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# Effects of Dietary Sugars and Sugar Alcohols on Mortality and Thermal Tolerance in *Culex quinquefasciatus* and *Aedes aegypti*

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**EFFECTS OF DIETARY SUGARS AND SUGAR ALCOHOLS ON  
MORTALITY AND THERMAL TOLERANCE IN *CULEX*  
*QUINQUEFASCIATUS* AND *AEDES AEGYPTI***

A Thesis

Submitted to the Graduate Faculty  
of the Louisiana State University and  
Agriculture and Mechanical College  
in partial fulfillment of the  
requirements of the degree of  
Master of Science

in

The Department of Entomology

by  
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## Abstract

*Aedes aegypti* (L. 1762), the yellow fever mosquito, and *Culex quinquefasciatus* Say 1823, the southern house mosquito, are prevalent in tropical and subtropical areas worldwide and are responsible for the spread of a number of devastating diseases to humans and livestock. The development of new control methods, as well as continued study of mosquito biology, is vital for improving human health. This study aimed to examine the efficacy of sugar alcohols as a novel means for mosquito control, as well as how sugar alcohols and other factors are involved in thermal tolerance in mosquitoes.

No-choice tests with the sugar alcohols erythritol, sorbitol, and xylitol resulted in significant mortality in at least one species, with erythritol resulting in the highest mortality. A two-choice test between sucrose with or without added erythritol showed no significant preference between the two in either *Ae. aegypti* or *Cx. quinquefasciatus*. Based on our findings, erythritol and other sugar alcohols have good potential as novel mosquito toxins, and further study should be conducted into the efficacy of deployment in the field.

Thermal tolerance assays demonstrated that the consumption of sugar alcohols does not improve cold tolerance in *Cx. quinquefasciatus*, but that consumption of mannitol can decrease heat tolerance. We observed similar levels of cold tolerance between all diets tested. However, we found that *Cx. quinquefasciatus* was inherently significantly more cold tolerant than *Ae. aegypti*, while *Ae. aegypti* had improved heat tolerance compared to *Cx. quinquefasciatus*. There were no differences in thermal tolerance between sexes within either species. Our results suggest that although dietary factors such as sugar alcohols and sugars may play a role in thermal tolerance in mosquitoes, there are likely physiological and genetic factors that can have a greater influence on the limits of thermal tolerance within a species.

## Chapter 1. Literature Review

### 1.1. Mosquito Biology

#### 1.1.1. Mosquitoes as Vectors

Mosquitoes are often referred to as nature's deadliest animals due to their ability to vector a myriad of debilitating and sometimes life-threatening parasites and pathogens. Mosquitoes in the genus *Anopheles* transmit the malarial parasites (*Plasmodium* spp.) that resulted in 216 million cases of malaria and 445,000 deaths in 2016 alone (Baird 2000, Centers for Disease Control 2018). Outside of the genus *Anopheles*, the species *Aedes aegypti* (L. 1762) and *Culex quinquefasciatus* Say 1823 are two of the most notable vectors, due to both their widespread ranges throughout the world as well as the nature of the pathogens and parasites they are capable of transmitting. *Aedes aegypti* is considered the most important vector of dengue virus (Bhatt et al. 2013), which infects as many as 400 million people a year and is one of the leading causes of illness and death in the tropics and subtropics (Centers for Disease Control 2016). Additionally, *Ae. aegypti* is a competent vector for yellow fever (Barrett and Higgs 2007), chikungunya (Vega-Rua et al. 2014), and Zika viruses (Ioos et al. 2014). *Culex quinquefasciatus* acts as a vector for *Wuchereria bancrofti* in the Western hemisphere, with long-term infections resulting in lymphatic filariasis (Centers for Disease Control 2013). In the southern United States, *Cx. quinquefasciatus* acts as the primary vector for West Nile virus (Hayes et al. 2005). Knowledge of the biology and ecology of these two species is vital for understanding the risk of infectious diseases for people in all parts of the world.



### 1.1.2. *Aedes aegypti* Life History

*Aedes aegypti*, the yellow fever mosquito, likely originated from Africa but in the past several centuries has expanded its range to encompass most of the world between the 35° northern and the 35° southern latitudes (Nelson 1986). In the United States, persistent populations are largely limited to the southern-most states, such as Florida, Louisiana, Texas, Arizona, and southern areas of California (Hahn et al. 2017). *Aedes aegypti* mosquitoes are highly associated with urban environments, and larvae are commonly found in man-made container habitats such as used tires, water storage containers, gutters, and buckets (Nelson 1986). Adults feed preferentially on humans (Scott and Takken 2012) and are voracious feeders, often taking multiple bloodmeals for each gonotrophic cycle (Scott et al. 1993).

*Aedes aegypti* females deposit their eggs singly in water-filled container habitats just above the water line; once embryonic development is complete, eggs become highly resistant to desiccation for months to potentially over a year (Nelson 1986, Sota and Mogi 1992). Upon immersion in water, eggs hatch within 24 to 48 hours, with greater success in water low in dissolved oxygen (Gjullin et al. 1941) and containing bacteria or organic matter (Christophers 1960). Larvae filter feed on microorganisms and detritus suspended in the water and progress through four larval instars before reaching the pupal stage (Christophers 1960). The rate of development is highly dependent on a variety of factors such as temperature, nutrition, and population density (Christophers 1960), but generally lasts from seven to ten days (Tun-Lin et al. 2000, Mohammed and Chadee 2011). Upon emergence, both males and females will consume plant sugars for energy for flight and mating; several days after emergence females will seek a blood meal and begin the gonotrophic cycle (Christophers 1960).

### 1.1.3. *Culex quinquefasciatus* Life History

*Culex quinquefasciatus*, the southern house mosquito, is one of the most prolific mosquito species in the world and is a common and medically important species in the southern United States. Like *Ae. aegypti*, *Cx. quinquefasciatus* is likely native to Africa but was spread throughout the globe through human travel and activity (Lounibos 2002). In North America, it is often considered together with its counterpart, *Culex pipiens pipiens*, the northern house mosquito; adults of these species are morphologically indistinguishable and interbreed in regions where their distributions overlap (Burkett-Cadena 2013). *Culex quinquefasciatus* larvae can withstand and thrive in highly organic water and are often associated with man-made habitats such as sewage effluent (Su et al. 2003, Calhoun et al. 2007, Metzger et al. 2008). Although many *Culex* species are considered primarily avian feeders, *Cx. quinquefasciatus* adults are opportunistic and often feed on both mammals and birds (Zinser et al. 2004, Reisen 2012), likely due to their association with cosmopolitan habitats.

*Culex quinquefasciatus* females lay their eggs in rafts on the surface of water, preferring to oviposit in nutrient-dense habitats such as septic tanks and ditches (Clements 1992). Like *Ae. aegypti* and many other larval mosquitoes, *Cx. quinquefasciatus* larvae are filter feeders, subsisting on detritus and microorganisms (Merritt et al. 1992). Development is dependent on factors such as crowding (Roberts and Kokkinn 2010) and temperature (Rueda et al. 1990), but takes between five and eight days under optimal conditions (Gerberg et al. 1994). After emerging, adults will seek sugar sources and mate. Females then begin host-seeking, securing the blood meal required to develop viable eggs (Clements 1992). The gonotrophic cycle lasts an average of 2-3 days (Elizondo-Quiroga et al. 2006), with females able to lay up to five egg rafts in a lifetime (Gerberg et al. 1994).

#### 1.1.4. Overwintering

Mosquitoes utilize a diversity of overwintering strategies varying with genus, species and environment. Mosquitoes in the genus *Aedes* diapause in the egg stage, with hatch occurring with the arrival of adequate temperatures and rainfall. *Aedes aegypti* mosquitoes are active year-round throughout most of their range; however, in more temperate areas they may spend colder months in the egg stage (Vezzani et al. 2004, Fischer et al. 2011). For those species that cannot enter egg diapause, the most common overwintering method is dormancy in adult females, especially for species in the genera *Anopheles* and *Culex* (Clements 1992). In temperate areas, *Culex* mosquitoes will often seek out hibernacula in which to undergo dormancy; hibernacula can include natural areas such as tree hollows or animal burrows, or anthropogenic structures such as barns or tunnels (Mitchell 1979, Reisen et al. 1986b, Spielman 2001, Wallace 2008). Dormancy can range from complete diapause to quiescence, depending on environmental conditions and the species in question (Eldridge 1968, Schaefer et al. 1971, Reisen et al. 1986a). *Culex quinquefasciatus* females do not enter true diapause, instead exhibiting quiescence based on environmental temperatures (Nelms et al. 2013, Thareja et al. 2016). In order to survive through the winter season when feeding opportunities are scarce, mosquitoes are reliant on lipid reserves (Clements 1992).

While some species develop lipid reserves from blood (Ramsdale and Wilkes 1985), most species, including most *Culex*, are thought to use sugars to enhance overwintering capabilities (Schaefer and Miura 1972, Reisen et al. 1986b, Jaenson and Ameneshewa 1991, Bowen 1992). Feeding behavior of overwintering females is additionally dependent on climate. In areas where winter temperatures go below 0°C, females are likely immobile and do not feed at all during dormancy (Jaenson 1987, Spielman 2001). However, in areas where mean

temperatures are above freezing, females often remain vagile and have been found to feed on sugars and occasionally take blood meals (Mitchell 1979, Reisen et al. 1986a, Nelms et al. 2013). The vast majority of field studies on overwintering diets in *Culex* mosquitoes have occurred in California on *Cx. tarsalis* and occasionally *Cx. pipiens pipiens*, leaving the habits of *Culex* mosquitoes in the southeastern United States relatively unknown.

## 1.2. Diets

### 1.2.1. Flight Energy and Reproduction

The diets of mosquitoes consist of two food sources: sugars and protein. In most species, females are obligate blood-feeders in order to secure the protein needed to produce eggs, but also utilize sugars for other sources of energy; males of all species rely on sugar as a sole food source (Clements 1992, Foster 1995). Mosquitoes can utilize sugar sources, such as decaying fruits or honeydew, but most commonly obtain sugars from the floral nectaries of plants (Joseph 1970, Clements 1992, Russell and Hunter 2002). Nectar composition differs among plants, but primarily consists of sucrose, D-fructose, or D-glucose in varying proportions (Wykes 1952). Carbohydrates from plant sugars are thought to be the main source of energy for flight in both males and females, with lipids derived from blood meals being a less efficient source (Nayar and Sauerman 1971, Nayar and Van Handel 1971). However, there is evidence that some populations of highly anthropophilic species such as *Ae. aegypti* feed minimally on nectar in nature (Edman et al. 1992, Van Handel et al. 1994), and laboratory studies support an ability to maintain reproductive success provided ample blood meals are available (Scott et al. 1997, Gary and Foster 2001, Braks et al. 2006). However, *Ae. aegypti* is likely more the exception than the rule; the literature largely supports that in most species, females continue to sugar feed throughout their lifetimes (Magnarelli 1978, Vargo and Foster 1984, Reisen et al. 1986b).

While blood may provide energy for other activities, its main purpose in the mosquito diet is for the development of eggs. Although autogeny does occur in wild mosquito populations (Trpis 1977, Strickman and Fonseca 2012), in most cases a blood meal is essential for the successful completion of the gonotrophic cycle. After finding a sugar meal and mating, females will engage in host-seeking behavior, using cues such as carbon dioxide and octanol (Takken and Kline 1989, Costantini et al. 1996) to locate a suitable host. Consumption of a blood meal initiates ovarian development and provides the protein necessary for the formation of eggs, leading to oviposition several days later (Clements 1992). In order to reach maximum fertility throughout a lifetime, most female mosquitoes must continuously locate appropriate sources for both sugar and blood meals.

#### 1.2.2. Effects of Diet on Thermal Tolerance

While field observations, as well as laboratory experiments, support an important role of sugars in preparing mosquitoes for overwintering, direct measures of how diet influences cold tolerance are largely absent from the literature. In other insects, diet seems to have the ability to significantly alter certain parameters for cold tolerance (Andersen et al. 2010, Owen et al. 2013, Li et al. 2014). These have been particularly well-studied in *Drosophila melanogaster*. Shreve et al. (2007) found that a cholesterol-augmented diet increased survival after cold shock and improved cold-hardening ability; Andersen et al. (2010) saw faster chill coma recovery of adult flies when reared as larvae on carbohydrate-rich diets as compared to protein-rich diets. However, Colinet et al. (2013) observed that increasing the concentration of sugars in adult diets increased recovery times and negatively affected cold tolerance. While there are still many questions regarding the effects of diet on *Drosophila* cold tolerance, there are even more

regarding mosquitoes, the answers to which could play an important role in understanding the overwintering biology and behavior of these insects.

The influence of feeding on heat tolerance is even less explored in mosquitoes; this is generally true regarding other insects as well, with the exception of a few species. Certain sugars, such as sucrose, as well as sugar alcohols, have been demonstrated to increase the thermal stability of proteins (Back et al. 1979). In insects, sorbitol, a sugar alcohol, increases tolerance to heat stress in whiteflies (Wolfe et al. 1998, Salvucci 2000), and mannitol acts in a similar manner in aphids (Hendrix and Salvucci 1998). Whiteflies derive sorbitol from fructose (Salvucci et al. 1998), and whiteflies given access to sorbitol-supporting diets show increased survival at high temperatures (Salvucci 2000). Andersen et al. (2010) found somewhat differing results in *Drosophila melanogaster*, which had increased heat tolerance when reared on a protein-rich diet as compared to a carbohydrate-rich diet. Understanding if and how mosquitoes can increase heat tolerance by dietary means is an unknown and perhaps relevant part of their life history and feeding habits.

### 1.3. Sugar Alcohols

Sugar alcohols are polyols with the general formula  $\text{HOCH}_2(\text{CHOH})_n\text{CH}_2\text{OH}$  found abundantly throughout nature. They are similar to sugars in structure, but have two additional hydrogen atoms, and tend to exist as chains instead of rings. They occur in a wide variety of plants; in particular, galactitol, mannitol, and sorbitol are found abundantly throughout the angiosperms in both leaf tissues and fruits (Loescher 1987, Moing 2000). Mannitol and sorbitol, as well as xylitol, are found in the fruits of numerous plant species, particularly within the Rosaceae (Lee 2015). This includes many fruits commonly cultivated for human consumption, such as strawberries, apples, pears, plums, and cherries.

Sugar alcohols impart a sweet taste to fruits, while generally containing less calories than sugars; this has led to the production and commercialization of sugar alcohols as low-calorie sugar alternatives. While sugar alcohols can be extracted from natural sources, they are more commonly synthesized industrially through the process of hydrogenation of sugars (Park et al. 2016). Although sugar alcohols have been determined as safe for human consumption by the U.S. Food and Drug Administration (Department of Health and Human Services 2018), recent research indicates that certain sugar alcohols and non-nutritive sweeteners may have toxic effects on insects.

Baudier et al. (2014) provided the first evidence for the toxicity of sugar alcohols using *Drosophila melanogaster*. They compared survival of adult flies on five non-nutritive sweeteners; the sugar alcohol erythritol resulted in the greatest mortality. In *Drosophila suzukii*, erythritol and its sugar counterpart erythrose also result in significant mortality, while mannitol, sorbitol and xylitol do not (Choi et al. 2017). The tephritid fly *Bactrocera dorsalis* displays increased inactivity and mortality when fed non-nutritive sweeteners, with the greatest effect seen with erythritol (Zheng et al. 2016). Although the mechanism through which mortality occurs in these various fly species is not fully understood, there is evidence that erythritol cannot be metabolized, and consumption results in greatly elevated osmotic pressure due to accumulation in the hemolymph, leading to death (Tang et al. 2017).

