.742, $P \leq$ 0.001). Precipitation, water table depth, barometric pressure, standing water presence, invertebrates, or other amphibian species did not correlate with the temporal distribution of *Cx. territans* larvae using either of these analyses.

On average, *Cx. territans* larvae appeared in sites 2.36 $\pm$ 0.67 SE weeks after the first appearance of *R. clamitans* within the same site. This trend was seen in permanent water habitats (4 $\pm$ 1.5 SE) vernal pools (1.67 $\pm$ 1.2 SE) and autumn pools (1.8 $\pm$ 0.92 SE). Of the 71 collections of *Cx. territans* larvae, 15 did not occur concurrently with *R. clamitans*. All collections of *Cx. territans* larvae occurred in a site that either currently or had previously contained *R. clamitans*.

Laboratory studies

The average number of days from egg to adult [D_a=62.7-1.8 (t ^oC)] for *Cx. territans* was 15.7 $\pm$ 0.76 SE, and decreased as the temperature increased. Developmental time from egg to adult was 13.8 days $\pm$ 0.37 SE at 27.3 ^oC and 13.8 days $\pm$ 0.2 SE at 26.8 ^oC, but was longer (19.6 days $\pm$ 0.6) for 23.9 ^oC (F=62.3, df=2,12, R^2=0.87 P<0.001).

As temperature increased, the number of days required from blood feeding to oviposition decreased (Y=-0.998x + 34.75), (R^2=0.96, F=1003, P < 0.001). The average length of the gonotrophic cycle ranged from 6 to 26 days depending on temperature (Fig. 2.2), differing significantly every 2 to 3 ^oC (F=1886, df=8,81, P<0.001). The regression of ovarian development rate against temperature was used to calculate the thermal minimum and length of gonotrophic cycle (Fig. 2.3). The slope and intercept were used

to determine the thermal minimum of the gonotrophic cycle as well as the number of degree-days needed for oviposition. In order to complete one gonotrophic cycle, 192.3 degree-days (*k*) above the thermal minimum (t_0) 3.9°C are required. Therefore, the rate of ovarian development can be calculated as *V*= (t-3.9)/192.3. *Culex territans* did not oviposit at temperatures of 3.2 and 6.5˚C, despite a mean longevity for females of 31 days.

Using the thermal heat summation model, the number of degree-days and days to complete the gonotrophic cycle can be determined using the average temperatures (Fig. 2.4). Based on these data, the date of blood feeding can be extrapolated from field-collected larvae. The first larval collections of *Cx. territans* occurred on 6 May 2004. The mean monthly temperatures during March, April, and May were 3, 9.5, and 17.6˚C respectively. *Culex territans* requires 192.3-degree-days to complete its gonotrophic cycle. By adding mean monthly temperatures to the formula for *D*, the first larvae collected were derived from a female which blood fed 33 days prior. This indicates that *Cx. territans* took its first bloodmeal the last week of March or first week of April, corresponding to initial collections of amphibians in the same area.

To test our model, we applied mean daily temperatures to $\Sigma(t\text{-}3.9)=192.3$ for all days above 3.9˚C. The first date above 3.9˚C was 29 March 2004. Based on our model, the first oviposition should have occurred in nature on 4 May 2004, which corresponds to our first collection of *Cx. territans* larvae on 6 May 2004.

Discussion

The presence of *R. clamitans* was the only variable that could spatially predict the presence of *Cx. territans* larvae. Crans (1970) found *Cx. territans* readily blood feeding

on *R. clamitans* in nature. If *Cx. territans* are obtaining bloodmeals from *R. clamitans,* then they are ovipositing in sites containing their preferred hosts. The advantage of ovipositing where there are *R. clamitans* is that as adult mosquitoes emerge, females will be close to bloodmeals. *Rana clamitans* prefer to remain in the same habitat throughout the course of the summer (Breder et al. 1927). Therefore, by ovipositing where hosts will be available, female *Cx. territans* aid in the survival of their offspring.

Occasionally *R. clamitans* will move outside of its home range during precipitation, breeding activities, and overwintering (Martof 1953). This behavior allows *R. clamitans* to forage for prey, build lipid reserves for overwintering, and establish new breeding and overwintering sites (Lamoureux et al. 2002). This foraging behavior occurs in the late summer and fall (Lamoureux et al. 2002) when vernal pools begin to hold water for extended periods. Vernal pools by definition are wetlands that are temporarily flooded during the winter and spring and dry throughout the summer (Mitsch and Gosselink 2000), but can retain water during periods of heavy rain. As trees begin to lose foliage and water demand is reduced, rainfall persists in pools for longer durations. The autumn pools at our study sites held water for 10.5 ± 1.6 weeks from September to November. In weeks where pools became re-flooded, amphibians were found in all pools immediately after rainfall. As *R. clamitans* colonized new habitats, *Cx. territans* larvae appeared in the same sites two weeks later. Use of the pools by *Cx. territans* always occurred after amphibians were already present. Therefore, both amphibians and mosquito larvae were taking advantage of new habitats as they became available.

Our study showed that *Cx. territans* females oviposit in permanent and vernal habitats. Early populations of *Cx. territans* are found in permanent water habitats, where

subsequent populations of *Cx. territans* use permanent water and late summer temporary pools. Vernal pools were not used in the spring because they were almost dry when *Cx. territans* females initiated oviposition. *Culex territans* larvae require at least two weeks (15.7 days ± 0.8SE?) to complete development from egg to adult. The earliest frogs collected on 14 March, *R. sylvatica*, occurred in vernal pool habitats. If *Cx. territans* were using *R. sylvatica* for bloodmeals, they would have needed to migrate after the bloodmeal to find a permanent water site for oviposition. *Culex territans* has been observed bloodfeeding from *P. crucifer* (Crans 1970), which uses both permanent and vernal pool habitats, and may provide the first bloodmeals of the season.

The spatial distribution of *Cx. territans* larvae did not correlate with soil characteristics. *Culex territans* larvae occurred in sites with a range of pH values, slopes, elevations, soil types, and wetland characteristics. Udevitz et al. (1987) showed that *Cx. territans* larvae occurred in water with a wide range of water chemistry profiles, such as pH and other ion concentrations. This lack of preference may explain why *Cx. territans* larvae have been reported from many types of habitats including permanent water, containers, ditches, marshes, ponds, streams, and tires (Joy and Clay 2002).

Low temperatures may impose the greatest physical barrier to *Cx. territans* existing temporally with their amphibian hosts. Near freezing temperatures can cause reduced oxygen consumption as well as increased risk of desiccation in terrestrial arthropods (Soemme 1999). If *Cx. territans* are using early amphibians as a bloodmeal source, they would need to be able to digest bloodmeals below 5°C, when amphibians are exiting diapause in New Jersey. *Culex territans* has a thermal minimum of 3.9ºC, in which females can digest a bloodmeal. This value is lower than for *Cu. melanura*

(Coquillett) (Mahmood and Crans 1997), *An. culicifacies* Giles (12.6ºC) (Mahmood and Reisen 1981), or *An. stephensi* Liston (8.9ºC) (Mahmood and Reisen 1981). These data indicate that *Cx. territans* has a threshold lower than other species of mosquitoes and likely has thermoregulation mechanisms that allow them to function at lower temperatures. In northern New Jersey, the first amphibians begin to appear in March and April. At that time, the mean daily temperature is rising from 2.9 to 8.8ºC. *Culex territans* females have been collected in resting boxes as early as late March in northern New Jersey as part of the NJ State Surveillance and Vector Surveillance programs. The mean monthly temperatures in northern New Jersey for the month of March average around 3.5˚C, with an average of 4.2˚C in the past decade (NJ Weather and Climate Network, Office of the NJ State Climatologist). If females are exiting diapause at this time, then they may be able to take advantage of early season bloodmeals. Our data support that females would have taken their first bloodmeal around the last week of March, when temperatures were approximately 3ºC. *Culex pipiens* with a thermal minimum of 10ºC would not be able to digest bloodmeals until late April or early May. Lobkova (1980) found that female *Cx. p. pipiens* left hibernacula when the temperatures were 10 to 16ºC, corresponding to the temperatures in which they are capable of undergoing a gonotrophic cycle. Therefore, it would be a disadvantage to exit diapause early, if they are not capable of digesting bloodmeals.

Temporal distribution did not overlap with precipitation events, number of wet sites, or fluctuations in water table. Because they use available sites of varying types, their distribution would not be affected by precipitation events. However, based on climatological data from New Jersey, 2004 was neither an unusually wet or dry year.

Habitat quantity can affect the oviposition behavior of mosquitoes (Reiskind and Wilson 2004), suggesting that extreme drought or rainfall could alter the oviposition behavior of *Cx. territans*.

Many parasites synchronize their life cycle to maximize the opportunity of finding a suitable host, which can be highly dependent on both biotic and abiotic factors (Cattadori et al. 2005). Temperature and weather conditions greatly affect populations of *R. clamitans* (Martof 1956). If *Cx. territans* are synchronizing their life cycle to match that of *R. clamitans*, then their distribution should also correlate with temperature and weather. However, the first amphibians to exit diapause include *R. sylvatica* and *P. crucifer*, both of which have freeze tolerance mechanisms (Schmid 1982). To synchronize its lifecycle to its amphibian hosts, *Cx. territans* adapted by completing its first gonotrophic cycle of the season at near freezing temperatures. However, by ovipositing in sites that contain hosts, females maximize their offspring's potential to locate a host after emergence. Our results support the hypothesis that *Cx. territans* coordinate their life cycle events with their amphibian hosts, specifically to *R. clamitans*.

By temporally and spatially co-occurring with their amphibian hosts, they are more likely to find bloodmeals, eliminating the need to travel long distances in search of a host. However, in order to coexist with their potential hosts, *Cx. territans* have adapted to lower temperatures, by being able to undergo a gonotrophic cycle at near freezing temperatures.

