Exploiting Sugar Feeding Behaviors For Mosquito Control

Lauren Hope Fryzlewicz

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Chloé Lahondère, Chair

Sally Paulson

Clément Vinauger

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Abstract

Mosquitoes are the deadliest animal on the planet killing about a million people a year. These insects are competent vectors of multiple pathogens (e.g., *Plasmodium sp,* filarial worms, and arboviruses). In most species, females are blood feeders, and must consume a blood meal to complete a gonotrophic cycle. Extensive research has been conducted on hematophagy and host-seeking behaviors, but relatively little is known about phytophagy. Sugar feeding is an essential aspect of mosquito biology. Both male and female mosquitoes must consume sugar as a primary fuel source. Mosquitoes use olfactory and visual cues among other cues to find suitable food sources. Abiotic factors, such as temperature and humidity, have been shown to impact mosquito behaviors, including sugar feeding. Recently, sugar feeding has been identified as a promising control target for multiple species of mosquitoes. Attractive toxic sugar baits (ATSBs) attract both male and females through the use of plant derived volatiles. In this work, we first examined the effects of temperature and humidity on the survival of sugar fed *Aedes aegypti.* We showed that sugar feeding greatly increases longevity in optimal conditions and that humidity impacted survival while temperature less so. Second, we aimed at developing an ATSB for controlling *Aedes j. japonicus* mosquitoes. Overall, this work sheds light on the importance of abiotic factors and sugar feeding on mosquito survival and lays the groundwork for controlling an invasive mosquito species.

Exploiting sugar feeding behaviors for mosquito control

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General Audience Abstract

Mosquitoes are the deadliest organisms on the planet killing about a million people each year due to the multitude of pathogens they may transmit (e.g., malaria parasites, Zika virus, dengue virus). Female mosquitoes transmit pathogens by taking blood meals to obtain essential nutrients needed to develop eggs. If knowledge on host-seeking behavior and blood-feeding is extensive, comparatively less is known about sugar feeding. Sugar feeding is an important behavior displayed by both male and female mosquitoes to obtain energy for flying, reproduction, and survival. Mosquitoes feed on a variety of sugar sources including plant nectar and decaying fruits. They use multiple different cues in order to locate suitable meals including olfaction and vision. Environmental factors such as temperature and humidity affect mosquito activity, dehydration, and sugar feeding. In the present work, we first examined the effects of temperature and humidity on survival in the major disease vector species, *Aedes aegypti*. As the global temperatures are increasing, it is essential to better understand how mosquitoes adapt and deal with environmental stressors in a changing world. We then aimed at exploiting sugar feeding behavior by developing a novel method of control for another invasive mosquito species, *Aedes j. japonicus*. Together, these results help us have a better understanding of mosquito biology and ecology which is crucial for predicting future distribution of invasive species and designing new control strategies

Acknowledgments

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I would like to thank members of both the Lahondère and Vinauger labs. With special acknowledgements to Ashylnn VanWinkle and Nicole Wynne. Ash, for helping me conduct all of the experiments needed to produce this thesis. And Nicole, for encouraging me to apply to the BS/MS program as well as being my graduate student mentor while I was working in the Vinauger Lab. To all other members, thank you for your gracious support in and out of lab over the past two years.

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Table of Contents

Table of Figures and Table

GENERAL INTRODUCTION

Mosquitoes are the deadliest organisms on the planet, contributing to more than 1 million deaths and putting more than a billion people at risk for contracting diseases. Mosquitoes play a key role in transmitting a multitude of pathogens (*e.g.*, *Plasmodium sp.*, Zika virus, dengue virus, and filarial worms) that cause diseases in humans (CDC, 2019). Species of particular concern are *Aedes aegypti*, *Aedes albopictus* and, more recently in the United States *Aedes j. japonicus*. These species are capable of spreading disease and are highly invasive. Indeed, they can rapidly adapt to new environments and populations have, for some species, become established on all continents except Antarctica and are expected to expand their geographical ranges (Rochlin *et al*., 2013). Diseases that can be spread by these mosquitoes include Zika, dengue, chikungunya, and yellow fever for *Ae. aegypti* and *Ae. albopictus;* while *Ae. j. japonicus* can spread La Crosse virus, Japanese encephalitis, and West Nile virus. (Kaufman & Fonseca, 2014; Wilder-Smith *et al*., 2017). Mosquito population control is key to limiting spread of disease as many of those mentioned above do not have established treatments or vaccines.

Some *Aedes* mosquitoes have adapted to feed on humans (*Aedes aegypti* and *Ae. albopictus* being more anthropophilic than *Ae. j. japonicus).* While *Ae. aegypti* and *Ae. albopictus* have developed a strong preference for human blood, *Ae. j. japonicus* has been shown to feed mainly on large mammals; however they have been documented as nuisance biters in areas with high human population densities (Kaufman & Fonseca, 2014; Melaun *et al*., 2015; Schaffner *et al*., 2009). While only female mosquitoes will bite to consume a blood meal in order to obtain essential nutrients for egg development (Christophers, 1960), both female and male mosquitoes must consume carbohydrates *(i.e.*, sugar) as their primary energy source (Foster, 1995).