Although direct consumption of erythritol can be toxic for insects, some species synthesize and store erythritol for use as a cryoprotectant (Baust and Edwards 1979, Kostal et al. 2007). Other sugar alcohols, namely sorbitol and mannitol, also act as protectants against cold stress in a number of insects (Story and Storey 1983, Kostal et al. 2007, Michaud and Denlinger 2007). As discussed in detail previously, these compounds can confer protection against heat

stress as well. Sugar alcohols are usually not directly consumed, but metabolized from sugars such as fructose (Hendrix and Salvucci 1998).

Both the toxicity of sugar alcohols and their function in cold tolerance have not been explored in mosquitoes. Mosquitoes can produce sorbitol through the metabolism of fructose (Van Handel 1969), but whether or not it is utilized in thermoprotection is unknown. While the toxicity of sugar alcohols against mosquitoes is also not known, the consistent toxicity of erythritol within Diptera, and the non-specificity of its proposed toxic mechanism make it a likely candidate as a mosquito toxin. Toxic sugar alcohols may be a novel insecticidal compound that could be integrated into mosquito control techniques such as attractive toxic sugar baits, which combine sugar sources with orally ingested insecticides.

## 1.4. Thermal Tolerance in Mosquitoes

### 1.4.1. Developmental Temperatures

Temperature is possibly the most important factor governing the ranges, seasonality, and development times of mosquitoes (Clements 1992). *Aedes aegypti* has traditionally thought to be limited in range by a 10°C winter isotherm (Christophers 1960), although more recent research suggests a 15°C average yearly isotherm may be more appropriate for broad range predictions (Otero et al. 2006). Egg hatch is stimulated by submergence in water usually brought on by rainfall; however, egg hatch is limited by temperatures below 13°C (Christophers 1960).

Complete larval development occurs on average between 15°C and 35°C; at temperatures closer to 30°C, development usually takes less than a week, but lower temperatures can increase this to closer to three weeks (Tun-Lin et al. 2000, Costa et al. 2010, Carrington et al. 2013, Marinho et al. 2016). Adults can withstand prolonged exposure of temperatures as low as 7°C to 9°C (Nelson 1986), but become inactive and unlikely to feed at temperatures below 14°C to 16°C



(Christophers 1960, Yang et al. 2009). Temperatures above 35°C are unfavorable for adults, and blood feeding decreases as temperatures are increased; exposures above 40°C are usually fatal (Christophers 1960, Carrington et al. 2013).

*Culex quinquefasciatus* has a very comparable life history to *Ae. aegypti* in regards to temperature. In North America, the range of *Cx. quinquefasciatus* is generally south of the 36°N latitude line (Barr 1957), and for *Ae. aegypti* it is below the 35°N latitude line (Nelson 1986); temperature tolerances likely influence these similarities in geographic ranges. *Culex quinquefasciatus* larvae can complete development between 15°C and 34°C, with survival highest between 20°C and 30°C (Rueda et al. 1990); models by Ahumada et al. (2004) suggest that populations can persist when average yearly temperatures exceed 14.6°C. Adults cannot withstand long-term exposures of 5°C or lower (Tekle 1960) and blood feeding is limited below 15°C (Eldridge 1968). Adult survival drops off drastically at temperatures greater than 34°C (Rueda et al. 1990).

#### 1.4.2. Supercooling Point

The supercooling point (SCP) is the temperature at which the cells of an organism's body begin to freeze. To determine SCP, a thermocouple is placed on an insect, and the temperature is gradually lowered at a constant rate; upon the start of freezing the latent heat of crystallization initiates an exotherm, and the lowest temperature reached just prior to this point marks the SCP (Sinclair et al. 2015). SCP is often thought of as the starting point for determining cold tolerance; for most organisms it has limited ecological relevance and must be considered along with other cold tolerance parameters (Renault et al. 2002). There are three general categories of cold-tolerance strategies: chill-susceptible, freeze-avoidant, and freeze-tolerant (Sinclair et al. 2015). Chill-susceptible insects cannot survive any internal ice formation and die from cold exposure

well before the SCP is reached (Bale 1993). Freeze-avoidant insects adapt to freezing conditions by supercooling bodily fluids and therefore preventing ice formation; freeze-tolerant insects can actually withstand a substantial amount of internal freezing (Salt 1961).

The cold tolerance strategy of mosquitoes can vary depending on species, life stage, and acclimation. Copeland and Craig Jr (1990) found that *Orthopodomyia* and *Anopheles* species overwintering as larvae in tree-holes were freeze-tolerant, but only when acclimated to low winter temperatures. However, Liu et al. (2018) determined that all stages of *Culex pipiens pallens* were chill-susceptible, even though adults had significantly lower supercooling points than larvae and pupae. Eggs of *Ae. aegypti*, *Ae. triseriatus*, and *Ae. albopictus* are chill-susceptible as well, with mortality occurring at much higher temperatures than the SCP regardless of cold acclimation (Hanson and Craig 1995). Given what is known about their biology, both *Ae. aegypti* and *Cx. quinquefasciatus* are likely chill-susceptible species; however, as yet this has not been determined for adults.

#### 1.4.3. Critical Thermal Limits

The critical thermal maximum (CTMax) of organisms was first assessed in reptiles and was defined as “the thermal point at which locomotory activity becomes disorganized and the animal loses its ability to escape from conditions that will promptly lead to its death” (Cowles and Bogert 1944). Since this time, CTMax has been evaluated in a large variety of animals, including mammals (Erskine and Hutchinson 1982), amphibians (Zweifel 1957, Miller and Packard 1974), fish (Currie et al. 1998, Rajaguru 2002) and invertebrates (Poulton et al. 1989, Dallas and Ketley 2011, Vinagre et al. 2015). CTMax is determined by exposing an organism to a temperature regime with a constant rate of increase, until a physiologically relevant end point signaling a loss of control of function. While this endpoint for CTMax can vary greatly

depending both on experimental design as well as the organism being tested, the two most common endpoints used are loss of righting response and the onset of spasms (Lutterschmidt and Hutchison 1997).

The critical thermal minimum (CTMin) is defined as the point at which an organism enters into a reversible state of paralysis known as chill coma, which is often preceded by loss of coordination (Hazell and Bale 2011). In practice, the CTMin is determined by the point at which an insect loses the ability to move or respond to an environmental stimulus (Sinclair et al. 2015). To determine CTMin, an organism is exposed to a temperature regime with a constant rate of decrease, usually between 0.1 and 0.25°C per minute (Huey et al. 1992, Sinclair et al. 2015). The endpoint for entry into chill coma depends on the organism in question, but commonly used metrics include lack of response to prodding (Klok and Chown 1997), onset of inactivity (Cokendolpher and Phillips 1990, Andersen et al. 2015), or reduced motor function (Lyons et al. 2012).

Studies of critical thermal limits in mosquitoes in the literature are scarce. Lyons et al. (2012) thoroughly assessed thermal limits in *Anopheles arabiensis* and *Anopheles funestus*. For CTMin protocols, they determined that a mosquito had entered chill coma when it could no longer cling to the tip of a paintbrush and displayed reduced motor function, such as spasms. The endpoint for CTMax was defined as reduced motor function following a period of rapid flight. Their success with these methods supports the ability to use mosquitoes in thermal limits assays, and provides a basis for experimental design to test other species.

#### 1.4.4. Chill Coma Recovery Time

Chill coma recovery time (CCRT) is often coupled with CTMin experiments and measures the amount of time required for an insect to regain function after entering into chill

coma (Hazell and Bale 2011). After determining the CTMin, the organism is exposed to a temperature below this threshold for a set amount of time, usually determined by ecological relevance, and brought into a state of chill coma. The organism is then removed and allowed to recover at a warmer temperature, often room temperature; the average time for this process to take place is the CCRT (Sinclair et al. 2015). As chill coma is defined by paralysis and loss of muscle or nerve function (MacMillan and Sinclair 2011), the regaining of any coordinated movement usually marks the recovery of an organism.

Like thermal limits, investigations into CCRTs of mosquitoes are also largely absent from the literature. However, once the CTMin has been determined, CCRT experiments are a relatively easy extension that can provide further information on the cold tolerance ability of a species. The most important consideration for designing a CCRT experiment is the relevant exposure time to the CTMin. For mosquito species such as *Cx. quinquefasciatus* and *Ae. aegypti*, which live in tropical to temperate areas, a relevant exposure time would likely be more short-term, representing the overnight lows during colder months of the year.

#### 1.4.5. Lethal Temperatures

Lethal temperatures (LTs) quantify the temperature at which mortality occurs in an organism given a set exposure time and acclimation regime. Often, lethal temperatures are expressed proportionally, such as the LT<sub>50</sub> being the temperature at which 50 percent of individuals die (Sinclair et al. 2015). Like the CCRT, ecological relevance usually dictates how long insects are exposed to a temperature regime. This can range from acute exposures, modelling extremes likely to be experienced during a summer or winter, to more long-term exposures, such as an overnight exposure to an average daily low during the winter (Sinclair et al. 2015). Once exposure regime is determined, groups of insects are exposed to a range of

temperatures; ideally, this range should include at least five temperatures and must include groups with both 0% and 100% survival. Analysis using linear regression, similar to probit analysis, can yield a temperature-response curve that predicts the temperatures required for any proportion of survival (Sinclair et al. 2015).

Measures of lethal temperatures in mosquitoes are somewhat more common than other metrics of thermal tolerance. Both *Ae. aegypti* larvae and adults have a LT<sub>99</sub> close to 45 °C for short term exposures of ten to fifteen minutes; this temperature decreases to 41 °C for adults when the exposure time is increased to one hour (Christophers 1960, Mourya et al. 2004, Andersen et al. 2006). Adults can survive exposures of one hour at 4 °C, but longer exposures result in mortality (Christophers 1960). Upper and lower lethal temperatures for *Cx. quinquefasciatus* are largely unexplored. Lyons et al. (2012) examined lethal temperatures in *An. arabiensis* and *An. funestus* using a four hour exposure time to represent the period of daily peak temperatures. These kinds of more ecologically relevant exposure times have not been examined to determine LTs in either *Ae. aegypti* or *Cx. quinquefasciatus*.

## 1.5. Objectives

Our goal was to understand how consumption of sugar alcohols impacts mortality in mosquitoes, as well as how sugar alcohols and other factors impact thermal tolerance. We examined toxicity using the following objectives: 1) to determine mortality of *Ae. aegypti* and *Cx. quinquefasciatus* fed on the sugar alcohols erythritol, sorbitol, and xylitol, and 2) to determine the preference of these two species between sugar alcohols and sugars. To determine impacts of sugar alcohols, diet, species, and sex on thermal tolerance, we investigated an additional two objectives: 1) to evaluate and compare thermal tolerance in *Ae. aegypti* and *Cx.*

*quinquefasciatus*, and 2) to examine how thermal tolerance is impacted by consumption of sugars, sugar alcohols, and blood in *Cx. quinquefasciatus*.

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## **Chapter 2. Lethality of Sugar Alcohols against *Culex quinquefasciatus* and *Aedes aegypti***

### 2.1. Introduction

The mosquito is often considered one of the deadliest animals in the world; millions of people worldwide are at risk for mosquito-borne diseases that result in hundreds of thousands of cases of illness and death every year. *Aedes aegypti* (L. 1762) is the primary vector for a number of devastating viruses, including dengue, yellow fever, and chikungunya viruses (Barrett and Higgs 2007, Bhatt et al. 2013, Vega-Rua et al. 2014), while *Culex quinquefasciatus* Say 1823 is responsible for the transmission of *Wuchereria bancrofti*, the causative agent of lymphatic filariasis, and West Nile virus in the Americas (Hayes et al. 2005, Centers for Disease Control 2013). Both species have large global distributions and are found abundantly in most tropical and subtropical areas (Barr 1957, Nelson 1986, Farajollahi et al. 2011). Currently, reducing the disease burden caused by these mosquito species largely relies on reduction of mosquito populations.

Historically, mosquito control has relied heavily on chemical insecticides; however, effective chemicals are limited in number and many rely on the same modes of action. The consequential cases of resistance development, as well as environmental concerns, have led to the call for novel chemistries and methods for eliminating mosquitoes (World Health Organization 2012). Over the past few decades, a number of researchers have investigated the efficacy of attractive toxic sugar baits as a novel means of controlling mosquitoes over a broad range of genera and species (Muller et al. 2010a, Fulcher et al. 2014, Qualls et al. 2014, Revay et al. 2015, Scott-Fiorenzano et al. 2017).

Attractive toxic sugar baits (ATSBs) take advantage of mosquitoes' natural sugar feeding behavior in order to control them. Almost all mosquitoes seek sugar meals shortly after

emerging, and many continue to sugar-feed throughout their lifetimes, acquiring energy for flight and reproduction (Clements 1992, Foster 1995). ATSBs combine a sucrose-based attractant with toxins such as boric acid (Muller et al. 2010a, Muller et al. 2010b), spinosad (Muller et al. 2008), or eugenol (Revay et al. 2014). Deployment of ATSBs usually occurs either through bait stations, or as foliar sprays applied to vegetation (Fiorenzano et al. 2017). Studies have reported non-target feeding on ATSBs, particularly when applications occur on flowering vegetation; however, in general, the non-target effects of ATSBs seem to be low, with by far the greatest effects on mosquitoes and midges (Khallaayoune et al. 2013, Qualls et al. 2014).

In order to fully take advantage of this control strategy, finding toxins that have the least environmental impact and monetary cost is a priority. Sugar alcohols, a class of polyol compounds, have recently been found to have insecticidal activity against some fly species, with erythritol being especially toxic (Baudier et al. 2014, Zheng et al. 2016, O'Donnell et al. 2018). Sugar alcohols are found abundantly in nature in various plant species (Shindou et al. 1989, Pharr et al. 1995, Stoop et al. 1996, Lee 2015), and many are commercially available for human consumption as non-nutritive sweeteners. Due to their low cost and high human safety, sugar alcohols present a potential ideal toxin for use in ATSBs.

We examined the lethality of three sugar alcohols (erythritol, sorbitol, and xylitol) against adult female *Ae. aegypti* and *Cx. quinquefasciatus* mosquitoes. We hypothesized that feeding on sugar alcohols at high concentrations (30%) would significantly reduce survivorship compared to survival on sucrose. We additionally conducted choice tests to determine if mosquitoes exhibit a preference between sugars and sugar alcohols, with the hypothesis that mosquitoes would not exhibit a preference. This research will help determine if consumption of sugar alcohols results in adult mosquito mortality, and if mosquitoes are likely to feed on sugar alcohols in the



presence of alternative sugar sources. Ultimately, this study provides a basis for determining the efficacy of sugar alcohols for use in mosquito control through the method of attractive toxic sugar baits.

## 2.2. Materials and Methods

### 2.2.1. Mosquitoes

Mosquito colonies were obtained from East Baton Rouge Mosquito and Rodent Control. This included the Sebring strain of *Cx. quinquefasciatus*, which was originally collected in Sebring, Florida and colonized by staff at the USDA Agricultural Research Station in Gainesville, FL, as well as the Rockefeller strain of *Ae. aegypti*, originally collected in the Caribbean and colonized at the Rockefeller Institute in 1930 (Kuno 2010). The Sebring strain has been maintained in colony at the medical entomology lab at Louisiana State University since 2017, and the Rockefeller strain has been maintained since 2018.