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Table 2.1. A comparison of thermal minimum (°C) and gonotrophic duration (degree days) for eight mosquito species

Species	Thermal Minimum (°C)	Gonotrophic cycle (Degree-days)	Reference
Culex territans Walker	3.9	192.3	Current article
Cx. tarsalis Coquillett	*n/a*	123.5	Reisen et al. 1992
Culiseta melanura Coquillett	6.4	95.9	Mahmood and Crans 1997
Culicoides variipennis (Coquillett)	7.7	50.7	Mullens and Holbrook 1991
Anopheles stephensi Liston	8.9	43.4	Mahmood and Reisen 1981
Cx. pipiens pipiens L.	9.6	57.8	Madder et al. 1983
Cx. pipiens pipiens L.	10.0	71.0	Vinogradova 2000
An. culicifacies Giles	12.6	29.7	Mahmood and Reisen 1981

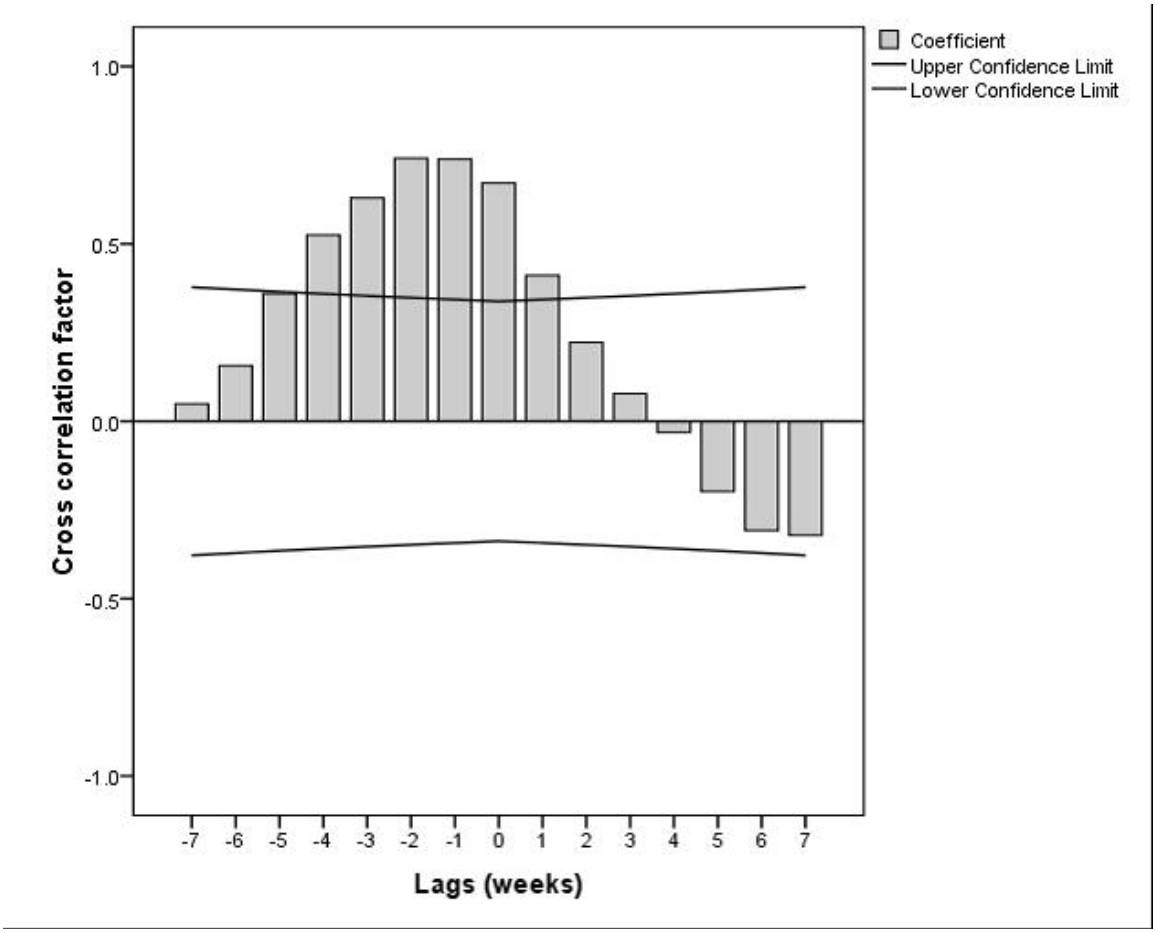

Fig. 2.1. Cross correlation functions (CCF) between the presence of *Culex territans* and the presence of *Rana clamitans*. Lag periods are in weeks. Upper and lower 95% confidence intervals are indicated by the two horizontal lines. Coefficients must cross the confidence interval to be statistically significant. The greater the CCF value, the stronger the correlation.

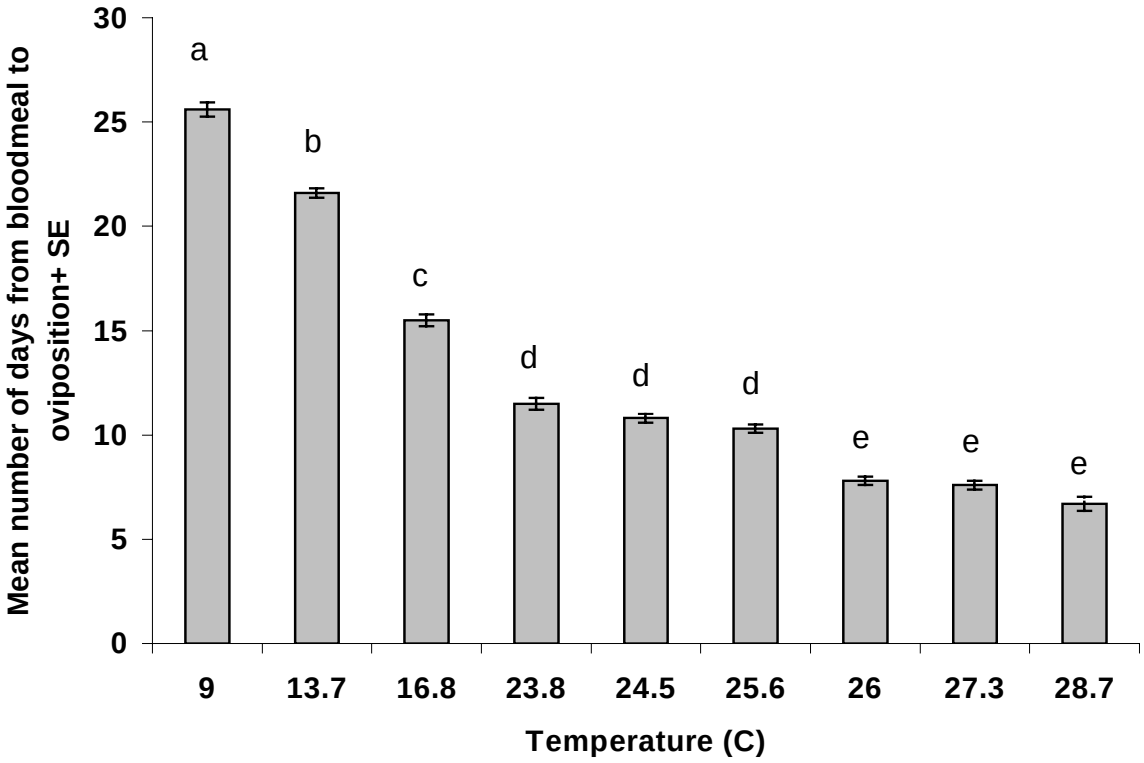

Fig. 2.2. Length of gonotrophic cycle (in days) at increasing temperatures (˚C) for *Cx. territans*. Temperatures were compared using an ANOVA. Bars followed by the same letter are not statistically significant (P=0.05) based on Tukey's post hoc test.

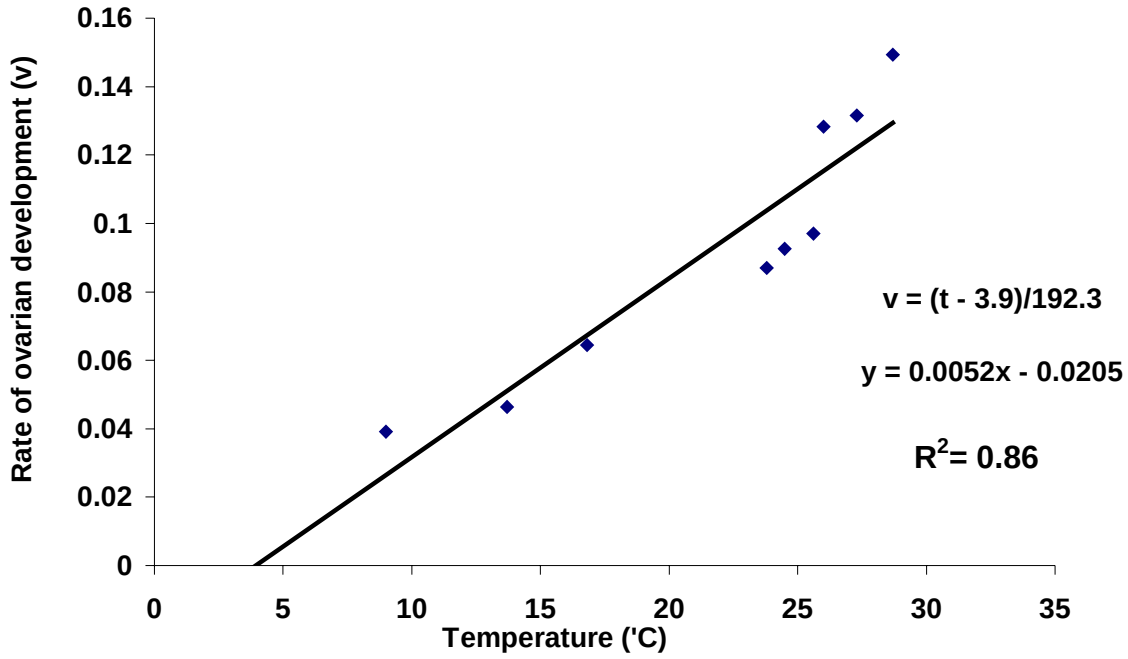

Fig. 2.3. Rate of ovarian development (V=(t-t_0)/k) for *Cx. territans*, correlated with increasing temperatures (˚C)

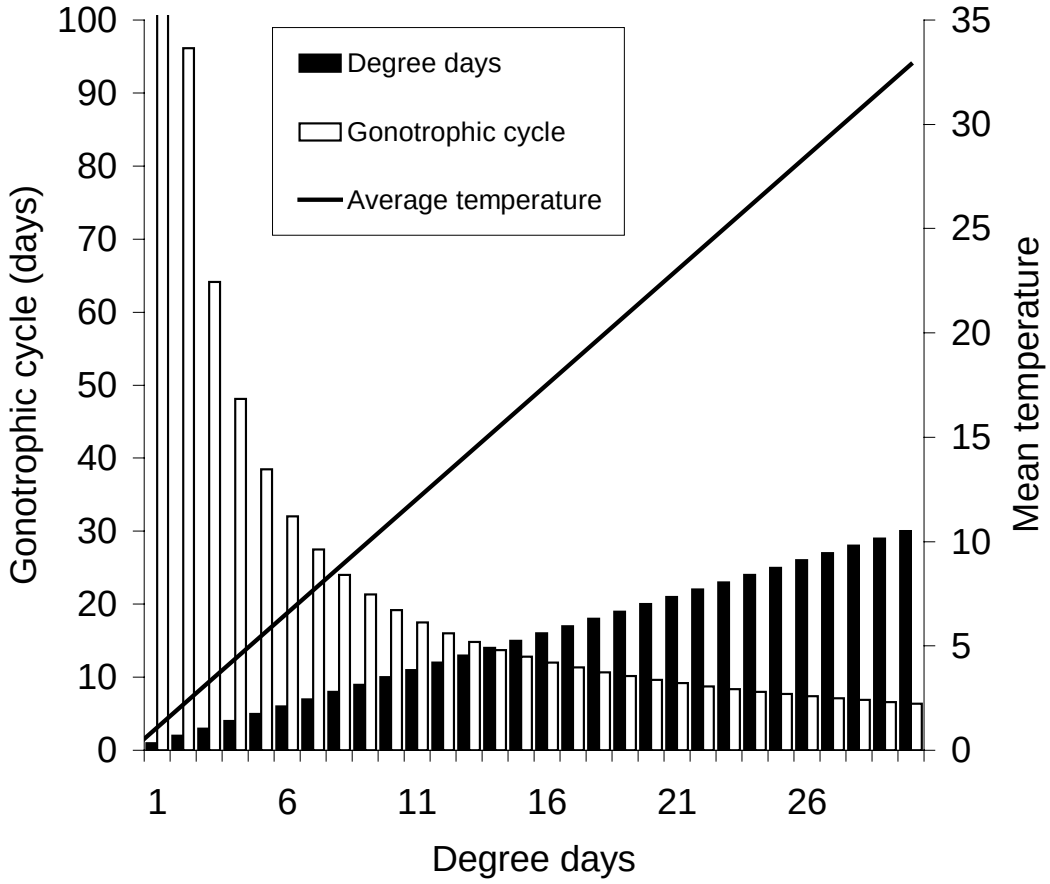

Fig. 2.4. Relationship among degree-days, gonotrophic cycle, and mean temperature for *Cx. territans*. The black line indicates the mean temperature. The white bar indicates how many days a female would take to complete one gonotrophic cycle at that mean temperature. The black bar indicates the number of degree days that accumulates at the mean temperature.