Research efforts have primarily focused on mosquito-host interactions (*e.g.*, host-seeking behavior, host preference), but sugar feeding behaviors have been historically overlooked. Sugar feeding has been identified as an essential component of the mosquito's life cycle (Foster, 1995). Without carbohydrate sources, mosquitoes are unable to complete essential activities such as flying, host-seeking, reproduction activities, or any activity without energy obtained from sugar sources (Nayar & Van Handel, 1971). Moreover, sugar feeding increases lifespan, reproductive output and thus has a potential impact on population dynamics (Upshur *et al.*, 2019). Sugar sources used by mosquitoes are species-specific. Many species primarily consume plant nectar, but some have also been documented feeding on decaying fruits, honeydew, and sap in the field (Foster, 1995.; Nayar & Van Handel, 1971). Mosquito species (*Aedes, Anopheles, Culex*) have been shown feeding on damaged fruits such as mangos, grapes, peaches, and melon (Joseph, 1970), as well as on sugar solutions produced by aphids *(i.e.*, honeydew), or ants (*i.e.*, ant regurgitation) (Clements, 1999; Edwards, 1932). Consequently, sugar feeding behavior constitutes an ideal target for mosquito control that can be exploited to limit populations.

Similar to finding hosts, mosquitoes will locate sugar sources using (primarily) chemical and visual cues. Mosquitoes possess antennae that have sensory structures called sensilla (Christophers, 1960). These sensory structures act as the mosquito's "nose" by the use of olfactory receptor neurons (ORNs) that can detect different chemical signals by expressing specific olfactory receptor proteins (Zwiebel & Takken, 2004). Electroantennography and behavioral assays have helped determine the detection of different scent profiles emitted by a host (plant or animal) and the various behavioral reactions that a chemical evoke (*i.e.*, neutral, attractive or repulsive) (Lahondère *et al.*, 2019; Meza *et al.*, 2020). When seeking a sugar meal chemical classes such as phenols, aldehydes, alcohols, ketones and terpenes of plant scents are known to be attractive

(Nyasembe & Torto, 2014). Chemicals such as lactic acid, ammonia, and carbon dioxide are particularly attractive in the context of blood meal seeking (Zwiebel & Takken, 2004). Carbon dioxide (CO_2) can also attract mosquitoes to sugar sources by fermentation of decaying fruits. Indeed, microorganisms, such as yeast, on the outside of fruits will utilize their sugar sources by way of fermentation, thus producing CO_2 (Peach *et al*., 2019). Visual stimuli are also used to locate suitable sugar sources. Like many other insects, mosquitoes have large compound eyes that allow them to see shapes and colors. Generally, mosquitoes are more attracted to dark colors during the day and light colors at night (Browne & Bennett, 1981). Previous studies have shown that mosquitoes are primarily attracted to black and red. This is a distinctive trait that separates mosquitoes from other flying insects such as pollinators (van Breugel *et al*., 2015). Exploiting these behaviors and biology of sugar feeding in mosquitoes may lead to novel techniques to limit populations.

Temperature has been shown to impact survival, blood and sugar feeding, and reproduction in insects. For example, mosquito activity increases with temperature and is higher if mosquitoes do not have access to sugar (Upshur *et al*., 2019). Moreover, mosquitoes will actively seek a blood or sugar meal in order to avoid dehydration and the risk of desiccation and death (Hagan *et al*., 2018). Temperature is also known to impact gonotrophic cycles, development time, and population densities. Within active temperatures, typically ranging from 15℃ to 32℃ with optimal activity from 26-30℃ for *Ae. aegypti*, mosquitoes will develop faster and produce more offspring (Reinhold *et al*., 2018). This is highly relevant as global surface temperatures are projected to continue to rise. Mosquitoes will be exposed to increasing temperatures and may actively and more often seek blood and / or sugar meals, thus increasing risk of transmitting pathogens. Importantly, arboviral transmission in mosquitoes can be accelerated when temperature increases. Indeed, viral replication is known to be temperature sensitive and pathogens may have shorter extrinsic incubation periods at higher temperatures (Hardy *et al*., 1983).

In this context, to decrease the risk and spread of diseases vectored by mosquitoes, control methods for larval and adult populations have been developed and implemented. These control strategies fall into these four categories -biological control, genetic modification, and insecticides/larvicides, and baits/traps methods (Huang *et al*., 2017):

- **Biological control**

Biological control methods include the use of a mosquito larvae predator or utilizing microorganisms to limit populations. Predators of mosquito larvae such as *Gambusia affinis* and *Toxorhynchites* mosquitoes have both been introduced to bodies of water that have large numbers of mosquito larvae. The mosquito fish, *Gambusia affinis*, do not have a specific diet and are predaceous to multiple aquatic larvae. After the introduction of these fish decreases in mosquito populations have been reported (Bence, 1988). *Toxorhynchites* mosquitoes have predaceous larvae that consume other insects during their aquatic stages. Once these mosquitoes mature into adults they are strictly phytophagous and only consume sugars (Bay, 1967; Huang *et al*., 2017; Mohamad & Zuharah, 2014). Like any control method this has its drawbacks, as many mosquito species such as *Ae. aegypti* and *Ae. j. japonicus* are highly adapted to laying their eggs in artificial containers where it is difficult to introduce these predators. It can also be very labor-intensive and high expensive to introduce such predators.