Prior to experiments, mosquitoes of both species were maintained at 27°C on a 14:10 L:D cycle. Adults were housed in 31cm<sup>3</sup> collapsible cages and provided 10% sucrose solution *ad libitum* from cotton dental wicks. Cages were draped with damp cloth covered with plastic bags to maintain humidity. Mosquitoes were provided blood once a week using an artificial feeding system (Hemotek® Ltd, England) using Parafilm M® (Bemis Company, Oshkosh, WI) as a membrane. *Culex quinquefasciatus* were provided defibrinated chicken blood and *Ae. aegypti* were provided defibrinated sheep blood (Rockland™ Immunochemicals, Limerick, PA). *Culex quinquefasciatus* were given small plastic cups containing aged DI H<sub>2</sub>O for oviposition; *Ae. aegypti* received the same cups lined with seed germination paper. *Culex quinquefasciatus* egg rafts or dried (> 1 week) *Ae. aegypti* eggs on seed paper were hatched in 1.9 L plastic hinged deli containers in 600 mL of aged DI H<sub>2</sub>O with 2.5 mL of bovine liver powder solution (60 g/L).

Larval densities were maintained at 100 to 150 larvae per container with more containers made as needed; each container was provided 1.5 mL of bovine liver powder solution every other day. Pupae were removed individually with plastic pipettes into small plastic containers with aged DI H<sub>2</sub>O and placed into cages for emergence.

### 2.2.2. Mortality Assays

Three sugar alcohols were assessed for lethality: erythritol, sorbitol, and xylitol. Each sugar alcohol was dissolved into a 10% sucrose-DI water solution at either 10, 20, or 30% concentration, with each sugar alcohol assessed at all concentrations simultaneously. Sugar alcohols were added to sucrose solutions instead of DI water alone, as preliminary trials showed improved feeding with sucrose, and tarsal contact with sucrose acts as a stimulant for feeding (Clements 1992). A 10% sucrose solution was used to assess control mortality. Each treatment was tested with three replicates of 20-25 mosquitoes each.

For use in mortality experiments, 3-5 day old *Ae. aegypti* or *Cx. quinquefasciatus* females reared on 10% sucrose were starved for 24 hours prior to the start of each trial. Mosquitoes were aspirated into 500 mL plastic cups containing 30 mL of their designated treatment solution in a small plastic cup with a cotton dental wick. One drop of blue food coloring was added to each solution so feeding could be observed throughout the trial by the presence of blue coloring in the abdomen. We did not attempt to quantify feeding, but observed high feeding rates, particularly during the first 24 hours. The 500 mL cups were covered with fine mesh secured with rubber bands and each was topped with a Petri dish to reduce evaporation. Each cup was assessed for mortality every 24 hours for 72 hours. Mosquitoes were disturbed by tapping the bottom of the cup, and any individual that did not display coordinated movement (flying or walking on all six legs) was recorded as dead.

### 2.2.3. Choice Tests

After determining the lethality of the sugar alcohol solutions, we selected the most effective solution for use in a two-choice test for both *Ae. aegypti* and *Cx. quinquefasciatus* to determine preference between sucrose alone and sucrose with an added sugar alcohol. We tested 1-2 day old never-fed mosquitoes, to eliminate any effects of previous feeding on sucrose on preference. Replicates of 10-15 mosquitoes were aspirated into 950 mL plastic cups covered with fine mesh secured with rubber bands. Each cup contained both 30 mL of 10% sucrose solution and 30 mL of 30% erythritol in 10% sucrose, provided in small plastic cups with cotton dental wicks. We added either red or blue food dye at 1% concentration to each solution; we used a total of ten replicates, five of combination A (red sucrose and blue sucrose-erythritol) and five of combination B (blue sucrose and red sucrose-erythritol). Mosquitoes were allowed to feed for a period of two hours, after which point they were killed by freezing for later examination. Each mosquito's abdomen was observed using a dissecting microscope and scored as red, blue, purple, or unfed. To determine preference, the following choice index (CI) was calculated for each replicate cup (Ignell et al. 2010):

$$CI_{red} = (n_{red} + n_{purple}) / n_{total}$$

$$CI_{blue} = (n_{blue} + n_{purple}) / n_{total}$$

The mean CIs for the sucrose and erythritol-sucrose solutions were calculated for both combination A and B; we compared mean CIs of the same solutions between combinations using a Fisher's exact test (JMP®, Version 14) to test for differences in color preference. We combined datasets that showed no statistically significant differences ( $P < 0.05$ ).

#### 2.2.4. Statistical Analysis

Mortality over time was analyzed separately for each species with generalized linear models with sugar alcohol (erythritol, sorbitol, and xylitol) and concentration (0, 10, 20, and 30%) as the effect variables (PROC GLIMMIX; SAS Version 9.4). We additionally produced generalized linear models for each sugar alcohol separately to determine LC<sub>50</sub> and LC<sub>99</sub> values for each species, with concentration as the main effect (PROC GLIMMIX; SAS Version 9.4). Estimation was based on Laplace approximation for all models. Several GLMs were assessed for fit (binomial with probit link, binomial with logit link, and beta with logit link), and Akaike's information criteria corrected for small sample size and Pearson's  $\chi^2$ / degrees of freedom were used to rank and select the most appropriate models. In *Cx. quinquefasciatus*, for all selected models the error distribution was binomial and the link was probit; in *Ae. aegypti*, the error distribution was beta and the link was logit. To analyze preference data, we calculated choice indices as described in the previous section for sucrose, sucrose-erythritol, and no-choice, and compared these indices with a one-way ANOVA (JMP® Pro Version 14). The assumption of normality of the residuals was upheld (Shapiro-Wilk Pr<W=0.86); as well as homogeneity and linearity, which were assessed by visual examination of the residual plot. Post hoc comparisons were performed using a Tukey test with a significance value of  $\alpha=0.05$ .

### 2.3. Results

#### 2.3.1. Mortality Assays

In *Ae. aegypti*, sugar alcohol was not a significant predictor of mortality ( $F_{2,137}= 0.65$ ,  $p=0.52$ ); however, concentration and the interaction term sugar alcohol x concentration were both significant ( $F_{1,137}=213.74$ ,  $p<0.01$ ;  $F_{2,137}=15.23$ ,  $p<0.01$ ; Figure 2.1). In *Cx. quinquefasciatus*, sugar alcohol was a significant predictor of mortality ( $F_{2,137}=5.06$ ,  $p<0.01$ ; Figure 2.2), in

*Aedes aegypti*

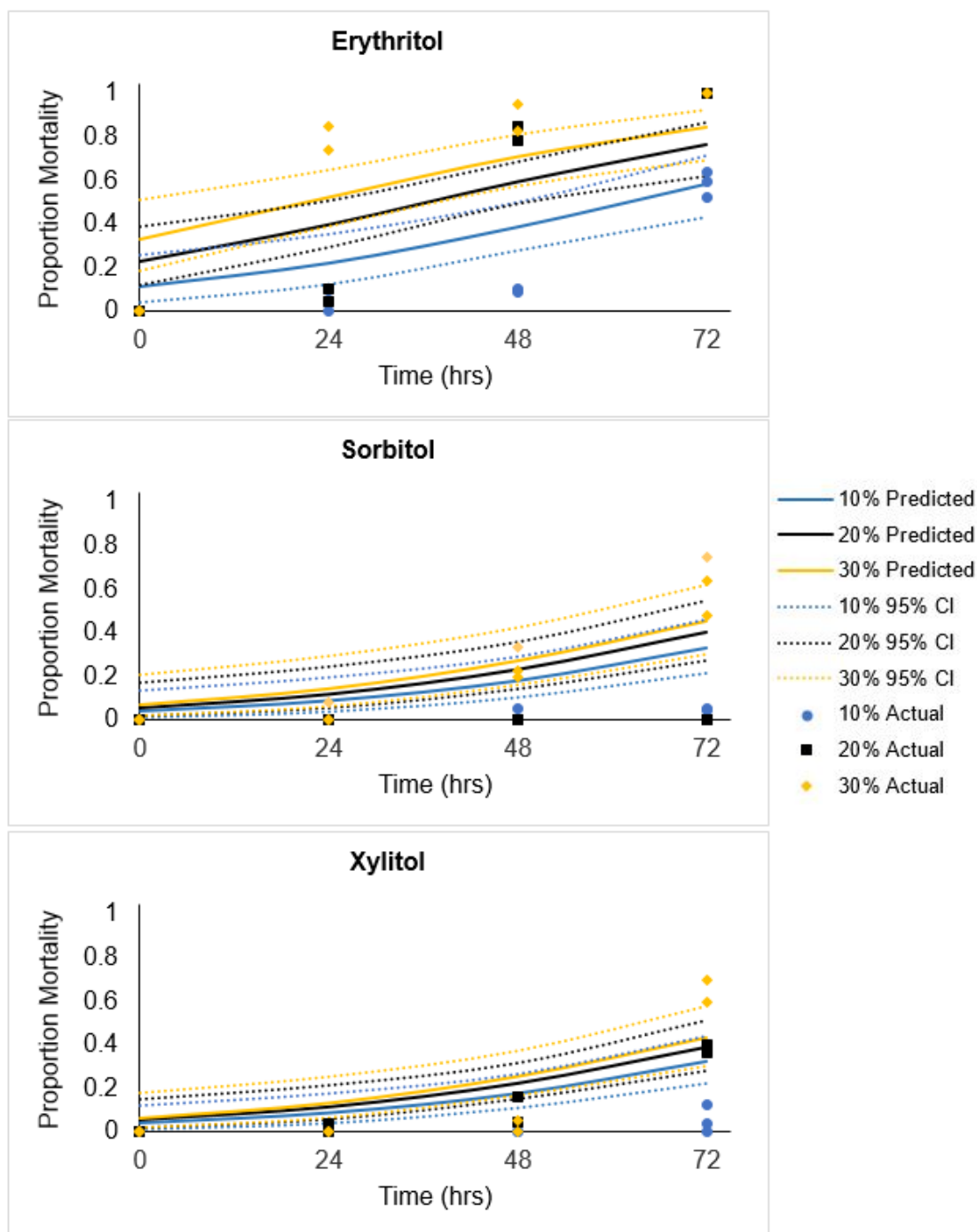


Figure 2.1. Predictions for mortality of *Ae. aegypti* adults over time fed erythritol, sorbitol, or xylitol at concentrations of 10, 20 and 30%. Predictions and 95% confidence intervals were calculated using generalized linear models with a beta distribution and logit link and appear as lines; symbols represent data obtained in mortality trials.

*Culex quinquefasciatus*

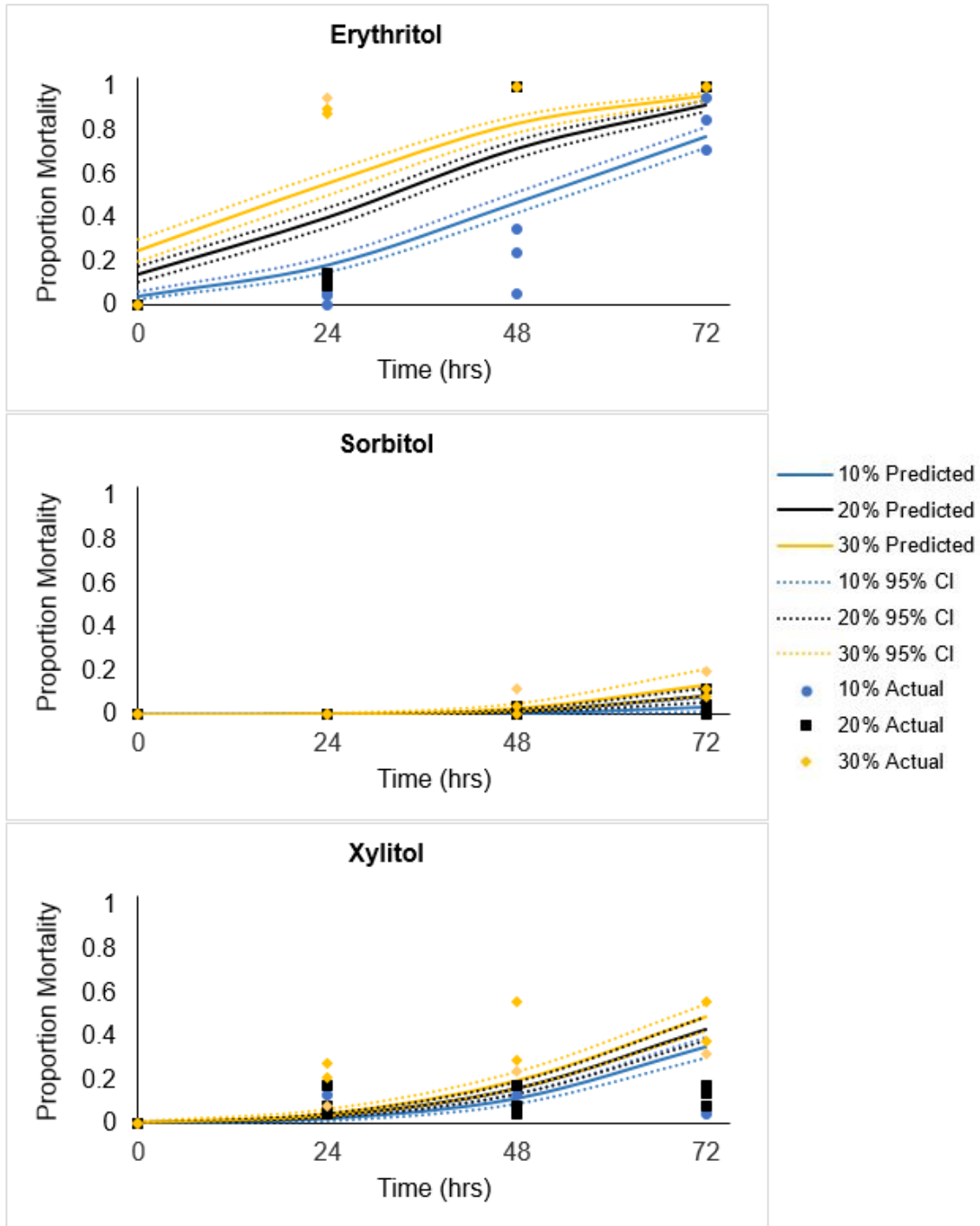


Figure 2.2. Predictions for mortality of *Cx. quinquefasciatus* adults over time fed erythritol, sorbitol, or xylitol at concentrations of 10, 20 and 30%. Predictions and 95% confidence intervals were calculated using generalized linear models with a binomial distribution and probit link and appear as lines; symbols represent data obtained in mortality trials.

addition to concentration ( $F_{2,137}=53.04$ ,  $p<0.01$ ) and sugar alcohol x concentration ( $F_{2,137}=29.67$ ,  $p<0.01$ ). There were significant differences between the effects of all three sugar alcohols, with erythritol having the greatest effect, followed by xylitol, and with sorbitol being the least effective (Figure 2.2).

Erythritol additionally had the lowest  $LC_{50}$  of the three sugar alcohols for both species (Table 2.1). For *Ae. aegypti*, the  $LC_{50}$  for xylitol increased over 4-fold, as compared to the  $LC_{50}$  for erythritol, and 5-fold for sorbitol. For *Cx. quinquefasciatus*, the  $LC_{50}$  values for xylitol and sorbitol were roughly 35 times that of erythritol, and both were far beyond the range of solubility for either compound.

Table 2.1.  $LC_{50}$  and  $LC_{99}$  values for three sugar alcohols for *Ae. aegypti* and *Cx. quinquefasciatus*, expressed as percentage concentration in sucrose solution.  $LC_{50}$  and  $LC_{99}$  values represent the concentration necessary to kill 50 and 99 percent of the population, respectively.