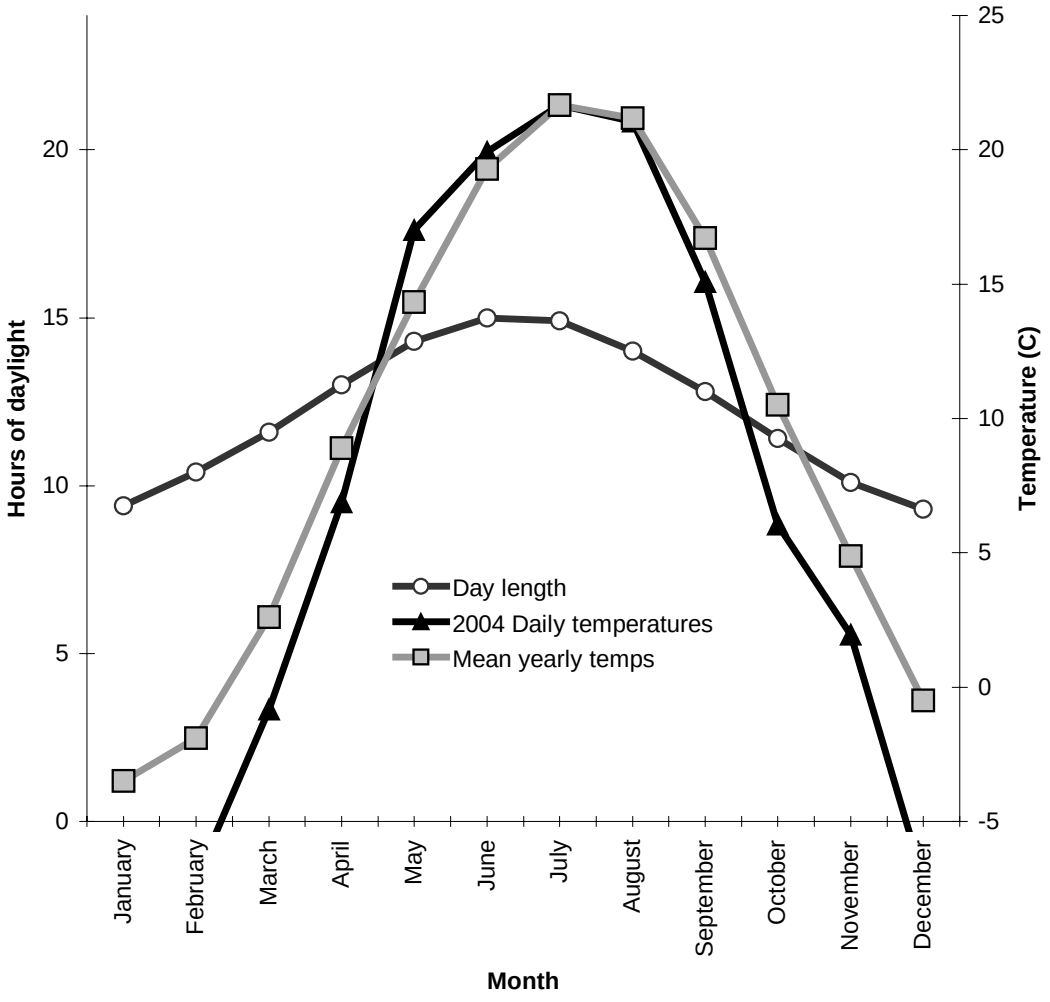

Figure 2.5. Day length, mean yearly temperatures, and mean temperatures for 2004, from the Sussex county weather station.

Chapter 3:

Vertebrate hosts and phylogenetic relationships of amphibian trypanosomes from a potential invertebrate vector, *Culex territans* Walker (Diptera: Culicidae)

Introduction:

Culex territans Walker (Diptera: Culicidae) is found throughout most of the Northern hemisphere (Knight and Stone, 1977). Females prefer amphibian bloodmeals (63%), feeding intermittently on Reptilia, Aves, and Mammalia (Savage et al., 2007). In New Jersey, *Cx. territans* occasionally feed on reptile and avian sources, but prefer amphibians (88.5%) (Crans, 1970). *Culex territans* is a competent vector for several amphibian parasites, including the nematode *Foleyella flexicauda* (Benach 1971). In New Jersey, bullfrogs (*Rana catesbeiana*) show high levels of co-infection with *F. flexicauda* and trypanosomes (Benach, 1971). Amphibian trypanosomes infect most anuran species (Bardsley and Harmsen, 1973). Barta and Desser (1984) found trypanosomes are prevalent parasites infecting amphibians in Ontario, an area where *Cx. territans* is common (Desser et al., 1973).

Trypanosomes are cosmopolitan vertebrate parasites that are transmitted by invertebrate vectors (Hamilton et al., 2004). Within the genus *Trypanosoma,* the biology, hosts, and mode of transmission are unknown for many species. Phylogenetic analysis is used to determine evolutionary origins and relationships of trypanosomes, to clarify fundamental questions on the biology of these species. Various Trypanosomatidae phylogenies show coevolution of trypanosomes with vertebrate hosts, invertebrate vectors, and biogeography. In most interpretations, the amphibian trypanosomes were among the earliest to diverge from monoxenous trypanosomatids (Hamilton et al., 2004). Amphibian trypanosomes are placed within a monophyletic group referred to as the aquatic clade (Hamilton et al., 2007), which are primarily leech transmitted. Trypanosomes within this clade also occur in terrestrial vertebrates, suggesting an insect

vector might transmit trypanosomes within the aquatic clade (Barta and Desser, 1984). A further understanding of vertebrate and invertebrate hosts of aquatic trypanosomes might shed light on unresolved phylogenies, and aid in understanding the evolution of parasitism within this group.

It has been proposed that *Cx. territans* might serve as a vector for amphibian trypanosomes, including *T. ranarum* (Barta and Desser, 1984). Transmission of amphibian trypanosomes has been demonstrated in Diptera, including Corethrellidae (Johnson et al. 1993), Culicidae (Ramos and Urdaneta-Morales, 1977) and Psychodidae (Anderson, 1968), indicating that leeches are not the only vectors of amphibian trypanosomes. Van Dyken et al. (2006) detected trypanosomes in unengorged *Cx. pipiens* and *Cx. tarsalis*, and suggested that trypanosomes might increase the vector competence of West Nile virus.

Parasitic trypanosomes rely on bloodfeeding vectors for transmission to new hosts (Hamilton et al., 2007). Trypanosome transmission occurs while blood feeding via saliva, fecal deposits being rubbed into wounds, or by host ingestion. Desser *et al*. (1973) found *T. rotatorium* development to the epimastigote stage in *Cx. territans,* but were unable to experimentally infect *Rana pipiens* with epimastigotes (Desser *et al.,* 1975). The authors did not attempt transmission via bloodfeeding, or with other species of trypanosomes. Trypanosomes require suitable host conditions, such as a specific pH, to initiate the development into the infective stage (Ucros et al., 1983); thus a species might require bloodfeeding to initiate development. Martin and Desser (1991) found that infective stage *T. fallisi*, which infects amphibians, migrated to the leech's proboscis during bloodfeeding. *Trypanosoma corvi* colonizes the midgut of *Cx. pipiens*, degrades

the stomodeal valve, and is later transmitted via regurgitation during feeding (Votypka et al., 2004).

Our goal was to further the understanding of amphibian trypanosomes and phylogenies, by examining the life history of a potential invertebrate vector, *Cx. territans*. The objective was to identify the vertebrate sources of bloodmeals, while concurrently examining *Cx. territans* females for trypanosomes. Adding a tritrophic study of trypanosome species, vertebrate, and invertebrate hosts to existing phylogenies can elucidate the evolution of parasitism within the Trypanosomatidae. Our hypothesis was that *Cx. territans* feeds predominantly on amphibian blood, and acquires amphibian trypanosomes during bloodfeeding. We predicted if trypanosomes coevolved with their invertebrate hosts, then all trypanosomes acquired by *Cx. territans* would be contained within the same monophyletic clade.

MATERIALS AND METHODS

Mosquito collections

Culex territans females were collected in New Jersey using resting box, light, and carbon dioxide-baited traps, from 2003 to 2007 through a state-wide vector surveillance program (Crans and McCuiston, 1993). Resting boxes were set out each year from May to October in Atlantic, Burlington, Camden, Cape May, Monmouth, and Salem counties. In 2005 and 2006, resting boxes were also set in early April in Bergen and Sussex counties. Mosquitoes were anesthetized using triethyl-amine, hand aspirated and placed in 10 ml glass vials within clear plastic bags in a cooler with dry ice. Blooded specimens of *Cx. territans* were identified to species on a chill table, and placed in an individually

labeled 1.5 ml microcentrifuge tube. Trap type and location, Sella stage of blood meal digestion (WHO, 1975), and date were recorded. Specimens were stored at –70 ºC.

Primer design

Primers were designed using Primer3 software (Rozen and Skaletsky, 2000). Random sequences were chosen for trypanosomes and amphibians from GenBank, and aligned using BioEdit Sequence Alignment Editor Version 7.0.5.3 (Hall, 1999) to create a consensus sequence. Primers were selected based on melting temperature, GC content, primer dimers, and hairpins. Mosquito sequences were added to the alignments to exclude amplification of mosquito DNA with the developed primers. Two primer sets were developed: one to amplify a 406 bp region of the cytochrome b gene in amphibians, and one to amplify a 426 bp region of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of trypanosomes (Table 3.1). The GAPDH gene was chosen due to its slow rate of molecular evolution (Hamilton et al., 2004). Primers for determining cytochrome b in mammal, bird, and reptile DNA were based on previous studies (Scott, 2003). Primers amplifying host DNA were tested on *Rana catesbeiana* (bullfrog), *R. clamitans* (green frog), *Bufo americanus* (American toad), *R. sphenocephala* (southern leopard frog), *R. virgatipes* (carpenter frog), *Chrysemys picta* (painted turtle), *Macrochelys temminckii* (snapping turtle), *Nerodia sipedon* (water snake), *Gallus gallus* (chicken), *Corvus brachyrhynchos* (American crow), *Equus caballus (*horse), *Procyon lotor* (raccoon), and *Sylvilagus floridanus* (Eastern cottontail *rabbit*) DNA.

Molecular techniques

Mosquitoes were pinned to a Styrofoam block, and the legs and wings were removed to minimize extraneous DNA. The mosquito's abdomen was cut from the

thorax, and the blood packet was rolled out using a probe. Blood packets were immediately transferred to a sterile 1.5 ml microcentrifuge tube at -70°C until further processing.