Another distinct biological control method consists in using microorganisms such as bacteria (*Bacillus thuringiensis israelensis* (*Bti*)) and entomopathic fungi which have been shown to effectively limit populations by producing toxins that disrupt the homeostasis of the mosquito (Huang *et al*., 2017; Lacey, 2007; Scholte *et al*., 2004). Unfortunately, some studies have shown that mosquitoes can develop a resistance to these toxins (Tetreau et al., 2013). It has also been proven difficult to have stable infection rates in wild populations.

- **Genetic Modification(s)**

Alternatively, there has been a surge in research and media coverage of the development of genetically modified mosquitoes. Genetically modified mosquitoes have had alterations within their genomes in order to transfer lethal, sterilizing, or antiviral genes to offspring. Antiviral genes will use the antiviral interference RNA and inverted repeats in order to inhibit the replication of specific viruses (Huang *et al*., 2017; Powers *et al*., 1996). The lethal gene (RIDL) is a dominant lethal gene that is controlled by transcriptional regulation of tetracycline. This gene will lie dormant or not expressed in lab reared mosquitoes as their diet is supplemented with tetracycline. Wild populations will mate with the lab reared mosquito and produce offspring where this gene will be expressed as there is no tetracycline in their diet, thus killing the offspring of the released mosquito (Huang *et al*., 2017; Phuc *et al*., 2007). Mosquito gene drives use CRISPR/Cas-9 (among other methods) to insert desired genes into mosquito genomes. Oftentimes these gene drives will disrupt fertility either by causing sterilization or selecting the Y chromosome over X so that more male mosquitoes are produced (Macias *et al*., 2017). But, this is often not easily sustained as male mosquitoes carrying these genes often have decreased sexual competitiveness and have to be released multiple times in order to have a large impact on populations (White et al., 2010) .

- **Insecticides/Larvicides**

Insecticides and larvicides (e.g., pyrethroids, DDT, malathion, naled, phenothrin, permethrin, temephos) were originally implemented around the 1940s. While the original result of these chemicals was a decrease in mosquito and other pest populations, it was not known that many of these harsh chemicals were detrimental to off target species such as honey bees and butterflies

(WHO, 2019;Brittain & Potts, 2011; Hemingway *et al.*, 2004). After many years of overuse of insecticides, multiple medically important mosquito species (e.g., *Aedes aegypti*, *Anopheles gambiae, Culex pipiens)* have become resistant to their toxic effects (WHO, 2019; Hemingway *et al.*, 2004; Liu *et al.*, 2015). The two most common ways insecticides are deployed are either by spraying, treated surfaces such as nets or by traps. Spraying or fogging of insecticides easily treats large areas but can also impact many off target species. It is also common to see indoor residual spraying (IRS) of insecticides used in conjunction in insecticide treated bed nets in Africa, to help prevent malaria transmission. Sadly, these control methods are not sufficient to completely eradicate malaria and are causing an increase of insecticide resistance among *Anopheles* species (vector of human malaria).

- **Baits and Traps**

Many traps can be equipped with specialized features (some utilize insecticides) that target mosquitoes in a specific physiological state (*i.e.*, oviposition, site-seeking, host-seeking, nectar seeking) (Kline, 2006). Oviposition traps lure gravid females looking for a site to oviposit. Traps that utilize host attractants (*e.g.*, BG-sentinel traps, CDC light traps) are targeting host-seeking female mosquitoes. These traps are baited with attractive host chemicals such as lactic acid and carbon dioxide to lure female mosquitoes to the trap (Wooding *et al.*, 2020). Lastly those that target sugar feeding, such as attractive toxic sugar baits (ATSBs), use a mixture of plant or fruit volatiles to attract both male and female mosquitoes. These traps are effective at limiting populations of mosquitoes with very little off target species interactions (Fiorenzano *et al.*, 2017; Wooding *et al.*, 2020).

The World Health Organization has recently called for the development of integrated vector management (IVM) to control populations of arbovirus vectors. IVM techniques promote novel control methods that are based on vector biology to have more efficient tools to limit mosquito populations (WHO, 2019). Among promising techniques are attractive toxic sugar baits (ATSBs) and toxic sugar baits (TSBs) as these are the only currently available traps that are targeting both female and male mosquitoes.