	<i>Ae. aegypti</i>		<i>Cx. quinquefasciatus</i>	
Sugar Alcohol	$LC_{50}$	$LC_{99}$	$LC_{50}$	$LC_{99}$
Erythritol	$7.59 \pm 44.3$	$44.3 \pm 1.82e6$	$3.94 \pm 9.16$	$37.9 \pm 2.41e11$
Sorbitol	$40.0 \pm 23.0$	$427 \pm 8.51e6$	$131 \pm 23.4$	$3790 \pm 6.10e4$
Xylitol	$25.5 \pm 20.5$	$235 \pm 2.14e7$	$150 \pm 17.8$	$323,000 \pm 4.34e14$

### 2.3.2. Choice Test

In both species tested, mosquitoes did not exhibit a preference between sucrose and sucrose-erythritol. Color did not affect preference in either *Ae. aegypti* ( $p=0.54$ ) or *Cx. quinquefasciatus* ( $p=0.16$ ) as determined by Fisher's exact test, allowing us to combine datasets from both choice-test combinations. Although one-way ANOVA showed significant differences between choice indices (CI) for both *Ae. aegypti* ( $F_{2,27}=4.05$ ,  $p=0.03$ ) and *Cx. quinquefasciatus* ( $F_{2,27}=24.32$ ,  $p<0.01$ ), post hoc tests revealed only the CI for non-fed mosquitoes was

significantly higher, with no significant differences between CIs for sucrose and sucrose-erythritol (Table 2.1). On average, greater than fifty percent of mosquitoes of both species did not feed during the trial (Figure 2.2). In *Ae. aegypti*, 22% of mosquitoes fed on a combination of both solutions, with the remaining mosquitoes split relatively evenly between sucrose (13%) and sucrose-erythritol (11%). This differed from *Cx. quinquefasciatus*, in which no mosquitoes fed on a combination of solutions. However, similarly to *Ae. aegypti*, preference was similar between sucrose (25%) and sucrose-erythritol (19%) (Figure 2.3).

Table 2.2 Average choice index (mean  $\pm$  SE) in a two-choice test for never-fed *Ae. aegypti* and *Cx. quinquefasciatus*. Means with different letters are statistically significantly different.

Average Choice Index (CI)		
Choice	<i>Ae. aegypti</i>	<i>Cx. quinq.</i>
No Choice	0.54 ( $\pm$ 0.04) A	0.56 ( $\pm$ 0.04) A
Sucrose	0.35 ( $\pm$ 0.04) B	0.25 ( $\pm$ 0.04) B
Erythritol	0.33 ( $\pm$ 0.01) B	0.19 ( $\pm$ 0.04) B

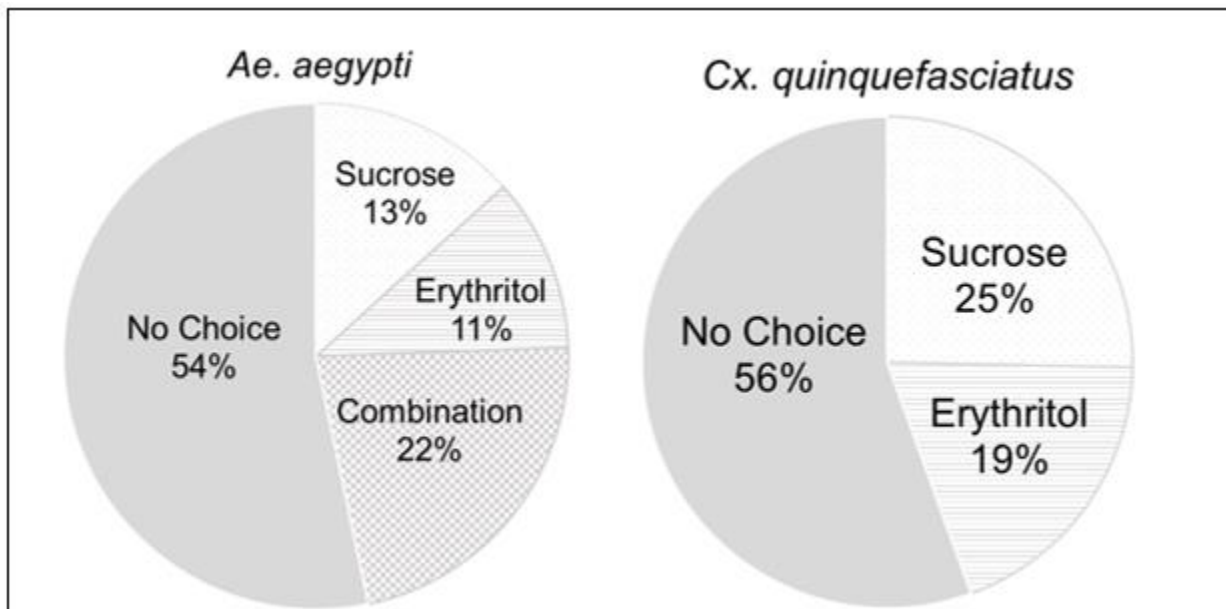


Figure 2.3 The percentage of *Ae. aegypti* and *Cx. quinquefasciatus* mosquitoes feeding on sucrose, erythritol (in sucrose solution), both, or neither in a two-choice test.



## 2.4. Discussion

Sugar alcohols, particularly erythritol, have been shown to have insecticidal properties in several different fly species, including *Drosophila melanogaster* (Baudier et al. 2014), *Drosophila suzukii* (Choi et al. 2017), and *Bactrocera dorsalis* (Zheng et al. 2016). The results of this study support that erythritol, as well as the sugar alcohols sorbitol and xylitol, can cause significant mortality in the two mosquito species evaluated, with dose-dependent and species-specific effects.

Sorbitol was the least effective sugar alcohol in models for *Cx. quinquefasciatus*, and had the highest LC<sub>50</sub> in *Ae. aegypti*. Within mosquitoes, there is a reversible pathway that converts sorbitol to fructose and vice versa with the use of a polyol:NAD oxidoreductase also found in rat liver; this differs from the more common aldose reductase pathway used by lepidopteran insects (Van Handel 1969). Furthermore, at a concentration of 10%, sorbitol has no negative effects on long-term survival of *Ae. aegypti* or the closely related species *Ae. taeniorhynchus*, and seems to even improve survival in comparison to sugar alone (Nayar and Sauerman 1971). The ability of these *Aedes* mosquitoes and potentially other species to metabolize sorbitol effectively may explain the results we obtained. Although we did not observe a significant effect of concentration of sorbitol on *Ae. aegypti* mortality, we found that increasing sorbitol concentration did significantly negatively impact *Cx. quinquefasciatus* survival. These results provide some evidence that at high concentrations, the sorbitol↔fructose pathway may not be efficient enough to break down large quantities of sorbitol. However, without direct evidence this remains a tentative hypothesis to account for the mortality caused by consumption of sorbitol.

Although the role of sorbitol in physiological processes in mosquitoes is largely unknown, in many insects and other organisms sorbitol is commonly stored and used as a

cryoprotectant (Story and Storey 1983, Wolfe et al. 1998, Salvucci 2000, Kostal et al. 2007, Lee 2010). Sugar alcohols and other polyol compounds can depress freezing points, stabilize cell membranes, and protect protein structures; xylitol and erythritol can perform these functions in some organisms, but sorbitol is far more commonly utilized across insects (Baust and Edwards 1979, Lee 2010). It is possible that mosquitoes also utilize sorbitol for thermal protection, and are therefore adapted to tolerate and even benefit from certain levels of sorbitol. Given the rarity of insects using xylitol or erythritol as thermoprotectants, we can hypothesize that mosquitoes do not utilize them, which could potentially help explain why we saw greater toxicity with xylitol and erythritol than with sorbitol.

Like sorbitol, xylitol is also sometimes present in insect tissues as a cryoprotectant, albeit less commonly (Storey and Storey 2004, Colinet et al. 2012). There is no direct evidence for a metabolic pathway for xylitol in mosquitoes or other insects; however, our results support that xylitol is at least partially metabolized in mosquitoes. Choi et al. (2017) found that *Drosophila suzukii* flies could survive as well on xylitol as compared to 1.0 M sucrose solution, as long as the concentration was above 0.05 M. However, survivorship on xylitol was lower than on sorbitol at similar concentrations. In mammals, xylitol is converted to glucose in the liver, although it is often taken up slowly and only partially by the gastrointestinal tract, resulting in gastric distress (World Health Organization). Although mammal and insect systems function drastically differently, xylitol may have similar effects in insects, being only partially absorbed and at high concentrations resulting in stress, leading to death.

Erythritol was highly effective in both species, with LC<sub>50</sub> values of less than 10% in both species. Although the mechanism by which erythritol causes death in flies is not fully understood, Tang et al. (2017) posit that accumulation of erythritol in the hemolymph causes

elevation of osmotic pressure which ultimately results in death. They observed in *D. sukuzii* that after consuming erythritol, flies had extremely high levels of erythritol in the hemolymph, as well as lower levels in frass; they hypothesize that erythritol cannot be metabolized and is instead transported directly from the midgut to the hemolymph without being broken down or stored. This potential mode of action suggests a broad target group that likely extends beyond just the two mosquito species tested in these experiments, making erythritol a good candidate for general mosquitocidal activity.

The results from our choice test experiments also indicate potential usefulness of erythritol as a mosquitocide. We observed no significant differences between preference for sucrose alone and a 30% erythritol solution in sucrose. In nature, mosquitoes use a variety of interacting cues such as plant odor and color in order to locate nectar sources, which can be highly dependent on the species and habitat, as well as other factors (Foster and Hancock 1994, Burkett et al. 1998, Nyasembe et al. 2012, Nikbakhtzadeh et al. 2014). Once an appropriate source has been located, contact of the proboscis or tarsi with sucrose or another sugar will stimulate a feeding response (Clements 1992). In our experiment, we saw no preference between the red and blue coloration of the different sugar solutions; from this, we can posit that consumption was likely randomly based on which solution each mosquito happened to encounter first. However, this is merely speculation without direct observation of the landing and probing behavior of the mosquitoes, a component which we did not include in this experiment.

Surprisingly, we found that in both *Ae. aegypti* and *Cx. quinquefasciatus* over 50% of mosquitoes did not feed on either sugar solution. It is possible that the two-hour period allowed for feeding was not long enough for mosquitoes to acclimate to their new environment, and that the stress of aspiration impacted the desire or ability to feed. Future experiments would benefit

from a set-up where the sugar solutions could be added to the arena instead of the mosquitoes. The experiment would also be improved by direct observation of the mosquitoes to determine if preference is actually based purely on chance encounter, or, more likely, is a more complicated combination of sensory factors. However, despite the potential for improvement, the results of this study provide evidence that mosquitoes will readily consume sucrose solution containing erythritol, even when pure sucrose solution is present.

The results of our choice test experiments, as well as no-choice survival experiments, indicate that erythritol has high potential for use as a mosquito toxin. Sorbitol and xylitol, though having some effects on mortality at high concentrations, were not nearly as potent as erythritol in either species of mosquito examined. If the mode of action of erythritol proposed by Choi et al. (2017) is indeed correct, erythritol likely exhibits broad toxicity across mosquito species, although this remains to be tested and cannot be concluded from the two species tested in these experiments. This potential broad toxicity also merits investigation into the effects erythritol may have on non-target insects, particularly pollinators. However, because erythritol has been deemed safe for human consumption and is already produced commercially, it is a great candidate for use in mosquito control. It would be particularly suited for use in attractive toxic sugar baits, which have already be shown to be effective in the field, and to generally have low non-target impacts (Fiorenzano et al. 2017). We conclude that erythritol has high toxicity when consumed by *Ae. aegypti* and *Cx. quinquefasciatus* mosquitoes, and that mosquitoes will voluntarily feed on erythritol even when other sugar sources are available. Further investigation of the efficacy of erythritol in the field when deployed in ATSBs should be conducted to determine if erythritol can be used in mosquito control.

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## Chapter 3. Thermal Tolerance in *Culex quinquefasciatus* and *Aedes aegypti*: Effects of Species, Sex and, Diet

### 3.1. Introduction

The mosquito species *Aedes aegypti* (L. 1762) and *Culex quinquefasciatus* Say 1823 are responsible for the spread of a number of debilitating pathogens and parasites around the world. *Aedes aegypti* is considered the primary vector for dengue (Bhatt et al. 2013), yellow fever (Barrett and Higgs 2007), and Chikungunya viruses (Vega-Rua et al. 2014), while *Cx. quinquefasciatus* contributes to the spread of *Wuchereria bancrofti*, the causative agent of lymphatic filariasis (Centers for Disease Control 2013), and West Nile virus (Hayes et al. 2005) in the Americas. These two species have similar ranges worldwide, with *Ae. aegypti* typically found between 35°N and 35°S latitudes (Nelson 1986), and *Cx. quinquefasciatus* populations inhabiting regions from 36°N down to 36°S (Barr 1957, Farajollahi et al. 2011). The ranges of these two species are thought to be largely temperature-dependent; *Ae. aegypti* has been found to be limited to areas within a 15°C average annual isotherm (Otero et al. 2006), while models for *Cx. quinquefasciatus* suggest populations can persist only in areas with average annual temperatures above 14.6°C (Ahumada et al. 2004).

While *Ae. aegypti* and *Cx. quinquefasciatus* mosquitoes occupy a mostly overlapping climatic range, their life history strategies for dealing with thermal stress are markedly different. *Aedes aegypti* does not overwinter throughout most of its range and is active year-round; however, in more temperate areas on the edge of its range it may spend colder months in the egg stage (Vezzani et al. 2004, Fischer et al. 2011). In tropical areas, *Cx. quinquefasciatus* populations are also active year-round, but in colder regions only adult females survive the winter, seeking hibernacula, such as tree hollows or barns, in which to undergo quiescence, but not entering a state of complete reproductive diapause (Eldridge 1968, Nelms et al. 2013). Due

to these differences in life history it is possible that *Cx. quinquefasciatus* females may require better cold tolerance strategies than *Ae. aegypti* adults or males of their own species.

Though different mosquito species potentially have inherent differences in cold tolerance, some can also alter their physiology through dietary means. Prior to entering diapause or quiescence, overwintering mosquitoes will often build up lipid reserves from blood meals (Ramsdale and Wilkes 1985) or, more commonly, from sugar meals (Schaefer and Miura 1972, Reisen et al. 1986b). In areas where temperatures go above freezing in the winter season, mosquitoes often remain vagile and continue to sugar feed and take blood meals sporadically (Mitchell 1979, Nelms et al. 2013). While both laboratory and field studies support an important role of diet in cold tolerance, direct measures of how specific diets affect tolerance are sparse in the literature, especially for non-diapausing species such as *Cx. quinquefasciatus*.

The diets of adult mosquitoes can be broadly classified into two categories: protein and sugar. Protein is acquired through blood feeding in most mosquitoes, with the exception of only a few species (Clements 1992). Sugars can be obtained from a wide variety of sources and include a variety of carbohydrates in different proportions. Nectar is the most common sugar source for most mosquito species (Clements 1955, 1992), and is primarily composed of sucrose, glucose, and fructose (Wykes 1952); in addition, mosquitoes can obtain sugars through rotting fruits (Joseph 1970, Clements 1992), which usually contain fructose as well as various sugar alcohols (Lee 2015). The sugar alcohols mannitol and sorbitol have been found to bolster thermal tolerance in a variety of insects (Hendrix and Salvucci 1998, Wolfe et al. 1998, Michaud and Denlinger 2007), and direct consumption of cryoprotectants has been shown to increase cold hardiness in some insects (Li et al. 2014). In *Cx. quinquefasciatus* and mosquitoes in general, the effects of different diets on thermal tolerance are largely unexplored.