Mosquitoes were homogenized in 140 µl of DNAzol® (Molecular Research Center, Cincinnati, OH, USA) using a sterile pestle, and placed in a heat block at 95ºC for 10 min. Tubes were centrifuged at 11,000 RPM for 10 min, supernatant removed, and 50 µl of 100% EtOH added to each tube. Samples were centrifuged twice for 10 min at 11,000 RPM, the liquid was decanted, and the pellet was resuspended in 35 µl of distilled H_20, and stored at 4°C.

The PCR sample consisted of 2 µl of DNA to 48 µl of master mix containing Takara *Ex* Taq Polymerase (Takara Bio Inc, Seta, Japan). Concentrations were based on manufacturer guidelines. Reactions were run on a GeneAmp® PCR System 9700 thermocycler for 50 cycles. Each cycle had 1 min denaturing (94°C), 30 sec annealing (54°C) and 1 min extension (72°C). *Trypanosoma cruzi* genomic DNA (ATCC 30266D, American Type Culture Collection, Manassas, VA) served as the positive control.

Amplified DNA was examined on a 1.25% low EEO agarose gel and modified TAE buffer. Samples were run with a 100 bp ladder (Promega Corp., Madison, WI) at 84V for approximately 60 to 100 minutes, stained using Ethidium Bromide, and visualized on a UV light table. Bands were cut from the gel, purified using Montage® DNA purifying kit (Millipore, Billerica, MA), and sequenced at the Rutgers University Biotechnology Center for Agriculture and the Environment.

Resulting sequences were retrieved and edited using the Chromas®Lite software. The chromatograms of sequences were further examined by aligning the forward and

reverse sequences. This allowed for filling in any missing base pairs. Sequences were compared to other known sequences in the GenBank database using BLAST® searches.

To determine the phylogenetic relationships of the amplified trypanosome DNA, results were aligned with Tyrpanosomatidae sequences using BioEdit Sequence Alignment Editor version 7.0.5.3 (Hall, 1999). The alignment consisted of a 393 bp region of the GAPDH gene. The alignment included 37 sequences from Trypanosomatidae, 9 sequences from field-collected *Cx. territans,* and two sequences from the *T. cruzi* control. The outgroup consisted of *Euglena gracilis* and *Bodo saltans.* Our original outgroup for the maximum likelihood analysis was *Lutzomyia longipalpis*. This species was chosen to represent Diptera DNA, as the GAPDH gene was not available for *Cx. territans.* Results showed that our amplified bands were *Trypanosoma* DNA, and not dipteran DNA, so the dipteran outgroup was removed from the analysis.

Maximum likelihood analysis of nucleotide alignments were carried out using PAUP* version 4.0b10 (Swofford, 2005). The model was determined using Modeltest 3.7 software (Posada and Crandall, 1998), and consisted of the general time reversible model (GTR+G) with 4 category gamma distributions. The parameters included a heuristic search with tree branch reconnections.

Bayesian analysis was performed using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). The general time reversible model was used for analyses of nucleotide sequence alignments. Rate variation across sites was modeled using a gamma distribution. The Markov chain Monte Carlo search was run with four chains for 2,000,000 generations, with trees sampled every 100 generations, until the average standard deviation reached 0.007.

Statistical analysis

Data were analyzed using SPSS software, Version 15.0 (SPSS Inc., 2005). For the bloodmeal analysis, a linear regression model was performed to determine if the rate of bloodmeal digestion affects the ability to amplify host DNA. Variables included Sella stage of development (WHO, 1975), and percentage of samples resulting in successful host identification. An Analysis of Variance (ANOVA) was performed to determine the affects of location on trypanosome prevalence rates. New Jersey was divided into three regions: northern (Bergen and Sussex counties), central (Monmouth, Ocean, and Mercer counties), and southern (Atlantic, Burlington, Camden, Cape May, and Salem counties), and the prevalence rates were compared. Five year means of prevalence rates and standard errors were plotted. A curvilinear regression was performed to determine the temporal pattern of trypanosome prevalence rates in *Cx. territans.*

RESULTS

We collected 119 bloodfed *Cx. territans* females from ten counties in New Jersey representing the north (Sussex and Bergen counties), central (Monmouth, Mercer, and Ocean counties), and southern (Atlantic, Burlington, Camden, Cape May, and Salem counties) parts of the state. We were able to amplify the cytochrome b region in 42 of the 119 specimens. Positive vertebrate host identifications were made in 29 of the females. *Culex territans* readily fed on amphibians (Fig. 3.1), including *R. clamitans* (48%), *R. catesbeiana* (24%), *R. sylvatica* (10%), *R. virgatipes* (7%), *Pseudacris crucifer* (4%) and *R.* spp. (4%). A single bloodmeal was identified as an unknown reptile (4%). Amplification of host DNA was most likely when bloodmeals were in an early stage of

digestion (Fig. 3.2). The ability to amplify host DNA decreased as bloodmeal digestion increased (R^2= 0.61, *F*=9.17, *P* = 0.02).

Vertebrate hosts varied throughout the state, and included *R. clamitans* (63%), *R. catesbeiana* (31%), and *Pseudacris crucifer* (6%) in Northern NJ, *R. clamitans* (100%) in central NJ, and *R. sylvatica* (42.8%), *R. virgatipes* (28.6%), *R. catesbeiana* (14.3%), and an unknown reptile (14.3%) in southern NJ. Sample size was too small to determine seasonal variation in bloodmeal hosts. *Rana clamitans* and *R. catesbeiana* were a bloodmeal source throughout the spring and summer.

Trypanosomes were detected in 24 (20%) of the bloodmeals examined. In six of the samples containing trypanosomes, there was also positive host identification, which included *R. clamitans* (50%), *R. catesbeiana* (16%), *R. virgatipes* (16%), and *R.* spp. (16%). Amplification of trypanosome DNA was not affected by the stage of bloodmeal digestion (R^2 = 0.05, *F*=0.33, *P*=0.58). Trypanosomes were amplified in females that were fully engorged (14.3%) to almost fully gravid (20%).

Samples containing trypanosomes were obtained in each of the ten counties where *Cx. territans* were examined (Fig. 3.3). Females with the highest prevalence of trypanosomes occurred in Atlantic (100%) and Monmouth (52%) counties, moderate rates occurred in Burlington (33%), Camden (33%), Mercer (25%), and Sussex (25%) counties, and low rates occurred in Ocean (14%), Salem (10%), Bergen (7%), and Camden (6%) counties. There was no significant difference (*F*=1.01, *P*=0.417, df = 2, 9) between prevalence rates throughout northern (13.2 $\pm$ 10.4), central (30.3 $\pm$ 8.5), and southern (17.1 $\pm$ 7.4) New Jersey.

The presence of trypanosomes in the bloodmeals occurred in a seasonal pattern (Fig. 3.4). The temporal distribution showed a quadratic trend (R^2=0.897, *P*=0.033), where the prevalence of trypanosomes within females increased in the spring, peaked in June, and then decreased towards the end of the season. The first infected *Cx. territans* was collected in April. A higher proportion occurred in females collected in June (37.5%), July (25%) and August (25%). No trypanosomes were collected in September, although 12% of the overall engorged females were collected during this month.

Nine trypanosome sequences were used in the phylogenic analysis, based on the quality of the returned sequence. Inferred phylogenetic trees from the Bayesian and maximum likelihood supported the placement of the aquatic and terrestrial clades. Results of our Bayesian analysis (Fig. 3.5) showed 100% bootstrap support that all of our samples from *Cx. territans* placed in the aquatic clade, and 100% support that eight our samples belonged to the group of amphibian trypanosomes, including *T. rotatorium, T. fallisi,* and *T. mega*. One of our samples was closely related to a trypanosome isolated from an aquatic leech.

In both the maximum likelihood and Bayesian analyses, the *Trypanosoma* formed a monophyletic group comprising aquatic and terrestrial species. The aquatic clade, which contains amphibian and fish trypanosomes, formed a distinct lineage from the terrestrial *T. brucei* and *T. cruzi* clades. Although samples from *Cx. territans* came from at least 3 species of amphibians, all isolated trypanosomes occurred within the aquatic clade. The sample Terr108, grouped with an aquatic leech. We were unable to identify the vertebrate host in this sample, but *Cx. territans* was at an early stage of bloodmeal digestion. Within the amphibian clade, our trypanosome samples grouped with *T. fallisi*

and *T. rotatorium*. A 100% bootstrap support combined Terr066 (*Rana virgatipes*), from southern New Jersey with *T. rotatorium*. Two trypanosomes collected in 2006 from Monmouth county, Terr086 (*Rana* sp.) and Terr087 (*Rana clamitans*), were grouped together (100% bootstrap support). These two trypanosomes clustered (92% bootstrap) with remaining samples (Terr107, Terr112, Terr108, Terr117 and Terr113) collected from central NJ in 2007. The *T. cruzi* control was sequenced twice. On both occasions, our sequences were correctly placed with *T. cruzi* in our phylogeny (100% bootstrap support).

DISCUSSION

We found *Cx. territans* readily bloodfed on frogs, including *R. clamitans*, *R. catesbeiana*, *R. sylvatica*, *P. crucifer*, and *R. virgatipes*. *Culex territans* has been observed blood feeding from each of these species in nature (Crans, 1970). Blood meals from non-anuran hosts include rabbit and rodent (Crans, 1970), horse, raccoon, American robin, and common grackle (Savage et al., 2007).

Host preference is affected by temporal and spatial abundance of potential hosts (Savage et al., 1993). Regardless of month and location, the highest proportions of bloodmeals were from *R. clamitans*. *Culex territans* exists both temporally and spatially with *R. clamitans* (Bartlett-Healy in press), suggesting that *Cx. territans* acquires bloodmeals from hosts near their oviposition site. Desser et al. (1973) found that *Cx. territans* were prevalent in their study areas around *R. clamitans*, suggesting that these mosquitoes could be vectors of anuran trypanosomes.

Barta and Desser (1984) reported infection rates of *T. ranarum* within *R. catesbeiana* (4%), and *R. clamitans* (10.5%), but infection rates of *T. rotatorium* were

high for *R. catesbeiana* (52%), and *R. clamitans* (43.9%). If *Cx. territans* were acquiring *T. rotatorium,* our results should mimic these high infection rates. Instead, our results are similar to those found with *T. ranarum.* This complex is made up of the giant anuran trypanosomes *T. mega, T. fallisi,* and *T. ranarum.* These species have been detected in diverse anurans, including *R. catesbeiana, R. clamitans, R. sylvatica,* and *B. americanus* (Barta and Desser, 1984). The potential vectors for many of the amphibian trypanosomes, including *T. ranarum* and *T. fallisi,* are still listed as unknown. Transmission studies using Diptera could elucidate this missing information.

Results showed a seasonal distribution of females infected with Anuran trypanosomes. This seasonal distribution has been documented in the literature for amphibian infections since the 1800s (Bardsley and Harmsen, 1973). In most cases, prevalence is highest in spring, and slowly decreases throughout the summer. Possible explanations for this distribution have included changes in photoperiod, temperature, amphibian glucose levels, and seasonal distribution of leeches (Bardsley and Harmsen, 1973). Leeches attach to amphibians beginning in May, and remain attached through June and July (Bardsley and Harmsen, 1973). By August, leeches are less abundant on amphibians, possibly explaining why our infection levels were highest in May, June and July. If seasonal variation was due exclusively to leeches, then trypanosome prevalence should be highest in all three months, and not show a rapid decrease in late summer. Another factor contributing to high infection rates in May is that frogs used as bloodmeals are mature adults that have been exposed to trypanosomes for over a year. As the season progresses, tadpoles are metamorphosing into adults, and by August there is a higher proportion of young adults.