In 1965, Lea and collaborators identified that baiting mosquitoes with sugar to administer oral insecticides could be a method for mosquito control giving rise to the idea of the toxic sugar bait or an ATSB (Fiorenzano *et al.*, 2017; Lea *et al.* 1965). These baits are lure traps that exploit the sugar feeding behaviors of male and female mosquitoes in order to limit populations. While toxic sugar baits (TSBs) and attractive toxic sugar baits (ATSBs) are inherently different they are both built upon this principle. TSBs will only use sugar as a phagostimulant to motivate mosquitoes to ingest oral toxins. This method is often limited by possible repellency of oral toxins, as well as limited attractiveness to baits as they must compete with natural sugar sources (Allan, 2011; Busvine, 1964; Fiorenzano *et al.*, 2017; Xue *et al.*, 2006). ATSBs use odorants as attractants. Many studies have utilized plant and fruit volatiles to develop ATSBs and have been shown to largely decrease mosquito populations when tested in the field (Beier *et al.*, 2012; Fiorenzano *et al.*, 2017; Müller *et al.*, 2010; Qualls *et al.*, 2014, 2015; Revay *et al.*, 2014; Sissoko *et al.*, 2019). These baits often consist of three elements an oral toxin, an attractive component, and sugar. Baits can be administered multiple ways (traps, or spraying) and still have minimal impacts on off target species, in addition to being inexpensive, sustainable, and can be possibly species specific (Fiorenzano et al., 2017).

Mosquito phytophagy has been historically understudied and remains poorly understood. Yet, these behaviors have a large impact on overall mosquito fitness, and by targeting both females and males, they constitute an ideal tool for mosquito control. The present work aimed at answering the following questions:

- How does temperature and humidity affect *Ae. aegypti* survival after sugar feeding?
- Do boric acid sugar solutions repel *Ae. j. japonicus?*
- What sugar solutions can be used to develop an ATSB against *Ae. j. japonicus?*

This thesis is organized into two chapters. **Chapter one** examines the effects of temperature and humidity on the survival of sugar fed *Aedes aegypti* mosquitoes by recording survival of mosquitoes that are placed in different climate conditions. **Chapter two** focuses on exploiting the sugar feeding behaviors to develop an attractive toxic sugar bait to control the newly invasive mosquito, *Aedes j. japonicus.* Various ATSB solutions were developed and survival rates were recorded showing that some solutions were more effective than others. Our results are discussed in the context of previous studies using attractive toxic sugar baits for mosquito control.

Chapter 1: Impacts of Temperature and Humidity on Invasive Mosquito Survival after Ingestion of a Sugar Meal.

Lauren Fryzlewicz, Ashlynn VanWinkle, Chloé Lahondère

In preparation for *Acta Tropica*

Abstract:

As global surface temperatures are rising it is thought that the distribution of the major disease vector *Aedes aegypti* will likely expand. Climate change will lead to changes in abiotic factors such as temperature and humidity. As mosquitoes are poikilotherms, their body temperature is greatly impacted by these factors. Behaviors such as blood feeding, host-seeking, sugar feeding, flying have all been shown to be impacted by temperature, while the effects of humidity are lesser known. Mosquitoes must constantly adapt to these changing factors or they can risk death due to overheating and desiccation. In the present study, we compared the survival of sugar fed male and female mosquitoes under various environmental conditions (cold temperature, 20-22℃; optimal temperature 26-27℃; low humidity (55-65% relative humidity); high humidity (80-90% relative humidity)). We found that humidity had a significant impact on the survival of *Ae. aegypti* mosquitoes while temperature affected them significantly less.

Keywords: *Aedes aegypti*, climate change, sugar feeding, abiotic factors, survival

1. Introduction

The globe's surface temperature has been increasing on average 0.15 degrees Celsius each decade and is projected to continue to increase throughout the next century (Hansen *et al*., 2010). As temperatures rise it is expected that the geographical range and length of infectious season of multiple disease vectors will also change. A major disease vector of concern is *Aedes aegypti*, a mosquito species that may vector pathogens responsible for causing diseases such as dengue, yellow fever and Zika (Wilder-Smith *et al*., 2017). Current global distribution and cases of dengue and yellow fever are the highest ever recorded (Barrett, 2018; CDC, 2019). *Aedes aegypti*'s geographic range is expected to continue to expand northward (Kraemer *et al.*, 2015) putting populations at risk for disease, ultimately increasing public health concerns. By 2050, more than half of the world's population will be at risk of contracting diseases vectored by mosquitoes (CDC, 2020; Monaghan *et al*., 2018).

Climate change will lead to changes in abiotic factors such as temperature and humidity. These factors can strongly influence mosquito life cycle and vector competence of mosquitoes, including *Ae. aegypti* (Reinhold *et al*., 2019). Mosquitoes are poikilotherms, meaning that body temperature is not internally regulated and is thus greatly impacted by the temperature of the surrounding environment (Hagan *et al*., 2018; Monaghan *et al*., 2018; Upshur *et al*., 2019). Recent studies extensively examined temperature effects on host-seeking, blood-feeding, flight, sugar feeding and water intake (e.g., Hagan *et al*., 2018; Reinhold *et al*., 2018; Upshur *et al*., 2019). Other studies have also shown that humidity can shape seasonal distributions, survival, and desiccation in mosquitoes. However, comparatively less is known about the impacts of humidity on mosquito feeding behavior, including blood and sugar feeding as well as water intake. Dehydration (potentially leading to desiccation) is one of the major limiting factors linked to mosquito survival. As temperature and weather patterns change, mosquitoes must be able to adapt and overcome dehydration challenges in order to survive (Hagan *et al*., 2018). Adult mosquitoes are typically composed of 40-90% water, and below this range, activity decreases, until death (Urbanski *et al*., 2010). Water can be lost through multiple mechanisms such as waste excretion, gas exchange, and cuticle transpiration.