Our goal was to understand how diet influences thermal tolerance in mosquitoes, as well as how inherent thermal tolerance differs between sexes and species. Because *Cx. quinquefasciatus* has a more northerly range than *Ae. aegypti* and is more prevalent throughout its northern border (Barr 1957, Nelson 1986, Farajollahi et al. 2011), we hypothesized that *Cx. quinquefasciatus* would have greater cold tolerance than *Ae. aegypti*. Additionally, we predicted that *Cx. quinquefasciatus* females would be the most cold tolerant due to their capacity for overwintering, and that increased cold tolerance would result in a trade-off of decreased heat tolerance. Because only *Cx. quinquefasciatus* females overwinter as adults, we selected them as our model to examine how diet influences thermal tolerance. We hypothesized that diet would have a significant impact on thermal tolerance, with diets enhanced with the cryoprotectants mannitol and sorbitol resulting in increased tolerance. We measured a variety of parameters, including supercooling capacity, critical thermal limits, chill coma recovery times, and lethal temperatures to examine how thermal tolerance is influenced by species, sex, and dietary regime in *Ae. aegypti* and *Cx. quinquefasciatus*. The information gained from this study could allow for a better understanding of differences in thermal tolerance between species with different life histories, as well as how diet can impact thermal tolerance in mosquitoes.

## 3.2. Materials and Methods

### 3.2.1. Mosquitoes

Mosquito colonies were obtained from East Baton Rouge Mosquito and Rodent Control. This included the Sebring strain of *Cx. quinquefasciatus*, which was originally collected in Sebring, Florida and colonized by staff at the USDA Agricultural Research Station in Gainesville, FL, as well as the Rockefeller strain of *Ae. aegypti*, originally collected in the Caribbean and colonized at the Rockefeller Institute in 1930 (Kuno 2010). The Sebring strain has

been maintained in colony at the medical entomology lab at Louisiana State University since 2017, and the Rockefeller strain has been maintained since 2018.

Prior to experiments, mosquitoes of both species were maintained at 27°C on a 14:10 L:D cycle. Adults were housed in 31cm<sup>3</sup> collapsible cages and provided 10% sucrose solution *ad libitum* from cotton dental wicks. Cages were draped with damp cloth covered with plastic bags to maintain humidity. Mosquitoes were provided blood once a week using an artificial feeding system (Hemotek® Ltd, England) using Parafilm® (Bemis Company, Oshkosh, WI) as a membrane. *Culex quinquefasciatus* were provided defibrinated chicken blood and *Ae. aegypti* were provided defibrinated sheep blood (Rockland™ Immunochemicals, Limerick, PA). *Cx. quinquefasciatus* were given small plastic cups containing aged DI H<sub>2</sub>O for oviposition; *Ae. aegypti* received the same cups lined with seed germination paper. Egg rafts or dried (> 1 week) egg papers were hatched in 64 oz plastic hinged deli containers in 600 mL of aged DI H<sub>2</sub>O with 2.5 mL of bovine liver powder solution (60 g/L). Larval densities were maintained at 100 to 150 larvae per container with more containers made as needed; each container was provided 1.5 mL of bovine liver powder solution every other day. Pupae were removed individually with plastic pipettes into small plastic containers with aged DI H<sub>2</sub>O and placed into cages for emergence. Newly emerged adults were taken from the colonies and used for experiments as needed.

### 3.2.2. Thermal Tolerance Metrics

To assess thermal tolerance, we used four experimental metrics: supercooling point (SCP), chill coma recovery time (CCRT), critical thermal limits, and lethal temperatures (LTs). SCP was defined as the point of intracellular freezing, determined as the body temperature of the insect directly prior to initiation of an exotherm (Sinclair et al. 2015). CCRT was defined as the length of time required to recover (stand on all six legs (Andersen et al. 2010)) from a six-hour

exposure to 0°C. The critical thermal minimum (CTMin) was defined as the point at which mosquitoes entered a reversible state of paralysis (chill coma) (Hazell and Bale 2011); the critical thermal maximum was defined as “the thermal point at which locomotory activity becomes disorganized and the animal loses its ability to escape from conditions that will promptly lead to its death” (Cowles and Bogert 1944). LTs were determined as the point of mortality for a four-hour temperature exposure.

### 3.2.3. Experimental Rearing and Acclimation Regimes

To determine the effects of species and sex on thermal tolerance, we measured supercooling points, chill coma recovery times, and lethal temperatures for males and females of *Ae. aegypti* and *Cx. quinquefasciatus*. For each experiment, we placed a small plastic cup with DI H<sub>2</sub>O containing 50 to 100 pupae of one species in a disposable 5 L cardboard cage. Cages were housed in the insectary at 27°C on a 14:10 L:D cycle, and after mosquitoes had emerged and were 1-2 days old they were provided a 10% sucrose solution containing 1% green food dye *ad libitum* for 72 hours. For use in cold tolerance experiments (supercooling, CTMin, chill coma recovery, and lower LT<sub>50</sub>), we acclimated mosquitoes for an additional 72 hours at 18°C on a 14:10 L:D cycle using an insect growth chamber (Caron® Model 6025-1, Marietta, OH). This temperature was chosen for acclimation because it mimics conditions at which quiescence and diapause may be triggered, but at which regular feeding still occurs (Eldridge 1968). Mosquitoes used in heat tolerance experiments (CTMax, upper LT<sub>50</sub>) continued to be maintained at 27°C in the insectary on a 14:10 L:D cycle, also for 72 hours. All mosquitoes were provided free access to sucrose solution during the acclimation period, with the exception of mosquitoes used in SCP experiments. Mosquitoes used in SCP experiments were starved for the last 24 hours of acclimation, as the presence of ice nucleators in the gut can affect SCP (Sinclair et al. 2015). For

all other experiments, recently fed mosquitoes (determined visually by green coloration of the abdomen) were used.

To determine the effects of diet on thermal tolerance, we examined SCPs, critical thermal limits, and CCRTs for female *Cx. quinquefasciatus* mosquitoes reared on five diet treatments: 10% fructose, 10% sucrose, 5% mannitol in 10% sucrose, 5% sorbitol in 10% sucrose, and 10% sucrose supplemented with one blood meal. For each diet treatment, we aspirated 10 to 20 mosquitoes into a 235 mL plastic cup covered with mesh netting. Each cup received 30 mL of the diet treatment with 1% green food dye in a cup with a cotton dental wick. Mosquitoes were acclimated according to the same procedures as described above, except all mosquitoes were removed from acclimation 48 hours before experiments for a period of two hours, at which time the mosquitoes receiving a blood meal as part of their diet treatment were offered defibrinated chicken blood using an artificial feeding system (Hemotek® Ltd, England). Any mosquitoes that did not blood feed at this time were removed and discarded. We used recently fed mosquitoes for all experiments with the exception of SCPs, as described above.

#### 3.2.4. Supercooling Points

To measure supercooling points (SCPs), mosquitoes were attached to type T thermocouples (TC6-T, HOBO®, Onset Computer Corporation, Bourne, MA) at the thorax using a small amount of high vacuum grease (DOW CORNING, Dow Corning Corporation, Midland, MI). Thermocouple wiring was threaded through the lid of a 2 mL cryogenic vial (Corning®, Corning Incorporated, Corning NY), and the thermocouple and attached mosquito were placed inside the vial. Four vials were placed into a freezing container (Corning® CoolCell® LX Freezing Container, Corning Incorporated, Corning NY), which was placed into a -30°C freezer, cooling the insects at a rate of -1.0°C per minute. Temperatures of individual

mosquitoes were recorded once every second using 4-channel thermocouple HOBO® data loggers (UX120-014M, HOBO®, Onset Computer Corporation, Bourne, MA). The SCP was determined as the lowest temperature reached before the initiation of the heat of exotherm (Sinclair et al. 2015). Replicates of 3 to 4 individuals per treatment were assessed for SCP simultaneously. SCPs were determined for 20 to 30 individuals per treatment, with each individual acting as a replicate.

### 3.2.5. Critical Thermal Limits

Measurements of upper (CTMax) and lower (CTMin) critical thermal limits were conducted using a Peltier thermoelectric plate (Model no: CP-200HTTT, TE Technology INC); temperature and ramping rate of the plate was controlled using a thermoelectric temperature cooler (Model no: TC-720, TE Technology INC, MI). We placed 2 to 3 mosquitoes per treatment onto the plate and covered them individually with 16 mm ethylene caps (TainerTop™, Fisher Scientific, Waltham, MA) with a thin mesh covering for observation. For both upper and lower limits mosquitoes were held at 20 °C for two minutes at the start of the trial. The temperature was then lowered to 10°C for CTMin and increased to 30°C for CTMax at a rate of 0.65°C per minute and then held for two minutes. At this point the temperature was decreased or increased at a rate of 0.25°C per minute (Lyons et al. 2012). The temperature of the plate was monitored using a thermocouple to determine the exact temperature of the plate at the time of entry into CTMin or CTMax. Mosquitoes were determined to have reached their CTMin when they lost the ability to cling to the sides of their covering and movement of legs and wings had ceased. The CTMax was established as the point at which mosquitoes ceased movement after a period of rapid flight (Lyons et al. 2012). Mosquitoes that reached their CTMin or CTMax were removed and allowed to recover at room temperature; any mosquitoes that did not recover were

excluded from analysis. The CTMin and CTMax were determined for 20 to 30 individuals each per treatment, with each individual acting as a replicate.

### 3.2.6. Chill Coma Recovery Time

To evaluate CCRT, mosquitoes were induced into chill coma by exposure to 0°C for a period of six hours. Three to five mosquitoes per treatment were aspirated into 120 mL plastic cups covered with fine mesh and then placed into an incubator. After six hours, mosquitoes were removed to room temperature (21°C); individuals were placed into plastic petri dishes with their dorsal side down and monitored every 30 seconds for recovery. An individual was determined as recovered when it was able to stand upright on all six legs without overturning (Andersen et al. 2015). A total of 20 to 30 mosquitoes per treatment were scored for recovery time, with each individual counting as a replicate.

### 3.2.7. Lethal Temperatures

Mosquitoes were exposed to a range of temperatures between -8°C and 4°C for lower or 34°C and 42°C for upper lethal temperatures at two-degree intervals in four-hour increments. These temperatures encompassed 0% to 100% mortality and allowed us to determine lower and upper LT<sub>50</sub> and LT<sub>90</sub> values. Groups of fifteen to twenty mosquitoes were transferred into 4 oz plastic cups covered with fine mesh using a mouth-operated aspirator and placed in an incubator set to the desired temperature. Temperatures inside the incubators were monitored using HOBO® data loggers. After a four-hour exposure period, mosquitoes were removed from the incubator, provided 10% sucrose solution from cotton dental wicks and allowed to recover for 24 hours in an incubator held at 20°C (±0.2°) for lower LT, or in the insectary at 27°C (±0.2°) for upper LT. After the recovery period, mosquitoes were scored for mortality by tapping the bottom



of the plastic cup; mosquitoes that did not display coordinated movement (either climbing or flying) were scored as dead. Three to six replicates per temperature were used per treatment.

### 3.2.8. Wing Lengths

We compared wing length measurements to SCP, CTMin, CTMax, and CCRT values to determine the impact of body size on thermal tolerance parameters, as body size can have effects on some of these metrics (Sinclair et al. 2015). Numerous studies have found a correlation between wing length and body size in mosquitoes (Briegel 1990, Lounibos et al. 1995, Koella and Lyimo 1996), and wing length is a commonly used metric for estimation of body size in the literature (Nasci 1986, Armbruster and Hutchinson 2002, McCann et al. 2009, Helinski and Harrington 2011). We stored whole mosquitoes in a freezer at -18°C after experimentation until wings were measured. We removed the right wing from each mosquito and placed it on a microscope slide with 70% ethanol covered with a glass cover slip. We examined wings using a Leica L2 microscope with EC3 camera attachment; we photographed each wing and determined wing length using the LAS EZ 3.4 software package (Leica Microsystems, Wetzlar, Germany). Wing length was defined as the distance between the base of the costal vein and the distal extreme of the R3 vein (Loetti et al. 2011).

We compared wing lengths between treatment groups using one-way ANOVA with post hoc Tukey test ( $\alpha=0.05$ ), and performed a linear regression analysis for each thermal tolerance parameter using wing length as the explanatory variable (JMP®, Version 14). We assessed the model assumption of normality of residuals with Shapiro Wilk tests ( $Pr W>0.05$ ) and the assumptions of homogeneity of variance and linearity by visual inspection of the residual plot. For all experiments, there were either no significant differences in wing lengths between treatments, or wing length was not a significant predictor of thermal tolerance. Therefore, we did

not include wing length as a variable in further analysis of differences in thermal tolerance parameters between treatments. Complete results from this study are included in the appendix.

### 3.2.9. Statistical Analysis

The residuals for SCP, critical thermal limit, and CCRT datasets were tested for normality using a Shapiro-Wilk test, and differences between treatments were analyzed using a Kruskal-Wallis test. Pairwise comparisons were performed using Wilcoxon rank tests, with a p-value of 0.05 corrected for multiple comparisons ( $p=0.05/K$ ,  $K$ =number of comparisons). For lethal temperature experiments, the data were analyzed using generalized linear models with probit link and  $LT_{50}$  and  $LT_{90}$  values were predicted from the model. Non-overlapping confidence intervals of  $LT_{50}$  and  $LT_{90}$  values indicated significant differences between treatments (Payton et al. 2003). All analyses were performed in JMP®, Version 14.

## 3.3. Results

### 3.3.1. Effects of Species and Sex

Supercooling points varied significantly between treatments ( $\chi^2= 49.98$ ,  $p<0.01$ ). Post hoc tests showed significant differences between species, but not between sexes within the same species (Figure 3.1). *Ae. aegypti* males had the highest average SCP ( $-10.7 \pm 0.27^\circ\text{C}$ , mean  $\pm$  SE), followed by *Ae. aegypti* females ( $-11.8 \pm 0.86^\circ\text{C}$ ). SCPs of *Cx. quinquefasciatus* males ( $-18.5 \pm 0.92^\circ\text{C}$ ) and females ( $-20.6 \pm 0.61^\circ\text{C}$ ) were significantly lower than those of *Ae. aegypti*, but not significantly different from each other. Unfortunately, wing length data were not available for this experiment, so we were unable to exclude this as a contributing factor for these results. Chill coma recovery times also varied significantly between treatments ( $\chi^2= 53.92$ ,  $p < 0.01$ ) (Figure 3.2). *Aedes aegypti* adults took on average more than three times as long as *Cx.*

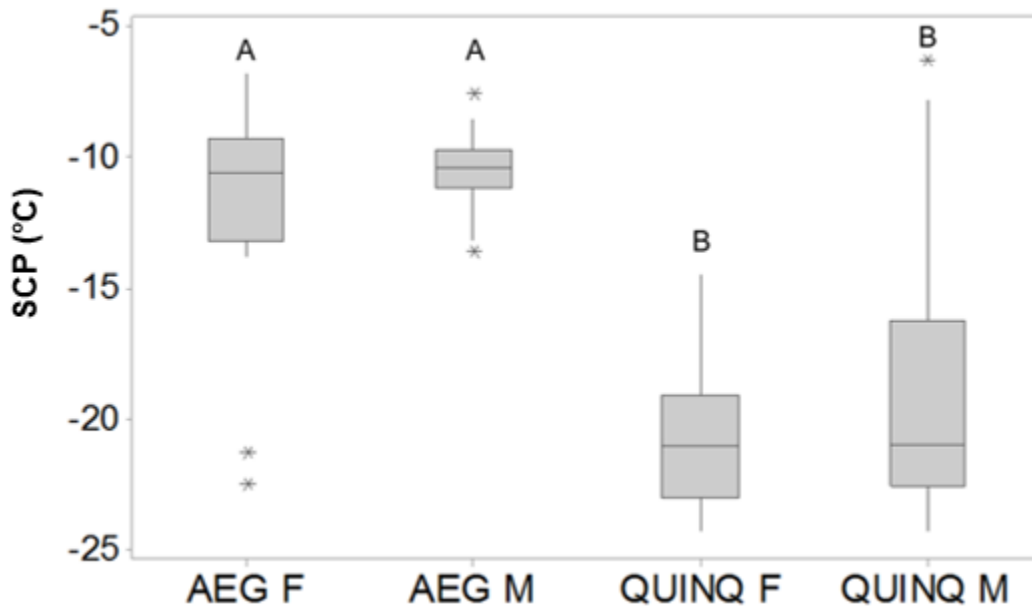


Figure 3.1. Supercooling points (SCP) (°C) for *Ae. aegypti* and *Cx. quinquefasciatus* females and males. Bars with different letters are statistically significantly different ( $\chi^2 = 49.98$ ,  $p < 0.01$ ).