Trypanosomes have been detected in all vertebrate classes. For many of these species of trypanosomes, the vector and mode of transmission remains unknown. Recent phylogenies have shown specific clades of trypanosomes, which may be similar by vertebrate host or invertebrate vectors. However, even within a particular clade, the vectors and modes of transmission may differ. Co-speciation does not occur between trypanosomes and their vertebrate and invertebrate hosts, suggesting that invertebrates are capable of transmitting a wide variety of parasites (Hamilton et al., 2007). Hamilton et al. (2007) attributes the evolution of trypanosomes to ecological host fitting, where organisms can colonize new hosts based on the traits they possess.

Based on our trypanosome phylogeny, one of the earliest divergences occurred at the aquatic clade. The first appearance of heteroxenous trypanosomes occurred in amphibians (Hamilton et al., 2007), which are primarily transmitted by leeches. Hamilton et al. (2004) proposed that parasitic trypanosomes evolved from monoxenous Trypanosomatidae 370 million years ago, which precedes the first bloodfeeding insect. Although the origins of leeches are proposed to be around 350 mya, the *Placobdella* leeches, which transmit several amphibian trypanosomes, did not evolve until 250 mya (Perkins et al., 2005). Insects in general arose 420 to 430 mya (Gaunt and Miles, 2002), were abundant 300 million years ago when amphibians were predominant, and are commonly infected with monoxenous trypanosomes. The split into a heteroxenous trypanosome might have occurred as amphibians were ingesting invertebrates infected with trypanosomes. Gut content analysis of 1,301 frogs and toads showed that insects composed 94% of their diets (Brown, 1974). Transmission of trypanosomes via ingestion of invertebrate host has been observed in birds (Votypka and Svobodova, 2004) and

amphibians (Anderson, 1968). Therefore, the origin of heteroxenous trypanosomes might have been the result of ingesting invertebrates containing monoxenous trypanosomes.

Trypanosoma grayi, a reptilian parasite, is basal to both the *T. cruzi* and the *T. brucei* clades. This suggests a reptilian trypanosome could be a common ancestor to both of these clades. Reptiles were abundant during the Mesozoic period, and were likely infected with trypanosomes before the split of Gondwanaland. As the continents divided, reptilian trypanosomes occurred on both South American and Africa, with new clades developing at different times on these two continents. The decline in reptiles can explain the decline in the number of reptilian *Trypanosoma* species. The majority of mammalian vertebrates in the Cenozoic era may reflect the predominance of mammalian trypanosomes. Although amphibians are not the dominant vertebrates, there are 6,184 described species (Frost et al., 2007). This profuse number of amphibian species might explain why there still exists a large group of amphibian trypanosomes.

The question arises as to what role *Cx. territans* plays in the trypanosome phylogeny. Leeches are not the only vectors of amphibian trypanosomes. Sand flies transmit trypanosomes to amphibians and reptiles in Brazil, (Ferreira et al., 2008), and development to the epimastigote stage in *T. rotatorium* has been shown within *Cx. territans* (Desser et al., 1973). The trypanosomes isolated from *Cx. territans* occurred within the aquatic clade, splitting from leech-transmitted trypanosomes. The origin of the Culicidae and Phlebotiminae occur at least 40 million years after the origin of *Placobdella* leeches, suggesting that dipteral transmission of aquatic trypanosomes diverged from leech-transmitted trypanosomes. Our phylogeny supports a new lineage of

Diptera transmitted anuran trypanosomes diverging from the aquatic clade. Diptera are suitable invertebrate hosts for trypanosomes, with 41% of the lower Trypanosomatidae occurring within this family (McGhee and Cosgrove, 1980).

Our goal was to elucidate existing phylogenies of trypanosomes using a tritrophic study of invertebrate hosts, trypanosome species, and vertebrate hosts identified from *Cx. territans* bloodmeals. Lack of host specificity increases the probability of being colonized by parasites (Price, 1980). *Culex territans* feeds on a diversity of amphibians, increasing the likelihood of host switching in trypanosomes. This high degree of host switching might explain why *Cx. territans* and their amphibian hosts show similar trypanosome prevalence rates. Our study shows that *Cx. territans* are most likely acquiring *T. ranarum* and *T. fallisi.* Considering the prevalence in which females are picking up parasites, there should be a selective advantage to those trypanosomes that can complete development within the mosquito. Trypanosomes were detected in *Cx. territans* at all stages of bloodmeal digestion, indicating this species is a suitable invertebrate host for trypanosome development. Trypanosomatidae from *Leptomonas, Crithidia, Herpetomonas, and Trypanosoma* have all been described from mosquitoes (Bates, 1949). Trypanosomes can complete development to the epimastigote stage in *Cx. territans* (Desser et al., 1973), indicating conditions are suitable for development. Our results show that future experiments on amphibian trypanosomes along with vertebrate and invertebrate host studies are necessary, and can shed light on the complex phylogenies of Trypanosomatidae.

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TABLE 3.1. Primers used to amplify regions of target DNA from engorged *Culex territans* field-collected in New Jersey, 2003-2007.

Target Region	Forward and Reverse Sequences	Amplicon size
Amphibian Cyt b	THC TNT CNG CHG CCC CVT A GAG CGD AGR ATN GCR TAR GC	402 bp
Herptile Cyt b	GGN TCR TCC AAC CCA AYW G TTT DGC DAD DGG DCG RAA N	518 bp
Animalia Cyt b	TGA GGA CAA ATA TCA TTY TGA GG AGT TTT CTG GGT CTC CTA	358 bp
Trypanosoma gGAPDH	GTG CAY GGC AAG TTC AAG TA GTA CGA GTG GAT CGT CGT CA	426 bp

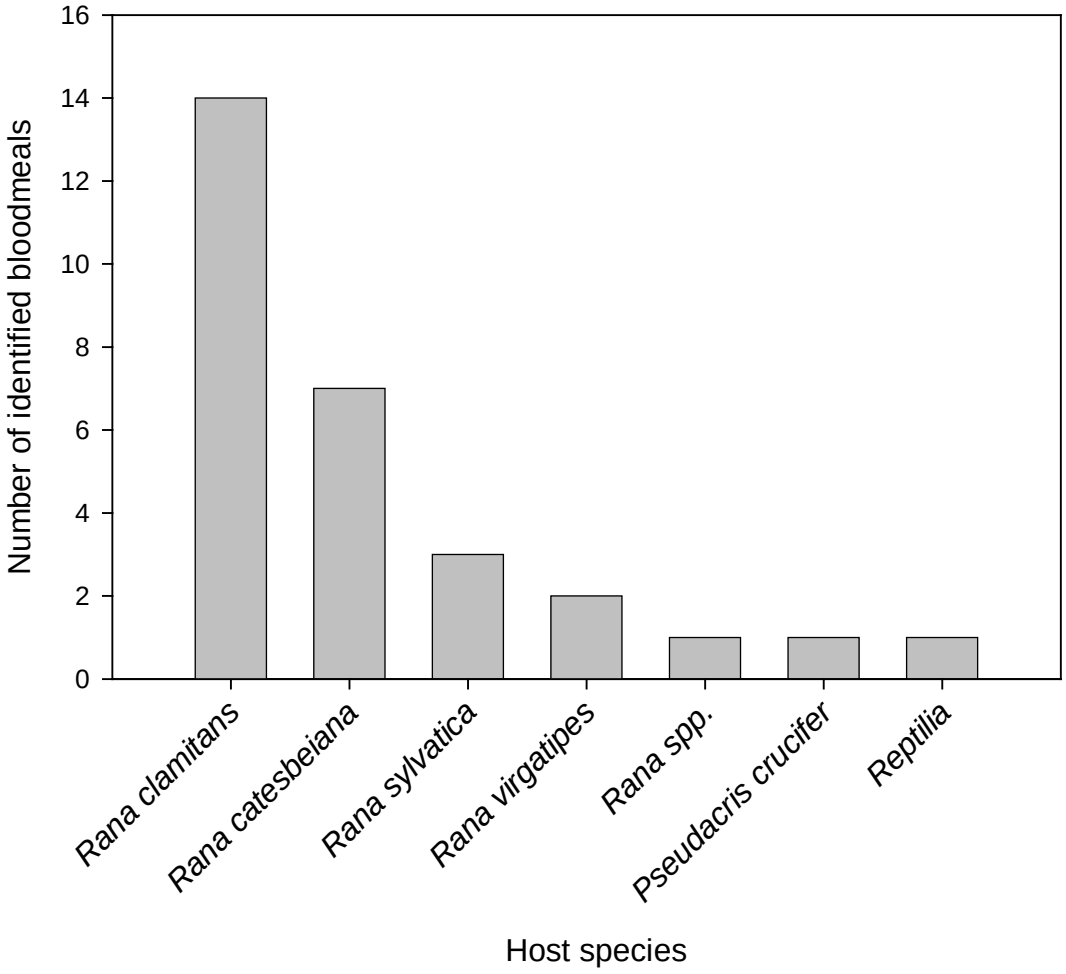


FIGURE 3.1. Identification of *Culex territans* blood meal hosts, determined by Polymerase Chain Reaction (PCR).