In order to combat dehydration mosquitoes can consume blood, sugar, or water (Benoit & Denlinger, 2007). Blood meals are imbibed only by female mosquitoes to obtain essential nutrients to produce eggs (Christophers, 1960). *Aedes aegypti* mosquitoes are anthropophilic and will actively seek blood meals when facing dehydration instead of sugar feeding (Edman *et al*., 1992). While blood feeding is different for each mosquito species, sugar feeding is a common behavior to all species and sexes of mosquitoes (Foster, 1995.). This behavior has a major impact on mosquito physiology, reproduction, survival, and overall fitness. These carbohydrates will provide mosquitoes with energy needed for reproduction, host-seeking, and flying (Foster, 1995). Upshur *et al*. (2019) reported that temperature impacts activity and survival of both females and males. Moreover, it has been shown that at higher temperatures, mosquitoes consumed more sugar which led to an increased survival rate compared to groups that were only provided with water (Upshur *et al*., 2019). Water consumption is a common behavior in male and female mosquitoes when other sources are not available. This behavior is commonly seen in arid environments and is an essential behavior in order to minimize dehydration (Benoit & Denlinger, 2007).

Studying how temperature and humidity affect the survival of invasive species is important as this can lead to changes in both sugar and blood feeding patterns which can have an impact on pathogen transmission. Sugar feeding behaviors have recently been targets for the development of new control strategies including attractive toxic sugar baits (ATSBs). These baits use sugar feeding behaviors to target male and female mosquitoes to limit their populations. Thus, to optimize this control strategy, sugar feeding behaviors must be fully understood, in particular in the context of a changing world to assess ATSB efficacy and efficiency. In this context, the present study aims at understanding the effects of temperature and humidity conditions on the survival of sugar fed *Ae. aegypti* mosquitoes to obtain a deeper understanding on mosquito biology that can be used to limit populations.

2. Materials and Methods

2.1. Insects

Laboratory strains of *Aedes aegypti* (Rockefeller, MR-734, MR4, AATCC®, Manassas, VA, USA) mosquitoes were used for the study. Larvae were reared in 26 x 35 x 4 cm covered trays filled with deionized water. These trays were kept in a climate chamber at 26 ± 0.5 °C, 60 ± 10% humidity and under a 12:12 hr light:dark cycle. Larvae were fed Hikari Tropic First Bites (Petco, San Diego, CA, USA) fish food until developed into pupae. For the experiments, about 50 pupae were placed into mosquito breeding containers (BioQuip, Rancho Dominguez, CA, USA) until emergence. Newly emerged mosquitoes were starved for 48 hours (*i.e.*, never previously fed mosquitoes) until the experiments were performed.

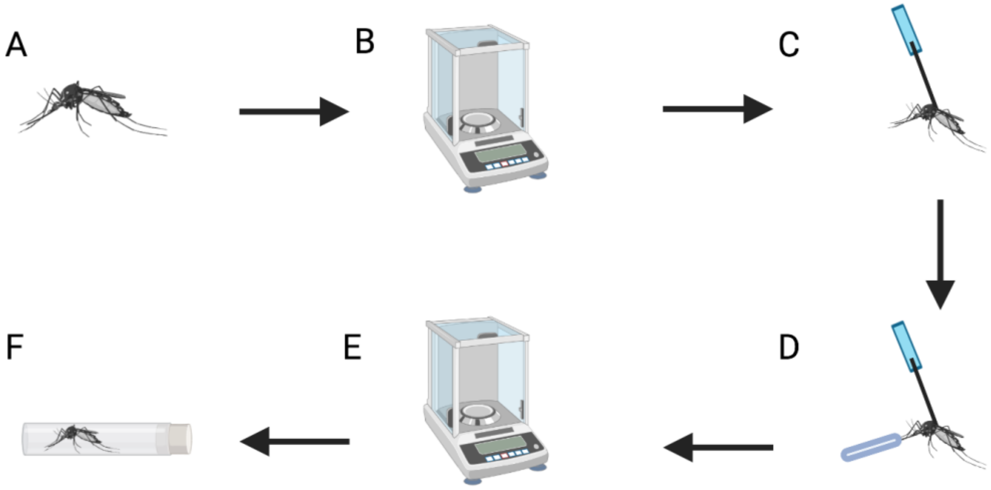
2.2. Effects of Tethering on Survival

We first assessed the potential impact of the tethering technique (i.e., application of the glue and removal) on mosquito survival. To immobilize mosquitoes for the force-feeding assays, a custom-made tungsten tether was glued to the mosquito thorax using a UV curable glue (Bondic, B0181BEHQU). We compared the survival rates of two groups of females and male mosquitoes:

tethered and non-tethered. Both tethered and non-tethered individuals were placed on ice for about 15 seconds until they were knocked down. Then each mosquito was placed on a micro balance (Metter Toledo, XA105) to obtain their initial weight. From here the tethered mosquitoes were tethered and then placed into a climate chamber for an hour, while the non-tethered individuals were placed directly into the climate chamber for the rest period. Once the rest period was up tethers were removed and each individual was weighed on the micro balance once more to obtain their "after weight". Each mosquito was placed into an individual fly tube (Genesee, cat: 50-207RED) in the optimal temperature (26-27 °C) and high humidity (80-90%) condition.