*quinquefasciatus* adults to recover. There were no significant differences between *Ae. aegypti* males ( $44.4 \pm 2.28$  min, mean  $\pm$  SE) and females ( $42.1 \pm 2.42$  min), or *Cx. quinquefasciatus* males ( $13.67 \pm 2.28$  min) and females ( $14.2 \pm 2.19$  min), respectively.

Lower  $LT_{50}$  ( $LLT_{50}$ ) values differed significantly between treatments;  $LLT_{90}$  values differed between species but not sexes within each species (Table 3.1). In *Cx. quinquefasciatus*, males had slightly lower average  $LLT_{50}$  and  $LLT_{90}$  values than females, but there were no significant differences between the sexes. This trend was reversed in *Ae. aegypti*, in which females had a significantly lower average  $LLT_{50}$  compared to males.  $LLT_{90}$  values were not significantly different between sexes in *Ae. aegypti*. Upper  $LT_{50}$  ( $ULT_{50}$ ) values differed significantly between all treatments (Table 3.2). *Ae. aegypti* females had the highest  $ULT_{50}$ ; based on the parameter of  $LT_{50}$  values, females were both more heat- and cold-tolerant males. This is not true of *Cx. quinquefasciatus*; in this species, females had a significantly higher  $ULT_{50}$

than males, the reverse of the trend observed in the LLT<sub>50</sub>. There were significant differences in ULT<sub>90</sub> values between sexes in *Ae. aegypti* but not in *Cx. quinquefasciatus*. Overall, *Ae. aegypti* had significantly higher ULT<sub>50</sub> and ULT<sub>90</sub> values than *Cx. quinquefasciatus*.

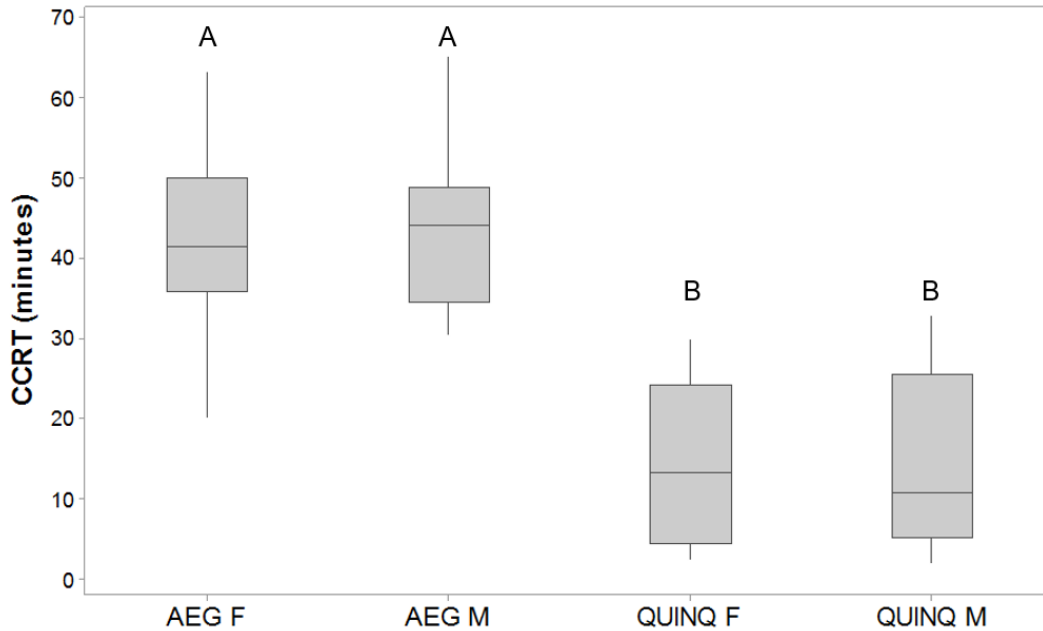


Figure 3.2. Chill coma recovery times (CCRT) (min) for *Ae. aegypti* and *Cx. quinquefasciatus* females and males. Bars with different letters are statistically significantly different ( $\chi^2= 53.92$ ,  $P < 0.01$ ).

Table 3.1. Lower LT<sub>50</sub> and LT<sub>90</sub> values for *Ae. aegypti* and *Cx. quinquefasciatus* females and males. LT<sub>50</sub> and LT<sub>90</sub> values represent the temperature (C°) required to kill 50 and 90% of the population during an exposure of 4 hrs. Differences in treatments are indicated by non-overlapping 95% confidence intervals (CI), marked by different letters. The chi-square value for the likelihood-ratio is the test statistic for the goodness-of-fit of the model compared to a null model (p-values for all models are <0.01).

Species	Sex	n	Slope $\pm$ SE	LT <sub>50</sub> (95% CI) C°	LT <sub>90</sub> (95% CI) ) C°	L-R $\chi^2$
<i>Cx. quinq.</i>	M	496	-0.55 $\pm$ 0.04	-3.59 (-3.91, -3.29) <b>A</b>	-5.91 (-6.50, -5.45) <b>A</b>	304.31
<i>Cx. quinq.</i>	F	499	-0.47 $\pm$ 0.04	-3.00 (-3.34, -2.69) <b>A</b>	-5.71 (-6.37, -5.20) <b>A</b>	302.34
<i>Ae. aegypti</i>	F	303	-1.7 $\pm$ 0.22	-1.61 (-1.84, -1.36) <b>B</b>	-2.90 (-3.37, -2.59) <b>B</b>	194.89
<i>Ae. aegypti</i>	M	359	-0.87 $\pm$ 0.08	-1.06 (-1.30, -0.83) <b>C</b>	-2.53 (-2.96, -2.21) <b>B</b>	249.52

Table 3.2. Upper LT<sub>50</sub> and LT<sub>90</sub> values for *Ae. aegypti* and *Cx. quinquefasciatus* females and males. LT<sub>50</sub> and LT<sub>90</sub> values represent the temperature (C°) required to kill 50 and 90% of the population during an exposure of 4 hrs. Differences in treatments are indicated by non-overlapping 95% confidence intervals (CI), marked by different letters. The chi-square value for the likelihood-ratio is the test statistic for goodness-of-fit of the model compared to a null model (p-values for all models are <0.01).

Species	Sex	n	Slope ± SE	LT <sub>50</sub> (95% CI) C°	LT <sub>90</sub> (95% CI) C°	L-R $\chi^2$
<i>Ae. aegypti</i>	F	324	0.95 ± 0.10	38.51 (38.25, 38.78) <b>A</b>	39.87 (39.55, 40.30) <b>A</b>	317.62
<i>Ae. aegypti</i>	M	303	0.81 ± 0.08	37.13 (36.88, 37.39) <b>B</b>	38.72 (38.72, 39.22) <b>B</b>	222.07
<i>Cx. quinq.</i>	F	400	1.53 ± 0.15	35.21 (35.01, 35.40) <b>C</b>	36.05 (35.84, 36.31) <b>C</b>	462.74
<i>Cx. quinq.</i>	M	379	0.52 ± 0.05	33.92 (33.62, 34.23) <b>D</b>	36.38 (35.92, 37.02) <b>C</b>	196.64

### 3.3.2. Effects of Diet

Supercooling points did not vary between the five diet treatments ( $\chi^2= 6.92$ , P=0.14) (Figure 3.3). Sucrose-fed mosquitoes had the lowest average SCP of all treatments ( $-19.2 \pm 0.85^\circ$ , mean  $\pm$  SE), followed by sorbitol ( $-18.1 \pm 0.96^\circ$ ), fructose ( $-17.7 \pm 0.95^\circ$ ), blood ( $-16.7 \pm 0.96^\circ$ ), and mannitol ( $-16.1 \pm 1.07^\circ$ ).

Critical thermal minima and chill coma recovery times also did not differ significantly between diet treatments ( $\chi^2= 2.12$ , p=0.72;  $\chi^2= 3.87$ , p=0.43) (Figure 3.4). For all treatments, the average CTMin was between 0 and 1°C. Mean chill coma recovery times ranged from 13.24 minutes ( $\pm 2.89$  minutes) for mannitol-fed mosquitoes to 16.23 minutes ( $\pm 2.22$  minutes) for fructose-fed mosquitoes.

Unlike cold tolerance parameters, we did observe significant differences between critical thermal maxima (CTMax) between diet treatments ( $\chi^2=28.09$ , p<0.01) (Figure 3.6). The average CTMax values for all treatments were between 38° and 42°C. Fructose-fed mosquitoes had the highest CTMax ( $41.60 \pm 0.33^\circ$ , mean  $\pm$  SE), followed by sorbitol ( $40.80 \pm 0.40^\circ$ ),

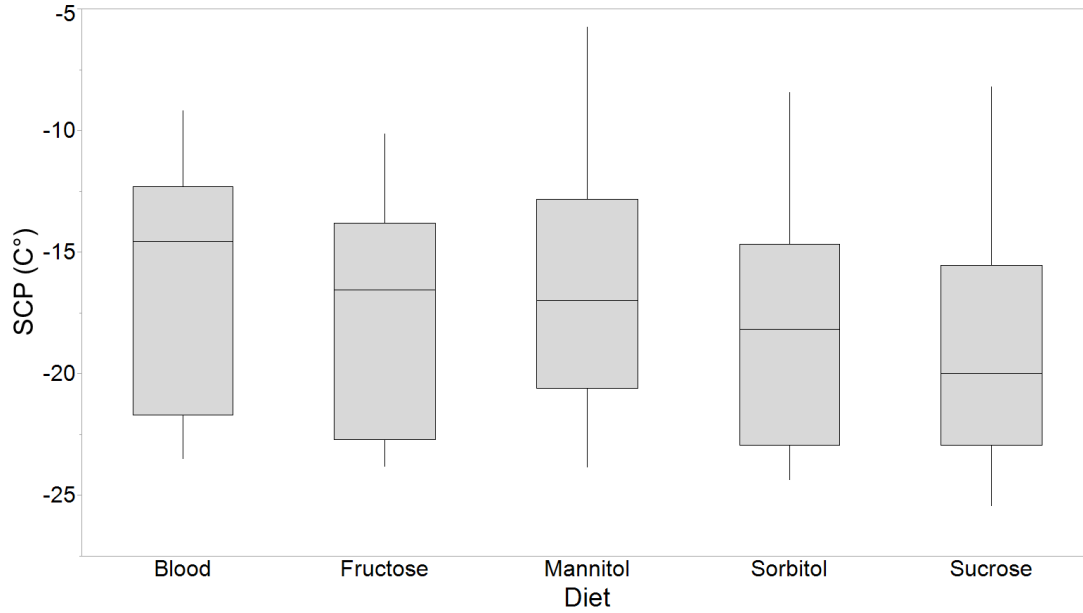


Figure 3.3. Supercooling points (SCP) (C°) for *Cx. quinquefasciatus* females reared on five diets: 10% sucrose supplemented with one blood feeding, 10% fructose, 5% mannitol in 10% sucrose solution, 5% sorbitol in 10% sucrose solution, and 10% sucrose.

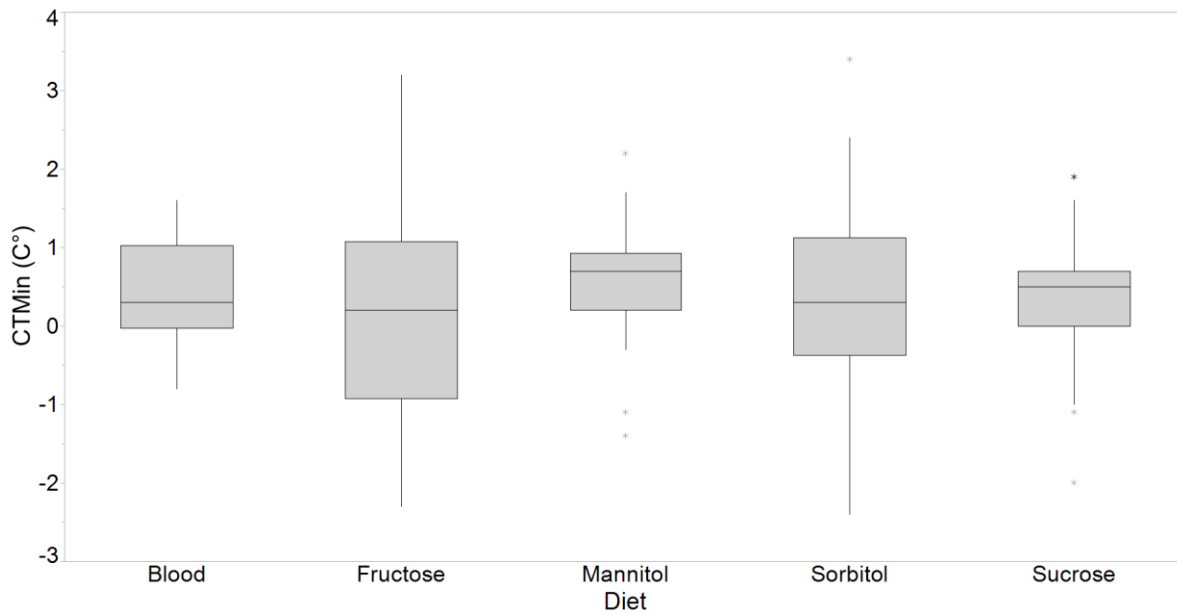


Figure 3.4. Critical thermal minima (CTMin) (C°) for *Cx. quinquefasciatus* females reared on five diets: 10% sucrose supplemented with one blood feeding, 10% fructose, 5% mannitol in 10% sucrose solution, 5% sorbitol in 10% sucrose solution, and 10% sucrose.

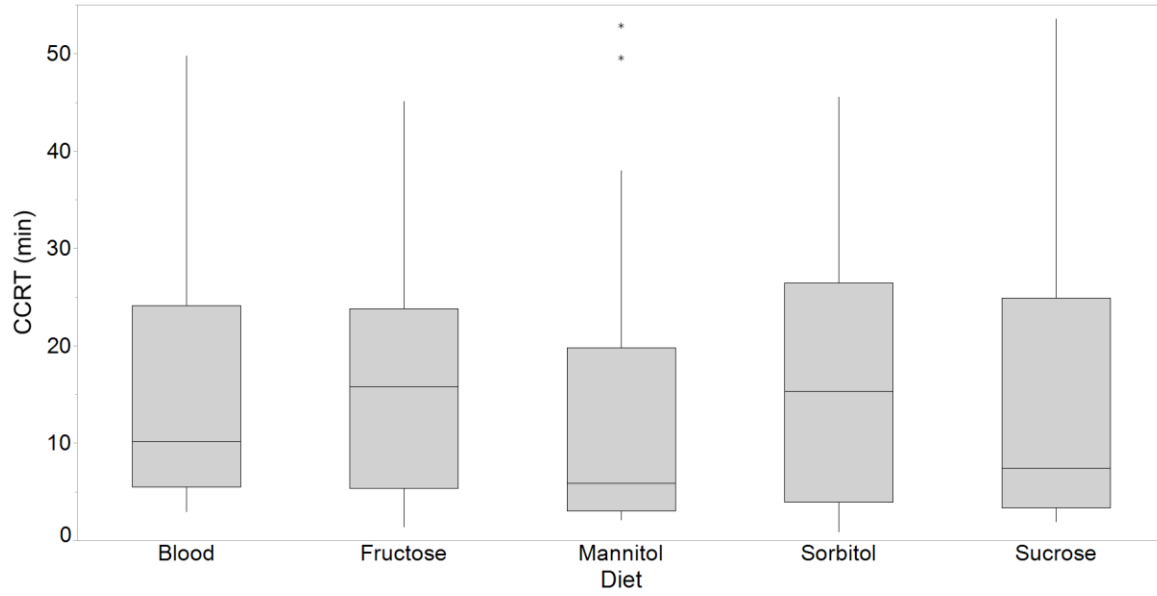


Figure 3.5. Chill coma recovery times (CCRT) (min) for *Cx. quinquefasciatus* females reared on five diets: 10% sucrose supplemented with one blood feeding, 10% fructose, 5% mannitol in 10% sucrose solution, 5% sorbitol in 10% sucrose solution, and 10% sucrose.