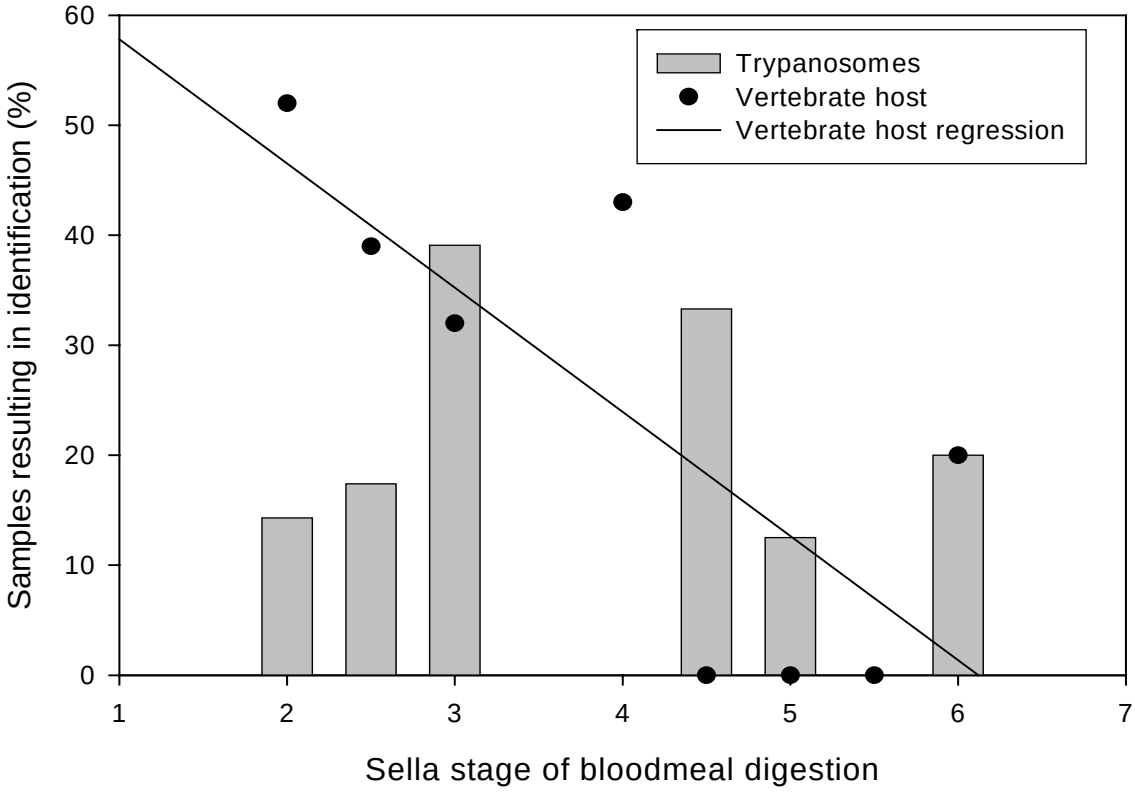
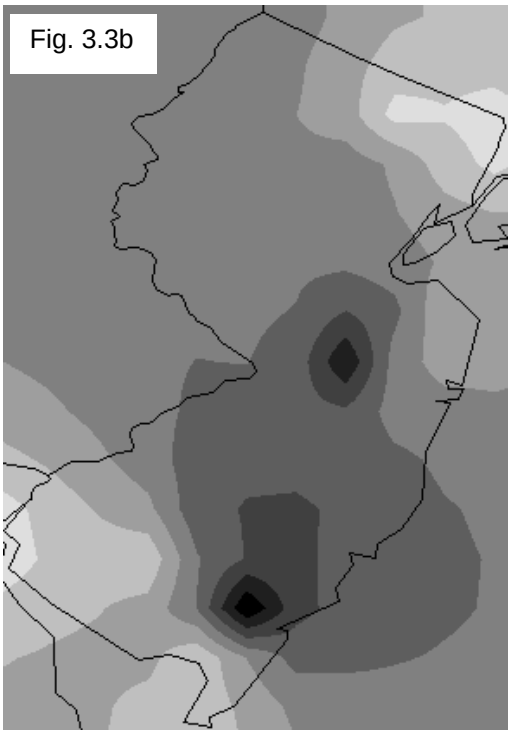
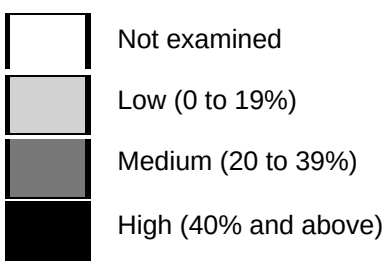


FIGURE 3.2. Number of successful trypanosome and vertebrate host identifications, from *Culex territans* bloodmeals, based on sella stage of bloodmeal digestion, determined by Polymerase Chain Reaction (PCR).





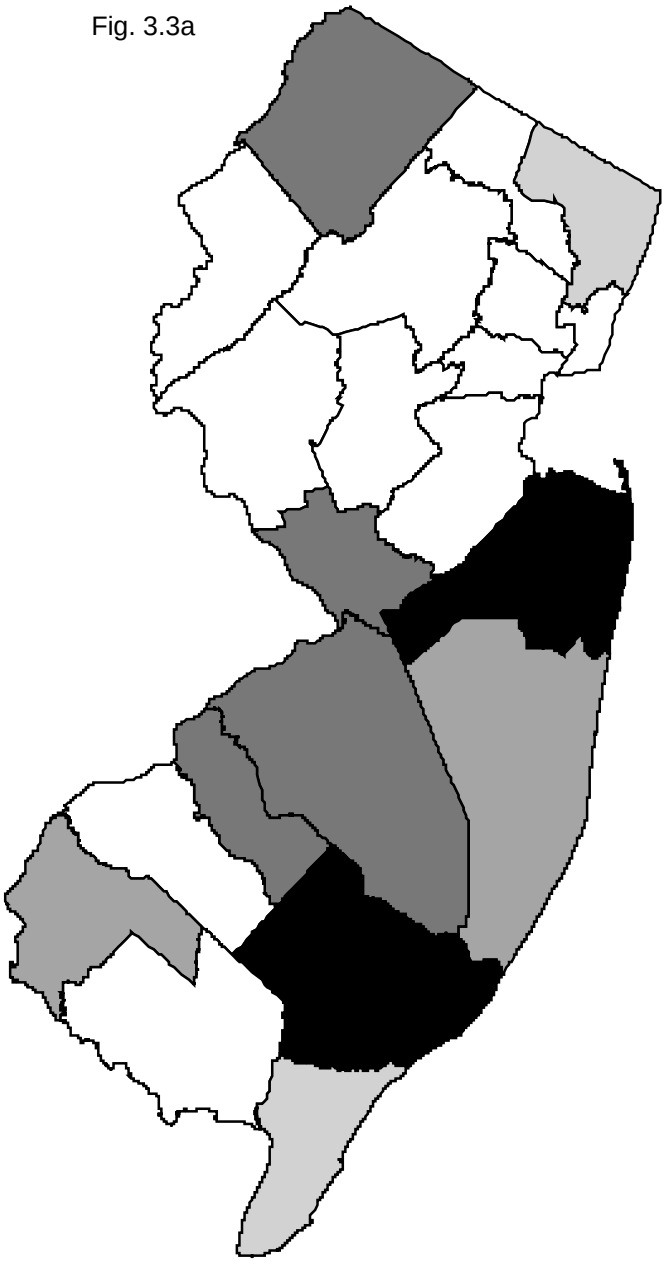


FIGURE 3.3. Distribution and prevalence of *Culex territans* trypanosomes. Figure 3.3 a shows the prevalence within each of the counties sampled. Trypanosomes were collected from each of the study sites sampled. In order to predict the prevalence of trypanosomes in all counties in NJ, a Kriging technique was peformed using ArcGIS 9.2 (Fig. 3.3b).

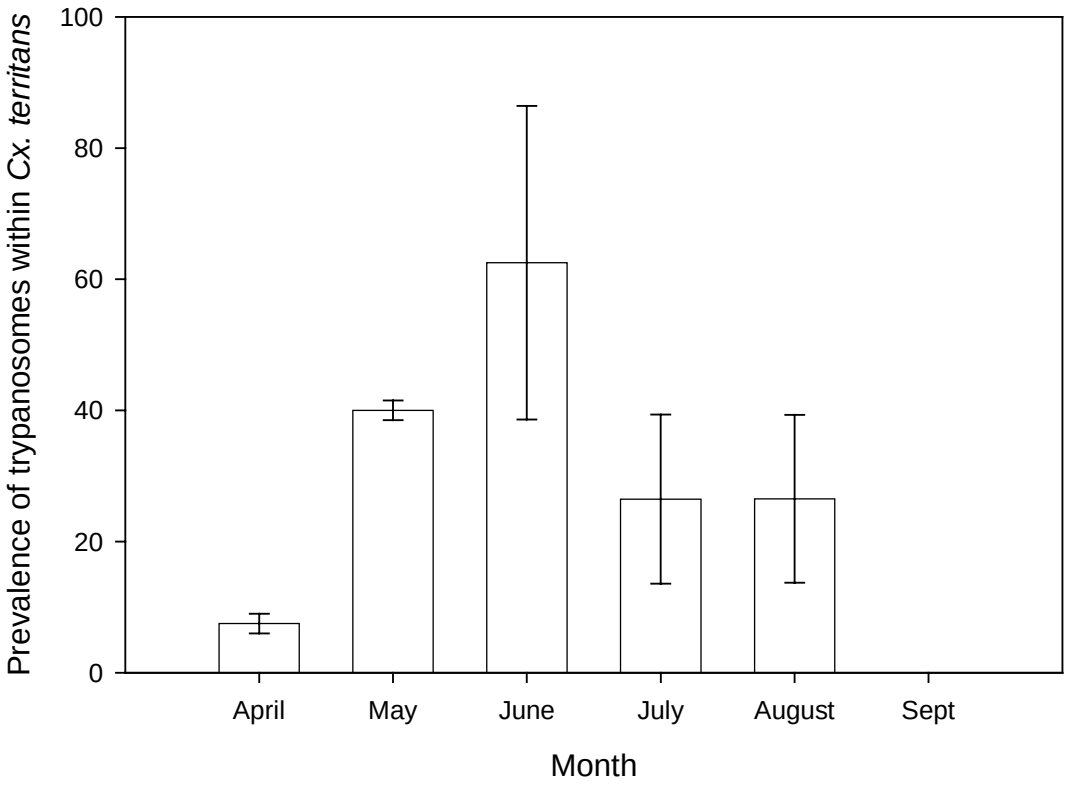
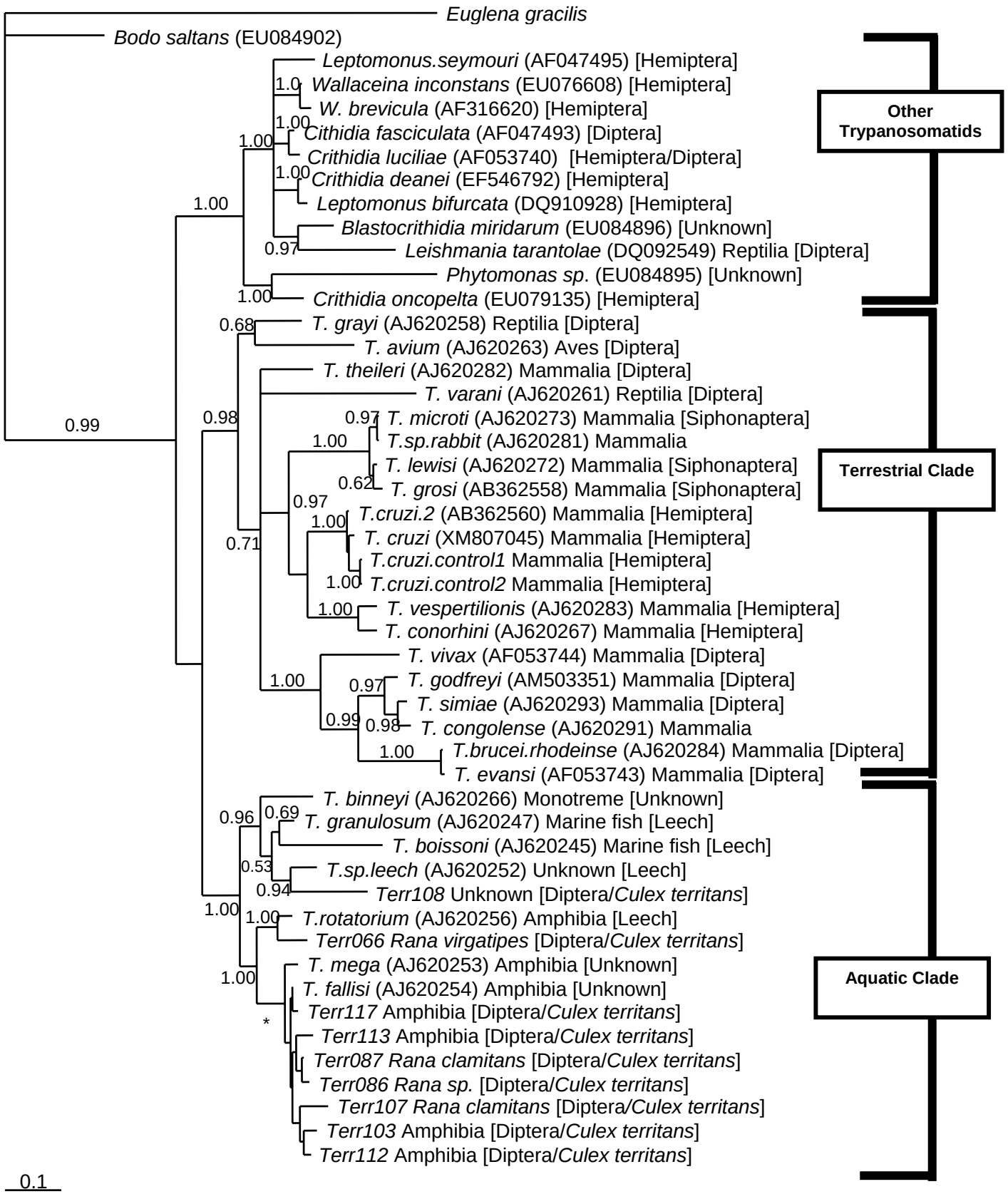


FIGURE 3.4: Prevalence (per 100) of trypanosomes within *Culex territans* by month. Results represent 5-year mean percentages and SE for 119 females collected from 2003-2007.