2.3. Force-feeding Assays

Mosquitoes were placed in a refrigerator (4°C) for 2 minutes to knock them down for sorting. Once sorted the mass of individual starved mosquitoes were recorded on a micro balance (Metter Toledo, XA105) one mosquito was then placed on a cold aluminum block, oriented on its ventral side pressed against the cold block. A small droplet of UV curable glue was placed on top of the thorax to adhere a custom-made tungsten tether. The tether immobilized the mosquito while allowing for free movement of the appendages. Individuals were allowed to rest in the climate chamber for one hour before the assay started. The mosquito proboscis was inserted inside a glass capillary (Sutter Instrument, USA) with a 0.7 mm inner diameter filled with a 30% nectar solution (composition: 15% sucrose, 10% fructose (D-(-)-Fructose ≥ 99%), 5% glucose (D-(+)Glucose ≥ 99.5%) (Sigma-Aldrich, Germany)) for 8 minutes. To ensure that the mosquito fed, its weight was recorded again after the tether had been carefully removed from the thorax; The residuals UV glue left on the thorax and any trace weight was considered negligible. If the mass of the mosquito did not increase the individual was discarded. If it increased, the mosquito was placed into an individual fly tube (Genesse, cat: 50-207RED) and then placed under the desired environmental condition: optimal (26-27 °C) or cold (20-22 °C) and low (55-65%) or high humidity (80-90%), which were monitored using ibuttons (Embedded, USA) (Table 1). Mosquito survival was monitored every 24 hours until the death of the individual.



<u>Figure 1:</u> Schematic diagram depicting the force-feeding assay methodology. **A.** Two-day old male and female *Aedes aegypti* mosquitoes were selected **B.** Individual mosquitoes were weighed using a microbalance **C.** Each mosquito was immobilized using custom made tethers that were glued to their thorax **D.** Their proboscis was inserted into a glass capillary tube filled with sugar solution for 8 minutes **E.** Mosquitoes were then removed from the tether and weighed once more **F.** Individuals were placed into a fly tube and placed into desired conditions. Survival was recorded every 24 hours. (Schematic created with Biorender).

3. Results

3.1. Effects of Tethering on Survival

The immobilization method of tethering did not affect the survival of male (tethered, n = 33; non tethered, n = 22) and female (tethered, n = 34; non tethered n = 25) mosquitoes (Two-tailed Student *t*-test, $p > 0.05$) (Fig. 2). However, it was found that the tethered males lost significantly more weight than non-tethered males (Two-tailed Student *t*-test, $p < 0.001$) while there was no significant difference in weight for the females (Two-tailed Student *t*-test, $p > 0.05$).

Tethered males lost on average 0.11 mg while not tethered males lost 0.05 mg compared to their initial bodyweight.

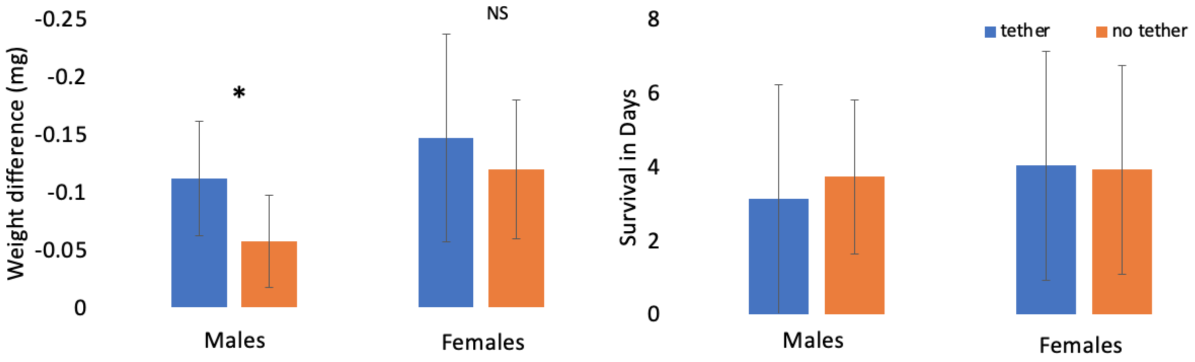


Figure 2: Average weight loss and survival (± standard error of the mean) of control tethered and non-tethered *Aedes aegypti* mosquitoes.