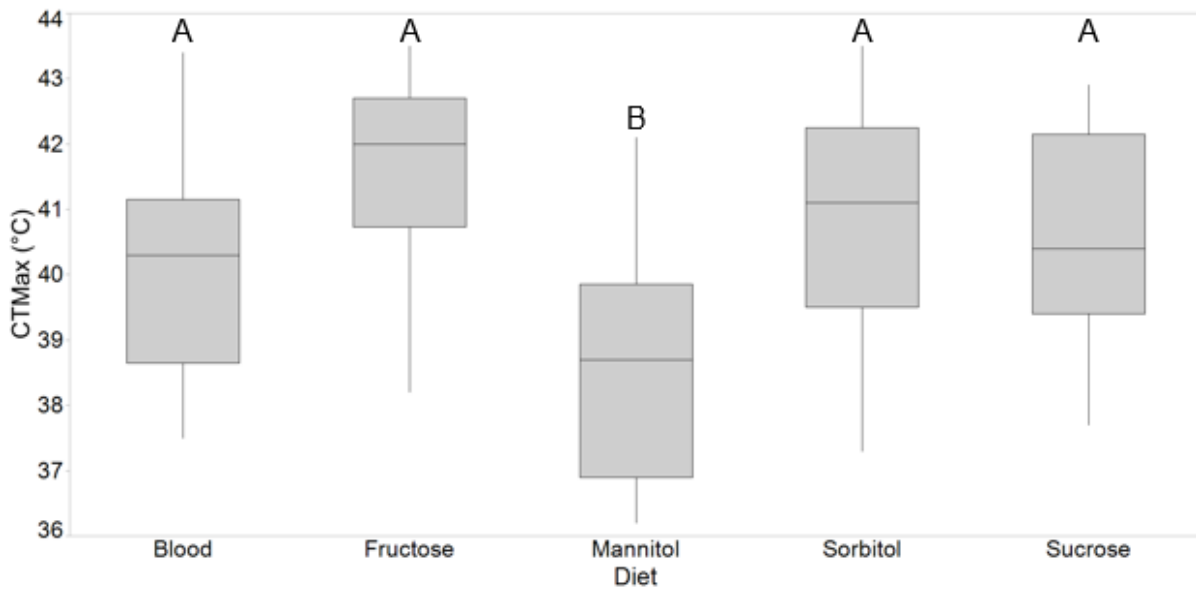


Figure 3.6. Critical thermal maxima (CTMax) (°C) for *Cx. quinquefasciatus* females reared on five diets: 10% sucrose supplemented with one blood feeding, 10% fructose, 5% mannitol in 10% sucrose solution, 5% sorbitol in 10% sucrose solution, and 10% sucrose. Bars with different letters are statistically significantly different ( $p < 0.01$ ).

sucrose ( $40.59 \pm 0.35^{\circ}\text{C}$ ), and blood ( $40.17 \pm 0.36^{\circ}\text{C}$ ); however there were no significant differences between these treatments. Mannitol-fed mosquitoes did have a significantly lower CTMax than all other diet groups ( $38.5 \pm 0.36^{\circ}\text{C}$ ).

### 3.4. Discussion

We determined that *Cx. quinquefasciatus* was significantly more cold tolerant than *Ae. aegypti* based on the parameters tested, and that this was accompanied by a lesser tolerance for heat, as measured by upper lethal temperatures. However, overall we did not see a strong effect of sex on thermal tolerance within either species, contrary to our hypothesis that *Cx. quinquefasciatus* females would be more cold tolerant than males. In *Cx. quinquefasciatus* females, we did not see any significant differences in thermal tolerance based on diet treatment, with the exception of the CTMax, which was significantly lowered when mosquitoes were fed mannitol.

We predicted that *Cx. quinquefasciatus* females would be the most cold tolerant of all sex-species combinations tested in the study. This hypothesis was based on distribution data that show that *Cx. quinquefasciatus* populations are present in colder geographic areas than *Ae. aegypti* (Nelson 1986, Farajollahi et al. 2011, Hahn et al. 2017), as well as the life history trait that *Cx. quinquefasciatus* females overwinter as adults in a quiescent state, unlike males or both sexes of *Ae. aegypti*, which cannot survive winter conditions in temperate areas (Christophers 1960, Almiron and Brewer 1996, Fischer et al. 2011). However, it was unclear if this hypothesized cold tolerance would be exhibited by lab-colonized mosquitoes, or if it is largely a product of environmental adaptation.

Conducting thermal tolerance studies under laboratory conditions allowed us to control for adaptation by standardizing rearing regimes and acclimation conditions for all groups. Based



on our results, adaptation alone cannot explain differences in thermal tolerance between species. We observed that *Cx. quinquefasciatus* adults were, on average, twice as efficient at supercooling, three times as efficient at chill coma recovery, and had LLT<sub>50</sub> values several degrees cooler than *Ae. aegypti*. However, *Ae. aegypti* males, and especially females, could survive significantly higher temperatures than *Cx. quinquefasciatus*. Although we did not directly examine the mechanisms behind these results, we propose several ways in which physiological processes may influence thermal tolerance in these mosquito species.

The mechanisms by which mosquitoes undergo cold hardening are largely unexplored, and most research on cold tolerance focuses on the physiological changes invoked upon exposure to diapause-inducing conditions. Most of this literature examines the same species, *Culex pipiens pipiens*, the northern house mosquito, which is often considered a model for mosquito diapause. *Culex quinquefasciatus* is very closely related to *Culex p. pipiens*; they are virtually morphologically indistinguishable and capable of interbreeding, and differ mainly in geographic range and overwintering strategies (Jupp 1978, Almiron et al. 1995, Urbanelli et al. 1997). While we cannot assume that the findings of research on *Cx. p. pipiens* can be applied to the species examined in our experiments, we can hypothesize some broader implications for the mechanisms by which mosquitoes in general may tolerate temperature stress.

Although some mechanisms for cold tolerance in *Cx. p. pipiens* occur only under diapause conditions, some result simply from exposure to lower temperatures. Kim et al. (2006) found that two actin genes are expressed in response to low temperatures regardless of diapause condition; these genes redistribute actin from clusters in the midgut to more evenly cover the cytoskeleton, hypothesized to fortify the insect body against cold conditions. However, gene expression was only triggered by below-freezing conditions that would likely be lethal to *Cx.*

*quinquefasciatus* or *Ae. aegypti*. Rinehart et al. (2006) found that rearing temperatures of 18°C significantly increased survival at low temperatures as compared to 25°C. They also observed upregulation of the heat shock protein *hsp70* in response to short-term cold shock but not to diapause conditions, a finding with implications for adaptation to cold stress in non-diapause states.

Heat shock proteins (HSP) are an important mechanism for stress mitigation in many organisms (Parsell and Lindquist 1993, Feder and Hofmann 1999), and are a major component in cold tolerance and overwintering across a diversity of insect orders and life stages (Rinehart et al. 2007). The (2006) Rinehart et al. study supports a crucial role for HSPs in cold tolerance of non-diapausing *Cx. p. pipiens* adults. As the name implies, HSPs are also an important physiological means by which organisms mitigate heat stress (Kregel 2002). *Aedes aegypti* upregulates a number of HSPs in response to acute heat stress, with the most highly upregulated being *hsp26* and *hsp83* (Zhao et al. 2009, Zhao et al. 2010). Interestingly, Benoit et al. (2011) found that *Ae. aegypti*, as well as *Cx. p. pipiens*, *Anopheles gambiae*, and the bed bug, *Cimex lectularius*, upregulate expression of *hsp70*, the same protein expressed in *Cx. p. pipiens* cold stress, upon ingestion of a blood meal, most likely to protect the midgut from heat stress. To date, there are no studies specifically examining HSP expression in *Cx. quinquefasciatus*, and none which examine the effects of cold stress on expression in *Ae. aegypti*. Investigation into whether differences in expression exist between the two species could potentially help explain the differing tolerances we observed in this study, and perhaps explain why we found differences only between species and not sexes.

We hypothesized that in addition to species- and sex-dependent differences in thermal tolerance, we would observe differences in thermal tolerance within the same sex and species

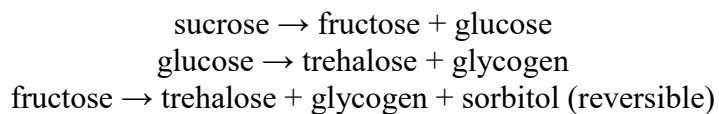
when groups were given different diet treatments. Of the five diet treatments tested, we did not see any significant effects of diet on cold tolerance, and only saw a significant difference in heat tolerance in mannitol-fed mosquitoes. There are several hypotheses to explain why we did not see effects of our diet treatments.

The accumulation of sugars and polyols is a common mechanism for promotion of cold tolerance in many organisms. These substances can directly prevent freezing by depressing the freezing point of bodily fluids; they can also serve to stabilize proteins and protect cell membranes, a likely more relevant function in mosquitoes, which are mostly not freeze-tolerant (Lee 2010). Consumption of sugars prior to overwintering is thought to be a critical component for survival for both diapausing and quiescent mosquitoes (Schaefer et al. 1971, Reisen et al. 1986a, Bowen 1992). However, whereas this behavior is known to be essential for accumulation of lipid energy stores (Clements 1992, Robich and Denlinger 2005), the extent to which sugars function in a true cryoprotective role is largely unexplored.

Whether or not mosquitoes utilize polyols for thermoprotection is also unknown. While it is reasonable to speculate that they do, given the widespread utilization of polyols throughout insects, there is no direct evidence to support this. Benoit and Denlinger (2007) found that in *Cx. p. pipiens*, neither the induction of diapause nor exposure to low temperatures (18°C) resulted in increases in sorbitol, glycerol, or trehalose. The authors of this study only compared the polyol content of mosquitoes reared at two temperatures: 25° and 18°C, so it is possible that more extreme conditions could have different results. Additionally, we cannot directly apply their findings to our study as they did not examine the same species as we did. However, with the current lack of evidence we have no basis to determine how important polyols are for

cryoprotection in mosquitoes, if at all, and the indirect results of our study do not give conclusive evidence one way or another.

It is possible that polyols are not a vital mechanism for cryoprotection in *Cx. quinquefasciatus*; however, it is also possible that the differences in the diets we tested were not distinct enough to impact thermal tolerance. Below is the expected breakdown of sugars in mosquitoes (Van Handel 1969, Clements 1992):



Essentially, since all groups were provided either sucrose or fructose, all mosquitoes would be expected to have some level of trehalose, glycogen, and sorbitol, although the amounts of these compounds would likely vary with diet. Additionally, we used a feeding period of six days; this length of time was chosen to be more field-realistic, as mosquitoes in the wild would likely spend considerable time sugar feeding before the on-set of winter. However, it also gave us less control over how much mosquitoes consumed, which additionally could impact the amounts of these compounds each mosquito stored. It is possible that all mosquitoes were able to obtain enough cryoprotectants to enhance cold tolerance capability to the same degree, and that additional stores did not confer additional protection. Colinet et al. (2013) found that fruit flies fed diets of sucrose, fructose, glucose, and trehalose in varying concentrations were able to effectively incorporate all sugars consumed into storage, but that cold tolerance was actually negatively impacted by diets with higher concentrations of sugars. This suggests that even though sugars and polyols are essential for cryoprotection in many insects, more does not necessarily equate to better. Our study did not directly measure sugar or polyol consumption and storage, so our results do not support or refute this hypothesis. However, our results do support

the idea that *Cx. quinquefasciatus* mosquitoes can tolerate cold conditions equally effectively with the consumption of either sucrose or fructose, and that tolerance is not affected negatively or positively by the consumption of a blood meal. Additionally, our results show that at the low concentration of five percent, direct consumption of polyols does not improve cold tolerance as compared to consumption of sugars alone.

While we did not see an effect of sugar alcohols on cold tolerance, we did observe decreased heat tolerance in mosquitoes that had fed on mannitol-enriched sucrose solution. We hypothesized that consumption of mannitol would have the opposite effect, based on its positive influence on heat tolerance in other insects, namely aphids (Hendrix and Salvucci 1998). However, in aphids mannitol is produced from fructose in response to heat stress and is not directly consumed. Mannitol metabolism is largely uncharacterized in animals, so the fate of mannitol consumed by insects is not known. Research has been conducted with human subjects: the majority of mannitol consumed is not metabolized, but when it is metabolized it is converted to glucose (Saunders and Wiggins 1981).

Although we may not understand how insects metabolize mannitol, research supports that mosquitoes are capable of effectively doing so. Nayar and Sauerman (1971) found that *Aedes taeniorhynchus* mosquitoes were able to survive as long on a ten percent mannitol solution as they did on the same concentration of sucrose. However, in *Ae. aegypti*, they found that survival on mannitol was significantly less than that on sucrose, fructose, or sorbitol. Unfortunately, there are no data available for *Cx. quinquefasciatus*, so their capacity for mannitol metabolism is unknown. However, we can speculate from Nayar and Sauerman's (1971) work that there are likely differences between species in their ability to metabolize different sugars and sugar alcohols, and that the effects of sugar alcohols may be variable. It is possible that in *Cx.*

*quinquefasciatus*, mannitol is not as effectively metabolized as other sugars and consumption may have negative effects. In *Droposhila suzukii*, it is believed that the sugar alcohol erythritol cannot be metabolized, and that this results in accumulation of erythritol in the hemolymph after it is not absorbed in the midgut (Tang et al. 2017). The accumulation of sugar alcohols elevates osmotic pressure within the hemolymph, eventually resulting in death of the organism in the case of the fly. In *Cx. quinquefasciatus*, the impartial absorption of mannitol could lead to osmotic stress, reducing the ability of the mosquito to combat other stresses such as extreme heat. However, mannitol-fed mosquitoes were as effective at tolerating cold stress as all other treatment groups, which does not support this hypothesis.

The results of our study suggest that overall, mosquitoes that have adequate access to sugar sources have an equal capacity for cold tolerance, regardless of the type of sugar or the addition of blood meal or sugar alcohols. However, different species of mosquitoes have inherent differences in their ability to tolerate cold stress, even when provided equal access to sugar sources and acclimated under the same conditions. This suggests that although environmental adaptation likely does play a role in thermal tolerance, there are underlying physiological or genetic differences that limit the abilities of certain species to adapt to thermal stress.

Investigation into the mechanisms behind these differences would greatly contribute to our understanding of how non-diapausing mosquitoes overwinter and tolerate thermal stress.