FIGURE 3.5. Phylogeny of trypanosomes, with placement of *Culex territans* trypanosomes based on Bayesian analysis. Each branch includes species (Accession number) vertebrate host, and [invertebrate host]. Bootstrap values are located at each node. * Indicates the branches are based on likelihood scores, and not bootstrap values.


Euglena gracilis
Bodo saltans (EU084902)
Leptomonus.seymouri (AF047495) [Hemiptera]
Wallaceina inconstans (EU076608) [Hemiptera]
W. brevicula (AF316620) [Hemiptera]
Cithidia fasciculata (AF047493) [Diptera]
Crithidia luciliae (AF053740) [Hemiptera/Diptera]
Crithidia deanei (EF546792) [Hemiptera]
Leptomonus bifurcata (DQ910928) [Hemiptera]
Blastocrithidia miridarum (EU084896) [Unknown]
Leishmania tarantolae (DQ092549) Reptilia [Diptera]
Phytomonas sp. (EU084895) [Unknown]
Crithidia oncopelta (EU079135) [Hemiptera]
T. grayi (AJ620258) Reptilia [Diptera]
T. avium (AJ620263) Aves [Diptera]
T. theileri (AJ620282) Mammalia [Diptera]
T. varani (AJ620261) Reptilia [Diptera]
T. microti (AJ620273) Mammalia [Siphonaptera]
T.sp.rabbit (AJ620281) Mammalia
T. lewisi (AJ620272) Mammalia [Siphonaptera]
T. grosi (AB362558) Mammalia [Siphonaptera]
T.cruzi.2 (AB362560) Mammalia [Hemiptera]
T. cruzi (XM807045) Mammalia [Hemiptera]
T.cruzi.control1 Mammalia [Hemiptera]
T.cruzi.control2 Mammalia [Hemiptera]
T. vespertilionis (AJ620283) Mammalia [Hemiptera]
T. conorhini (AJ620267) Mammalia [Hemiptera]
T. vivax (AF053744) Mammalia [Diptera]
T. godfreyi (AM503351) Mammalia [Diptera]
T. simiae (AJ620293) Mammalia [Diptera]
T. congolense (AJ620291) Mammalia
T.brucei.rhodeinse (AJ620284) Mammalia [Diptera]
T. evansi (AF053743) Mammalia [Diptera]
T. binneyi (AJ620266) Monotreme [Unknown]
T. granulosum (AJ620247) Marine fish [Leech]
T. boissoni (AJ620245) Marine fish [Leech]
T.sp.leech (AJ620252) Unknown [Leech]
Terr108 Unknown [Diptera/Culex territans]
T.rotatorium (AJ620256) Amphibia [Leech]
Terr066 Rana virgatipes [Diptera/Culex territans]
T. mega (AJ620253) Amphibia [Unknown]
T. fallisi (AJ620254) Amphibia [Unknown]
Terr117 Amphibia [Diptera/Culex territans]
Terr113 Amphibia [Diptera/Culex territans]
Terr087 Rana clamitans [Diptera/Culex territans]
Terr086 Rana sp. [Diptera/Culex territans]
Terr107 Rana clamitans [Diptera/Culex territans]
Terr103 Amphibia [Diptera/Culex territans]
Terr112 Amphibia [Diptera/Culex territans]
Other Trypanosomatids
Terrestrial Clade
Aquatic Clade
0.1

Chapter 4:

General conclusions regarding the factors contributing to the host specificity of *Culex territans* Walker (Diptera: Culicidae)

The objective of this research was to examine the factors contributing to the host specificity of *Culex territans* Walker (Diptera: Culicidae). *Culex territans* has often been called the frog-feeding mosquito due to its preference towards Anuran bloodmeals. Our results showed that *Cx. territans* readily bloodfeed on amphibians, share similar habitats with amphibians, are attracted to amphibian vocalizations, and readily acquire amphibian trypanosomes. Understanding the relationships between *Cx. territans* and amphibians can contribute to increased knowledge of potential causes of amphibian declines.

Our first goal was to determine if *Cx. territans* prefers Anurans to other potential bloodmeal hosts. Our results showed that *Cx. territans* readily acquired bloodmeals from green frogs (*Rana clamitans*), bullfrogs (*R. catesbeiana*), spring peepers (*Pseudacris crucifer*), carpenter frogs (*R. virgatipes*), wood frogs (*R. sylvatica*), and reptiles. One of the earliest sources of a bloodmeal was from a spring peeper. However, the most abundant source of blood was from greenfrogs. Our data suggests a preference for amphibian bloodmeals, particularly those species within the genus *Rana.* Regardless of date and location, the majority of bloodmeals came from *R. clamitans.*

Culex territans synchronized its lifecycle both temporally and spatially to match the green frog (*R. clamitans*) lifecycle. The high number of green frog bloodmeals from *Cx. territans* might be the result of the mosquito larvae existing in the same habitat in time and space as greenfrogs. *Culex territans* has fewer olfactory and carbon dioxide receptors than other species of *Culex* (McIver 1982). Therefore, ovipositing in the same habitat as a potential host, might maximize the female's chance of obtaining a bloodmeal. Therefore, host specificity might be determined by host availability within the same habitat.

Amphibians in New Jersey exit diapause when temperatures are close to freezing. If *Culex territans* is host specific, and takes advantage of these early season bloodmeals, then they must be able to digest bloodmeals at these temperatures. The results of our study show that *Cx. territans* can digest bloodmeals at temperatures above 3.9 °C, temperatures corresponding to the exit of amphibians from diapause. This ability to digest bloodmeals at this temperature suggest they are maximizing their ability to utilize amphibian bloodmeals as they become available. This supports our bloodmeal analysis, showing that *Cx. territans* have a preference for amphibian bloodmeals.

In early spring, *Cx. territans* are exiting diapause and searching for their first source of bloodmeal. Bloodmeal analysis indicated the first bloodmeals might be from *P. crucifer*. Spring peepers have a distinctive vocalization that can be heard from almost a mile from the source. We hypothesized that if *Cx. territans* is using spring peepers as an early season bloodmeal, then there would be a selective advantage to those females using sound to locate a host. Our results corroborated our hypothesis, as *Cx. territans* exhibited positive phonotaxis to amphibian vocalizations. The attractiveness of amphibian vocalizations contributes towards the host specificity of *Cx. territans* to amphibians.

In order for a bloodfeeding arthropod to be host specific, they must be capable of surviving the parasites and pathogens associated with that host. Our results showed that *Cx. territans* was readily acquiring amphibian trypanosomes during bloodfeeding. As bloodmeals were digested, the trypanosomes were likely developing within the mosquito. Results from Benach (1971) showed *Cx. territans* were capable of picking up and transmitting the nematode parasite *Foleyella flexicauda*. Benach (1971) showed that *Foleyella* did not show any degree of periodicity, and *Cx. territans* could bloodfeed and

survive infections throughout the day and evening. The ability to acquire and transmit amphibian parasites suggests a close relationship between *Cx. territans* and amphibians, which has contributed over time to a high degree of host specificity.

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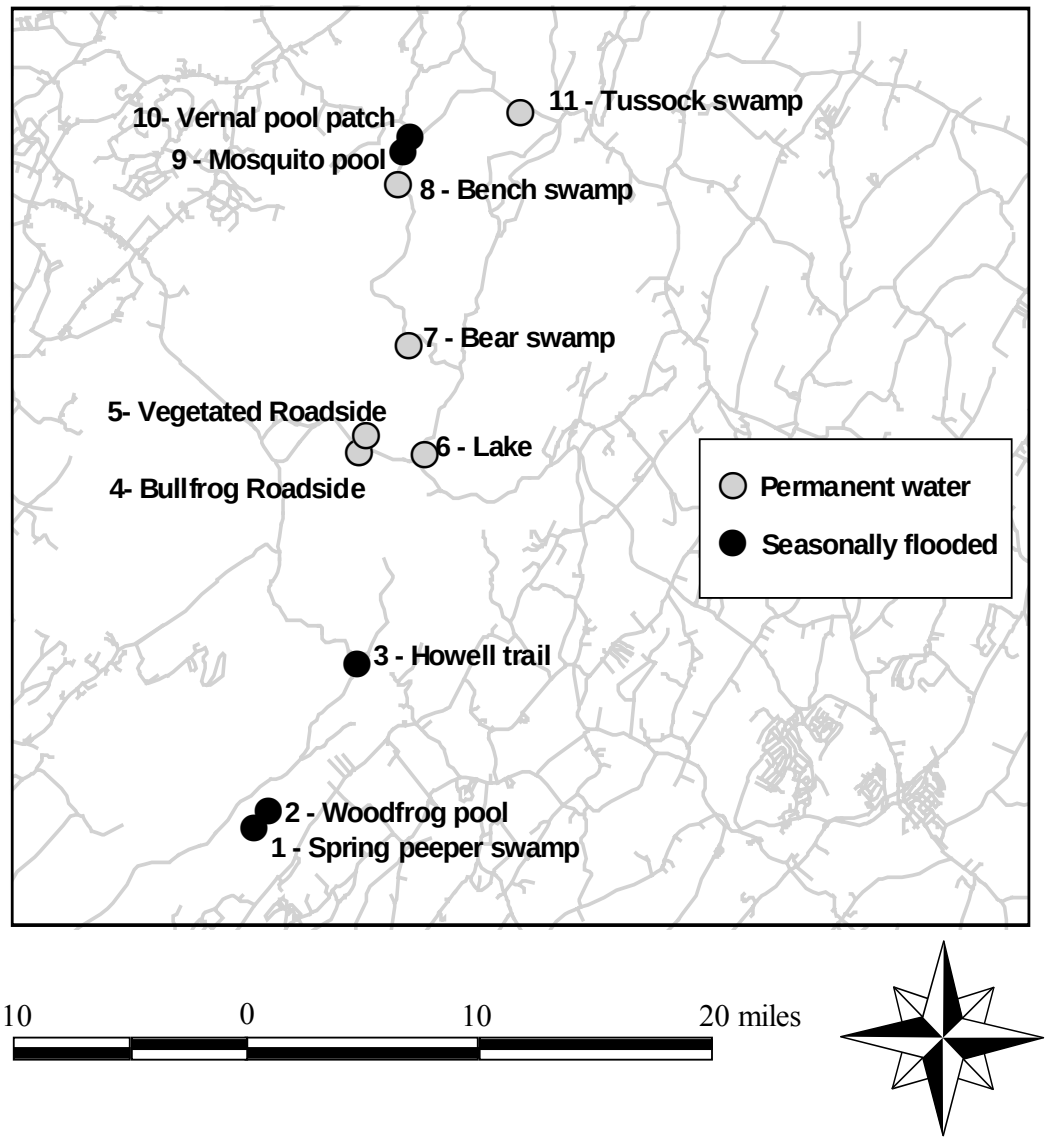