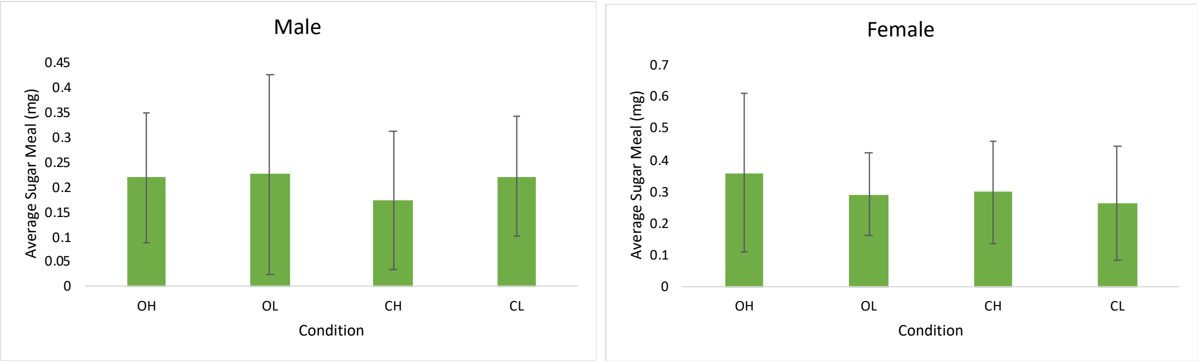
3.2. Force-feeding Assays

No significant difference in sugar intake was observed between males of each of the tested conditions (Two-tailed Student *t*-test, $p > 0.05$ for all comparisons). Non-significant results were also reported between females of each condition (Two-tailed Student *t*-test, $p > 0.05$ for all comparisons) (Fig. 2). This indicates that mosquitoes consumed a similar amount of sugar before being placed into their corresponding conditions. However, as expected, females consumed more sugar than males. Females on average imbibed 0.30 ± 0.18 mg of sugar solution while males consumed 0.21 ± 0.14 mg (Two-tailed Student *t*-test, $p < 0.05$). Non sugar fed mosquitoes exposed to optimal high humidity conditions lived on average 4.03 ± 3.12 and 3.12 ± 3.12 days for females (n = 34) and males (n = 33) respectively. This was significantly lower than that of sugar fed mosquitoes exposed to the same conditions which on average lived 9.31± 5.72 days and 10.13 ±

7.49 days for females (n = 13) and males (n = 15) (Two-tailed Student *t*-test, $p < 0.001$ for both comparisons).

<u>Table 1:</u> Average temperature and relative humidity of tested conditions

Condition	Average Temperature	Average Relative Humidity
Optimal Temp	27.4	
High Humidity		92.9
Low Humidity		65.7
Cold Temp	22.3	
High Humidity		90.5
Low Humidity		61.05



<u>Figure 3</u>: Average (± standard error of the mean) amount of sugar solution consumed of male (left) and female (right) *Aedes aegypti* mosquitoes. Conditions are as follows OH. Optimal temperature high humidity OL. Optimal temperature low humidity CH. Cold temperature high humidity CL. Cold temperature low humidity.

Female survival at optimal temperatures averaged 9.31± 5.72 and 2.0 ± 1.22 days for high and low humidity respectively. At cold temperatures survival averaged 6.26 ± 5.5 and 1.6 ± 0.88 days for high and low humidity (Table 1). Humidity had a significant effect on female survival (Log Rank test, $p < 0.001$ for both comparisons) when placed under both optimal and cold temperatures (Fig. 4.). Mosquitoes held in low humidity conditions died faster than those held at high humidity. No significant difference was found between the survival of mosquitoes at optimal and cold temperatures with constant humidity (Log Rank test, $p = 0.3$) (Fig. 4).