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## Chapter 4. Conclusion

Mosquitoes are some of the most important medical, veterinary, and economic pests globally; *Aedes aegypti* (L. 1762) and *Culex quinquefasciatus* Say 1823 are prevalent throughout much of the world and greatly contribute to the spread of debilitating viruses and pathogens. It is imperative to fully understand the life history and biology of these species in order to control them, and to consequently develop new and innovative methods for control.

This study sought to evaluate how sugar alcohols may be used as a method for mosquito control, and additionally how they and other factors may influence thermal tolerance in mosquitoes. We found that consumption of the sugar alcohols erythritol, sorbitol, and xylitol had negative effects on survival in adult female *Ae. aegypti* and *Cx. quinquefasciatus*, and that erythritol at concentrations of twenty percent or greater can result in complete mortality in both species. We additionally examined the preference of both species for sucrose with or without erythritol, and found that mosquitoes exhibited no preference between the two. We conclude that sugar alcohols, especially erythritol, have excellent potential for use in mosquito control techniques such as attractive toxic sugar baits, and that field trials should be performed to determine efficacy in wild populations.

The third chapter of this study examined how consumption of sugar alcohols, sugars, and blood affect thermal tolerance in *Cx. quinquefasciatus* females, as well as how the non-dietary factors species and sex may determine limitations to tolerance. We found that diet did not impact cold tolerance, while consumption of mannitol significantly lowered heat tolerance. We hypothesize that other factors such as heat shock protein regulation may be more important than sugar- or sugar alcohol-derived cryoprotectants in mosquitoes, or that our diet treatments all provided adequate cryoprotectant sources. However, we did find that species played a major role

in determining thermal tolerance, and that *Cx. quinquefasciatus* is more cold tolerant, while *Ae. aegypti* is more heat tolerant. Sexes within the same species had similar tolerance levels. We conclude that the type of diet consumed by *Cx. quinquefasciatus* likely does not greatly impact thermal tolerance, and that different mosquito species may have underlying genetic or physiological factors that are more important in determining thermal tolerance.

## Appendix. Wing Lengths

### Supercooling Points

#### *Diet Treatments*

Wing length was not a good predictor of supercooling point for *Cx. quinquefasciatus* females based on  $r^2$  and p-values for a simple linear regression analysis (Figure A.1).

Additionally, we did not observe any significant differences in average wing lengths between treatment groups in this experiment ( $F_{4, 104} = 1.09$ ,  $p = 0.36$ ).

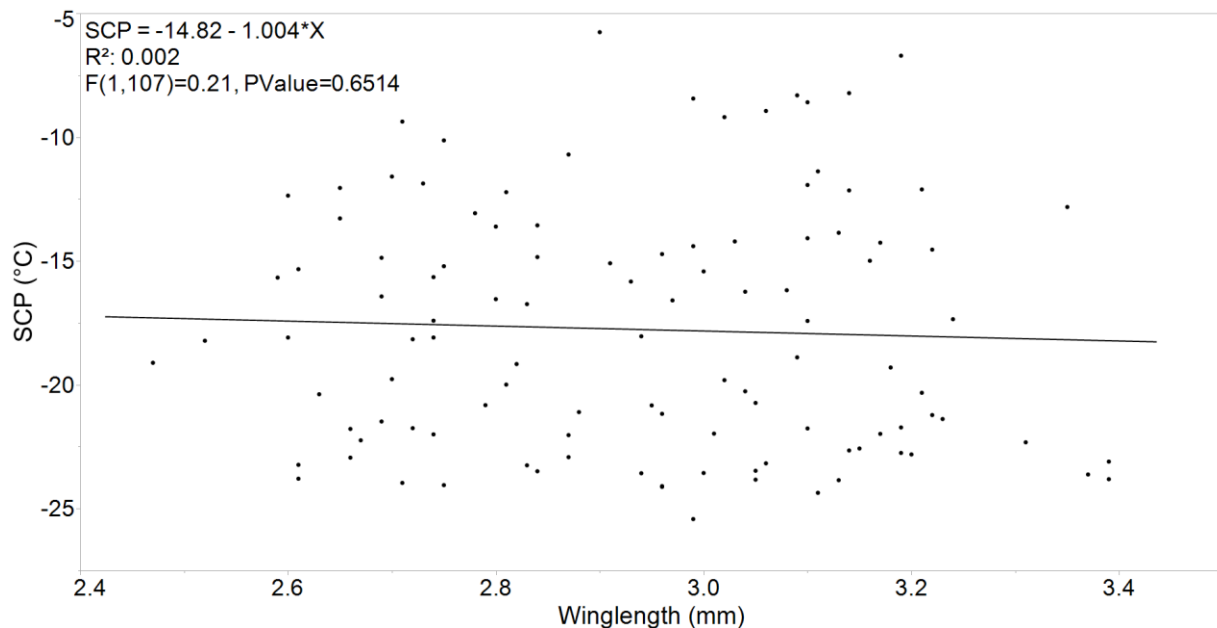


Figure A.1. Simple linear regression model with *Cx. quinquefasciatus* female wing length as the explanatory variable for supercooling point.  $R^2$  values indicate the amount of variation in SCPs explained by wing length, and F- and P-values give the fit of the model as compared to a null model.

### Critical Thermal Limits

#### *CTMin (Diet Treatments)*

The simple linear regression model with *Cx. quinquefasciatus* female wing length as an explanatory variable for CTMin was significant as compared to a null model; however, wing length did not explain a large amount of the variation in CTMin values we observed based on the

$r^2$  value (Figure A.2). Additionally, we did not observe any significant differences in average wing lengths between treatment groups in this experiment ( $F_{4, 97} = 0.63$ ,  $p = 0.64$ ).

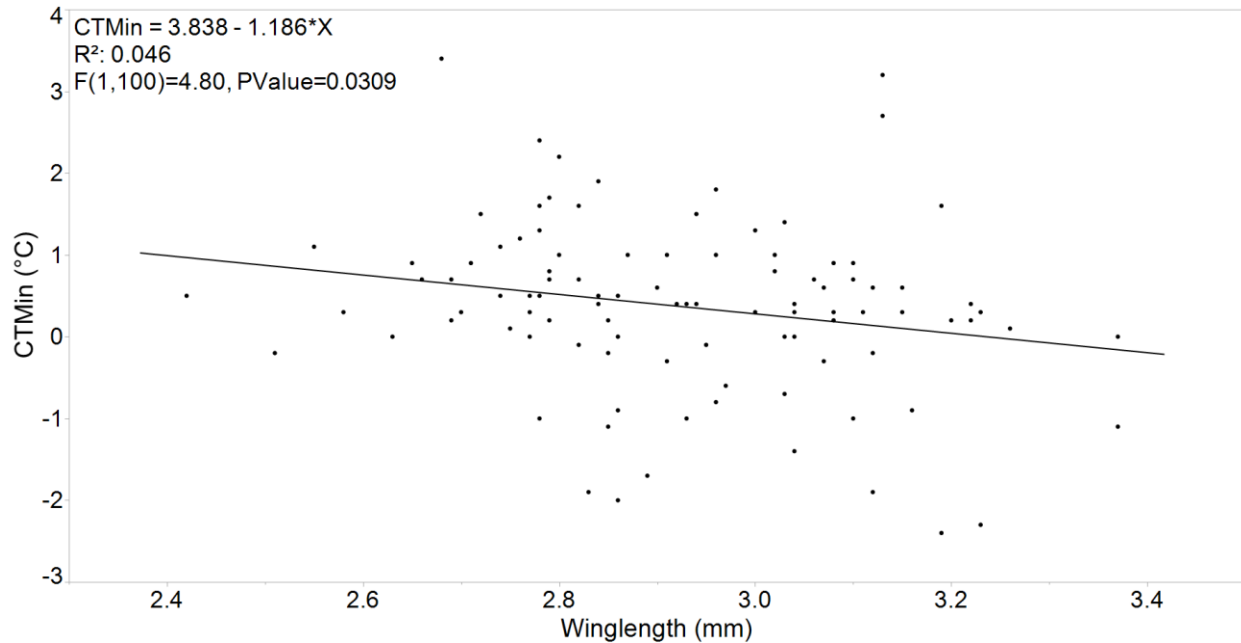


Figure A.2. Simple linear regression model with *Cx. quinquefasciatus* female wing length as the explanatory variable for CTMin.  $R^2$  values indicate the amount of variation in CTMin values explained by wing length, and F- and P-values give the fit of the model as compared to a null model.

#### *CTMax (Diet Treatments)*

Wing length was not a good predictor for CTMax for *Cx. quinquefasciatus* females based on  $r^2$  and p-values for a simple linear regression analysis (Figure A.3). We did not see any significant differences in average wing lengths between treatment groups in this experiment ( $F_{4, 80} = 0.33$ ,  $p = 0.86$ ).

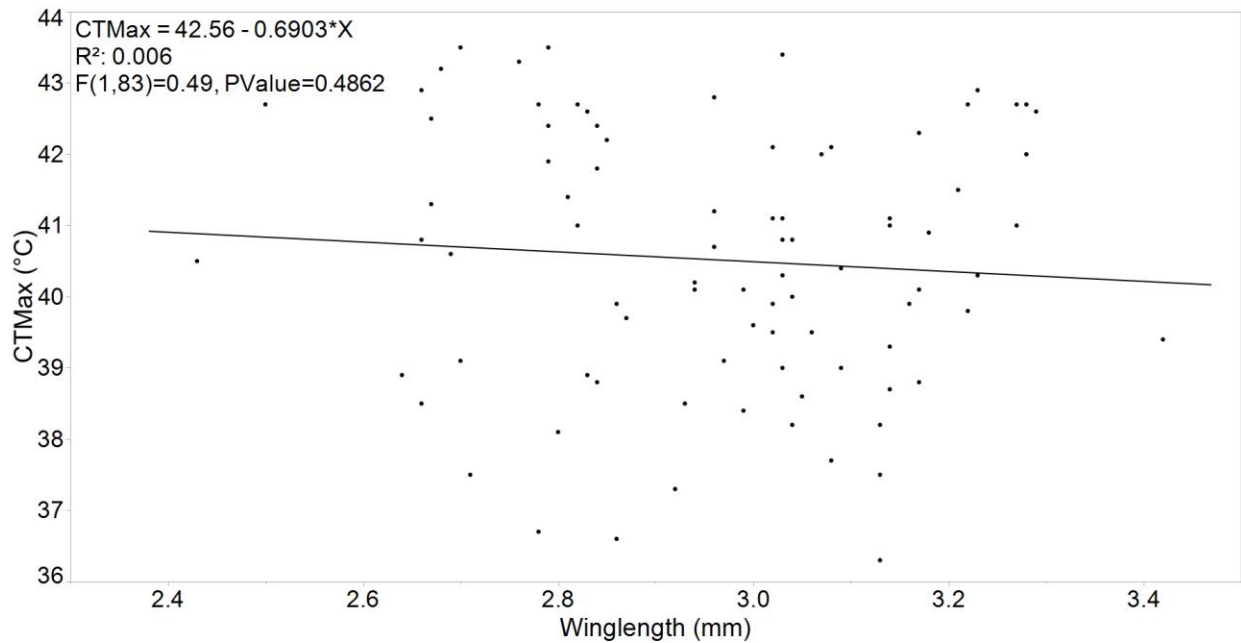


Figure A.3. Simple linear regression model with *Cx. quinquefasciatus* female wing length as the explanatory variable for CTMax.  $R^2$  values indicate the amount of variation in CTMax values explained by wing length, and F- and P-values give the fit of the model as compared to a null model.

## Chill Coma Recovery Time

### *Sex-Species Comparisons*

We did observe significant differences in average wing lengths between treatment groups in this experiment ( $F_{3,63} = 142.7, p < .001$ ) (Table A.1). However, wing length was not a good predictor for CCRT for *Ae. aegypti* and *Cx. quinquefasciatus* males and females based on  $r^2$  and p-values for a simple linear regression analysis (Figure A.4).



Table A.1. Mean wing length values for *Ae. aegypti* and *Cx. quinquefasciatus* males and females. Means with different letters are statistically significantly different.

Species	Sex	N	Wing Length (mean $\pm$ SE) (mm)
<i>Ae. aegypti</i>	F	16	2.99 $\pm$ 0.03 <b>A</b>
<i>Cx. quinq.</i>	F	15	2.76 $\pm$ 0.03 <b>B</b>
<i>Cx. quinq.</i>	M	20	2.29 $\pm$ 0.03 <b>C</b>
<i>Ae. aegypti</i>	M	20	2.26 $\pm$ 0.03 <b>C</b>

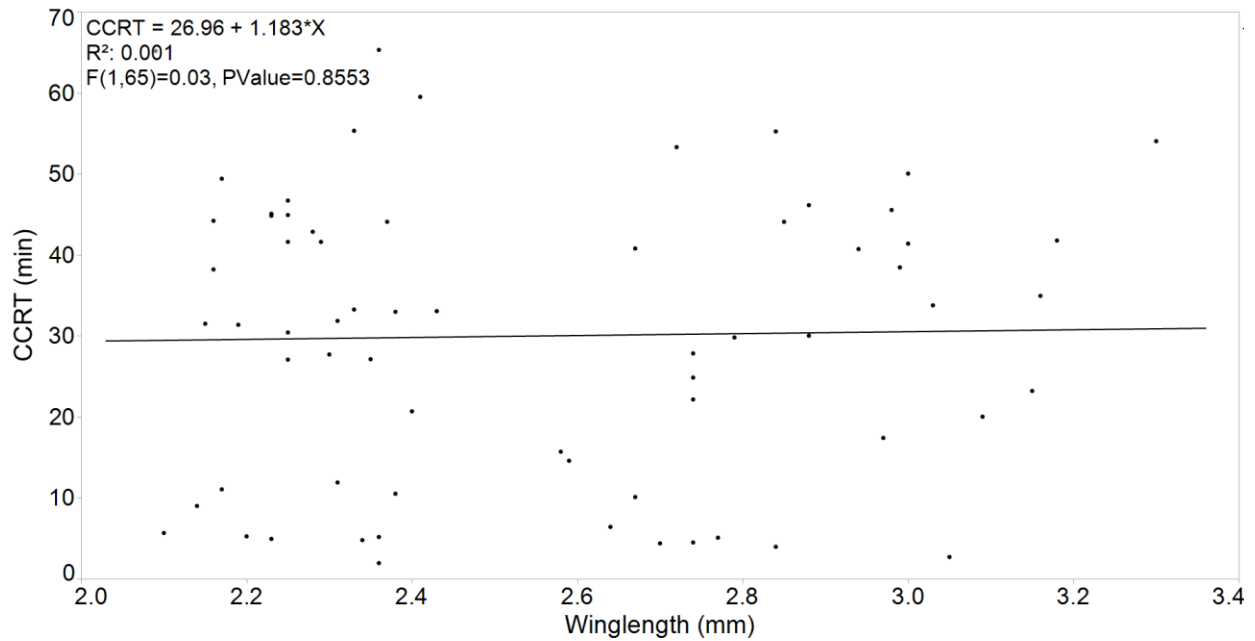


Figure A.4. Simple linear regression model with wing length as the explanatory variable for CCRT of *Ae. aegypti* and *Cx. quinquefasciatus* males and females.  $R^2$  values indicate the amount of variation in CCRTs explained by wing length, and F- and P-values give the fit of the model as compared to a null model.

## **Vita**

Madeleine Chura was born and raised in Newark, Delaware, where she also attended the University of Delaware. She received Bachelor of Science degrees in wildlife conservation and insect ecology in 2016. She developed an interest in mosquito research during an undergraduate research program at Fordham University as well as while working for Delaware's Mosquito Control Section. She is a candidate to graduate with a master's degree in entomology in the Spring of 2019, and plans to continue working in the field of medical entomology.