Figure A.1: Culex territans **field sampling locations. A detailed description of each of these sites can be found in the appendix.**

Site 1: Spring peeper swamp

Coordinates: 41.20158, -74.74843

Site 1 can be accessed along the road leading up to Sunrise Mountain. The Spring peeper swamp is 1.3 acres of Palustrine forested seasonally flooded wetland comprised mainly of broad-leaved deciduous trees. The soil type is Arnot-Lordstown Rock outcrop complex, and has a 5 to 35% slope. The top inch of soil is characterized by 70 to 100% organic matter, and has a combination of silt and sand below. The top 4 inches of soil has a 4.9 pH. The elevation of this site is 1240 ft. This site has a high surface run off from January to December. During 2004, this site was flooded 24 out of the 35 weeks of sampling. Adult spring peepers were frequently sampled at this site in both the spring and fall. Later in the spring, Northern gray tree frogs were abundant in the area. *Culex territans* larvae were only collected at this site in the fall.

Site 2: Woodfrog pool

Coordinates: 41.20410, -74.74448

The woodfrog pool is a 1.95-acre vernal pool habitat within a Palustrine scrub shrub seasonally flooded habitat. The soil type is an Arnot-Lordstown complex, and has a 0 to 15% slope. The top inch of soil is characterized by 70 to 100% organic matter, and has a combination of silt and sand below. The top 4 inches of soil has a 5.3 pH. The elevation of this site is 1280 ft. This site has a high surface run off from January to December. During 2004, this site was flooded 24 out of the 35 weeks of sampling. This site was utilized in the spring by wood frogs and *Aedes communis* mosquitoes. In 2004, many of the tadpoles using this site did not fully develop before the habitat completely dried down in the spring. In the fall, rainwater persisted in the wetland, and green frogs were frequently encountered. *Culex territans* larvae were only collected at this site in the fall.

Site 3: Howell trail

Coordinates: 41.22429, -74.71900

This wetland is located along the Howell trail along Sunrise Mountain. The wetland in this area is a 4.9-acre Palustrine forested seasonally flooded wetland, containing small collections of vernal pools. The soil type is a smartswood loam, and has an 8 to 15% slope. The top inch of soil is characterized by 70 to 100% organic matter, and has a combination of silt and sand below. The top 4 inches of soil has a 4.7 pH. The elevation at this site is 1200 ft. This site has a high surface run off from January to March, and November and December. The most commonly encountered frogs at this site included pickerel frogs. Rainfall did not persist in pools, and in 2004, this site was flooded only 17 of the 35 weeks. *Culex territans* was never found within this site.

Site 4: Bullfrog roadside

Coordinates: 41.25910, -74.71420

Site 4 is a large permanent water habitat, with very little emergent vegetation. This site occurs along the Deckertown Pike, and remains permanently flooded throughout the year. In 2004, this site was flooded 35 out of the 35 weeks. The soil type is a smartswood loam, and has an 8 to 15% slope. The top inch of soil is characterized by 70 to 100% organic matter, and has a combination of silt and sand below. The top 4 inches of soil has a 4.7 pH. The elevation at this site is 1080 ft. This site has a high surface run off from January to March, November and December. In the spring, salamanders were frequently encountered within the pools. Adult green frogs were commonly collected at this site throughout the summer, but only in small pockets on the far edges of the habitat. Throughout the summer and fall, bullfrogs were abundant as both adults and tadpoles. *Culex territans* were often collected at this site throughout the summer and fall.

Site 5: Vegetated roadside

Coordinates: 41.25980, -74.71500

Site 5 is directly across the street from site 4, but has ample emergent vegetation within the wetland. This site is a 1.9-acre Palustrine scrub shrub and emergent vegetation habitat that remains permanently flooded throughout the year. In 2004, this habitat was flooded 35 of the 35 weeks. The soil type in this habitat is a smartswood loam, and has an 8 to 15% slope. The top inch of soil is characterized by 70 to 100% organic matter, and has a combination of silt and sand below. The top 4 inches of soil has a 4.7 pH. The elevation at this site is 1100 ft. This site has a high surface run off from January to March, November and December. Although site 4 was across the street and rich in amphibian fauna, bullfrogs did not frequently use site 5. *Culex territans* larvae were occasionally collected at this site towards the end of the summer.

Site 6: The lake

Coordinates: 41.25871, -74.70582

The lake served as a control site to compare with the Palustrine forested and emergent wetlands. The lake is a 4.18-acre artificial lake that is permanently flooded throughout the year. The soil type is a smartswood loam, and has a 15 to 35% slope. The top inch of soil is characterized by 70 to 100% organic matter, and has a combination of silt and sand below. The top 4 inches of soil has a 4.7 pH. The elevation at this site is 1100 ft. This site has a high surface run off from January to March, November and December. Amphibians frequently found using this site included American toads, bullfrogs, pickerel frogs, and red spotted newts. *Culex territans* was never collected at this site, although there is a small patch of emergent vegetation along the perimeter.

Site 7: Bear swamp

Coordinates: 41.27597, -74.70675

After 2 miles along Park Ridge road, there is a small pull off and hiking trail on the left. Directly next to the trail is Bear swamp. Bear swamp is a 6.4-acre Palustrine scrub shrub emergent permanently flooded wetland. The soil type is an Alden silt loam, and has a 0 to 8% slope. The top 2 inches of soil are characterized by 70 to 100% organic matter, and has a combination of silt and sand below. The top 4 inches of soil has a 5.6 pH. The elevation at this site is 1200 ft. This site receives little surface run off. This site was permanently flooded throughout the year, and water was present 35 out of the 35 weeks. This was the most common location to collect *Cx. territans* larvae*,* which was collected at this site for 26 consecutive weeks. This was the last site where *Cx. territans* larvae were present at the end of the year, persisting in the habitat until frozen with ice. There was also a high diversity of amphibians, including green frogs, pickerel frogs, spring peepers, and red spotted newts.

Site 8: Bench swamp

Coordinates: 41.30207, -74.71189

Approximately 10 miles north of Bear swamp on Park Ridge road, there is a stone bench on the left of the road. At this site there is a 14-acre Palustrine scrub shrub emergent permanently flooded wetland. The soil type is an Alden silt loam, and has a 0 to 8% slope. The top 2 inches of soil are characterized by 70 to 100% organic matter, and has a combination of silt and sand below. The top 4 inches of soil has a 5.6 pH. The elevation at this site is 1200 ft. This site receives little surface runoff. This site is permanently flooded, and in 2004, water was present 35 out of the 35 weeks. Spring peepers were frequently collected at this site in the spring. Green frogs were abundant throughout the summer and fall. *Culex territans* were collected at this site, but dip counts showed they were fewer in number than at the Bear swamp.

Site 9: Mosquito pool

Coordinates: 41.30699, -74.70939

Site 9 is a 0.9-acre Palustrine forested wetland that is seasonally flooded. The soil type is a smartswood loam, and has a 0 to 8% slope. The top inch of soil is characterized by 70 to 100% organic matter, and has a combination of silt and sand below. The top 4 inches of soil has a 4.7 pH. The elevation at this site is 1200 ft. This site has a high surface run off from January to March, November and December. During 2004, this site was flooded 28 out of the 35 weeks. In the spring, mosquitoes such as *Aedes excrucians* were common in this habitat. During the fall, rainfall persisted in the habitat, and adult green frogs were commonly seen using this site. Approximately 2 weeks after the green frogs, *Culex territans* could be found in very large numbers in these vernal pool sites.

Site 10: Vernal pool patch

Coordinates: 41.31024, -74.70622

The Vernal pool patch is 2.15 acres of forested wetland comprised mainly of broad-leaved deciduous trees. The soil type is a smartswood loam and has a 0 to 8% slope. The top inch of soil is characterized by 70 to 100% organic matter, and has a combination of silt and sand below. The top 4 inches of soil has a 4.7 pH. The elevation of this site is 1200 ft. This site has a high surface run off from January to March, November and December. During 2004, this site was flooded 28 out of the 35 weeks of sampling. This site was similar to Site 9, in that green frogs could be frequently collected after fall rain events. *Culex territans* were abundant in this site approximately 2 weeks after the presence of green frogs.

Site 11: Tussock swamp

Coordinates: 41.31342, -74.68019

The Tussock swamp is a 24.5-acre scrub shrub emergent permanent water wetland. The soil type is an Alden silt loam, and has a 0 to 8% slope. The top 2 inches of soil are characterized by 70 to 100% organic matter, and has a combination of silt and sand below. The top 4 inches of soil has a 5.6 pH. The elevation at this site is 1300 ft. This site receives little surface water runoff. This is a permanent water habitat, and in 2004, water was present 35 out of the 35 weeks of sampling. In the spring, this site was dominated by spring peepers. Large numbers of salamander and wood frog eggs were observed in this habitat, but tadpoles and adults of these species were very rarely observed. *Culex territans* larvae were occasionally collected at this site, but dip counts were typically very low.

Curriculum vitae
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Education

Doctor of Philosophy, Entomology, Department of Entomology
Rutgers University, New Brunswick, NJ, January 2009

Master of Science, Entomology, College of Resource Development,
The University of Rhode Island, Kingston, May 2001

Bachelor of Science, Zoology, College of Arts and Sciences,
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Work Experience

Teaching Assistant (September 2003 – May 2008)
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Graduate Assistant (June 2001 – September 2003)
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Laboratory Assistant (December 2000 - May 2001)
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Mosquito Technician (May-Oct. 1997 and 1999)
Rhode Island Department of Environmental Management
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Museum Technician (June 1998-Sept. 1998)
United States Department of Agriculture (Contact Al Norrbom)
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Publications

Bartlett-Healy, K., W. Crans, R. Gaugler. 2008. Phonotaxis of *Culex territans* toward amphibian vocalizations. Annals of the Entomological Society of America. 101 (1): 95-103.

Bartlett-Healy, K., W. Crans, R. Gaugler. 2008. Spatial and temporal synchrony of Culex territans to amphibians. Journal of Medical Entomology. 45 (6): 1031-1038.

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Bartlett, K., S. R. Alm, R. LeBrun, and H. Ginsberg. 2002. The horse and deer flies (Diptera: Tabanidae) of Rhode Island. The Annals of the Entomological Society of America. 95(5): 547-551.

Bartlett, K. 2001. Thesis titled "The horse flies and deer flies of Rhode Island, trapping methods, and the use of RAPD's to distinguish cryptic species of Tabanidae"

Patents Pending

The Rhode Island Canopy Trap for Collecting Horse and Deer Flies US patent office serial number 09/840,056