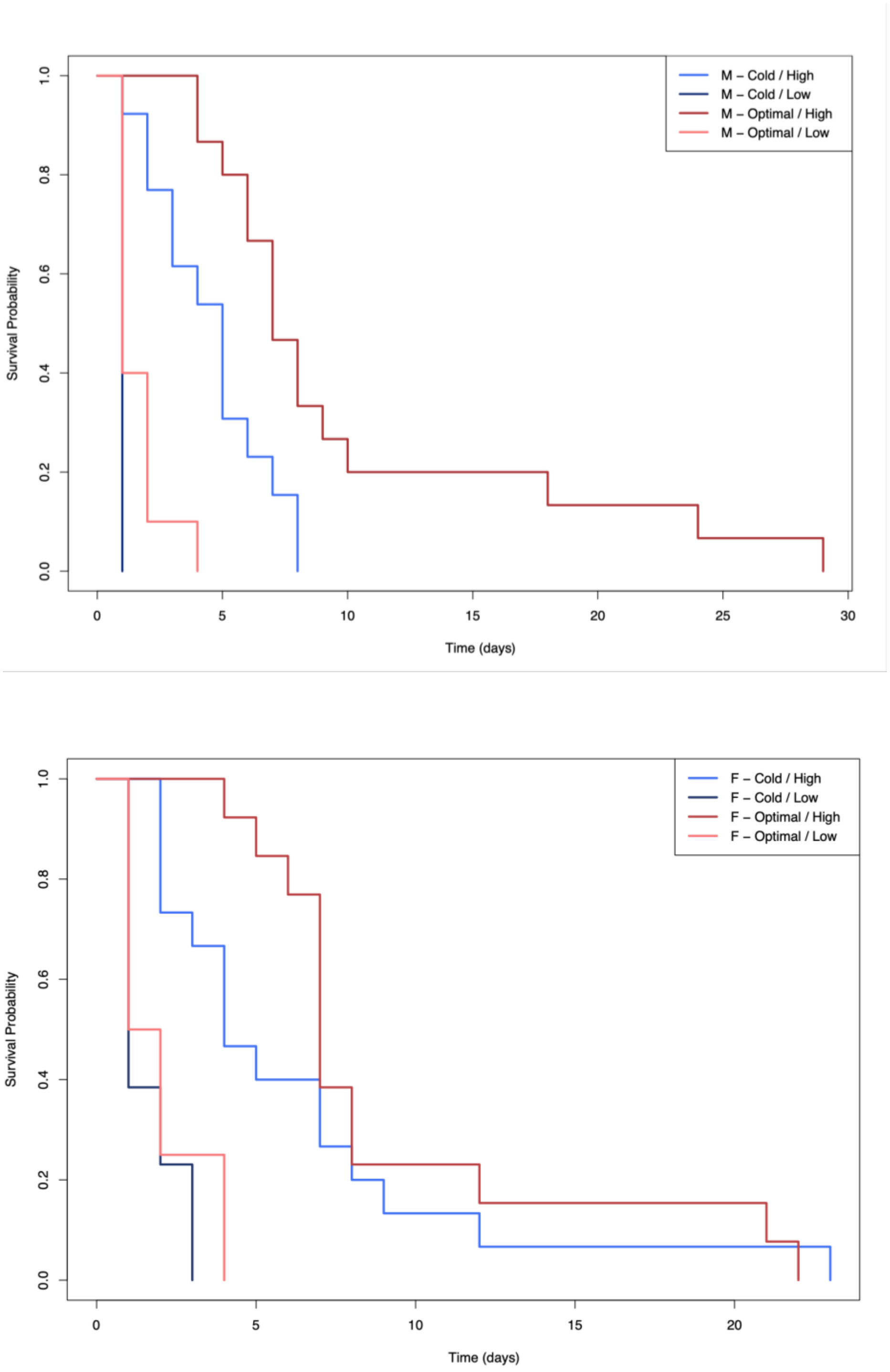


Figure 4: Raw Kaplan-Meier survival curves of males (top) and female (bottom) *Aedes aegypti* mosquitoes after being placed into different environmental conditions. **CH.** Cold temperature high humidity (males, n = 13; females, n = 15). **CL.** Cold temperature low humidity (males, n =10; females, n = 14) **OH.** Optimal temperature high humidity (males, n = 15; females, n = 13) **OL.** Optimal temperature low humidity (males, n = 10; females, n = 9)

Males survival at optimal temperature averaged 10.13 ± 7.49 and 1.6 ± 0.96 days for high and low temperatures respectively. At cold temperatures survival averaged 4.53 ± 2.30 and 1 ± 0 days for high and low humidity (Table 1). Similar to females, humidity conditions had a significant effect on survival at both optimal and cold temperatures (Log Rank test, $p < 0.001$ for both comparisons) (Fig. 4). Temperature had a significant effect on survival for males held in high and low humidity conditions (Log Rank test, $p = 0.004$ and $p = 0.03$ respectively) (Fig. 4).

Table 2: Average sugar consumption and survival (± standard error of the mean) of *Aedes aegypti* mosquitoes.

Condition	**Average normalized sugar consumption (mg) (±SEM)**	**Average Survival (days) (±SEM)**
Optimal temp. high humidity		
Female	0.259 ± 0.16	9.31 ± 5.72
Male	0.213 ± 0.13	10.13 ± 7.49
Optimal temp low humidity		
Female	0.215 ± 0.15	2.0 ± 1.22
Male	0.189 ± 0.12	1.6 ± 0.96
Cold temp high humidity		
Female	0.258 ± 0.21	6.26 ± 5.51
Male	0.233 ± 0.12	4.53 ± 2.30
Cold temp low humidity		
Female	0.265 ± 0.14	1.6 ± 0.88
Male	0.161± 0.11	1 ± 0

4. Discussion

Temperature and humidity are among the most important abiotic factors affecting mosquito biology and ecology (Reinhold *et al*., 2018). Dehydration is a limiting factor for mosquito survival, as maintaining water balance is imperative to prevent desiccation and the risk of death (Clements, 1999; Hagan *et al*., 2018). Mosquitoes will use many different mechanisms to avoid dehydration by decreasing excretion, reduced metabolic activities, and making behavioral adjustments to find more favorable microclimates (Kessler & Guerin, 2008). Variation in dehydration tolerance has been reported among different species of *Drosophila flies* (Hoffmann & Harshman, 1999) and mosquitoes (Gray & Bradley, 2005).

In the present study focusing on *Ae. aegypti*, we found that starved males (control groups) were more susceptible to water loss than starved females. In contrast, a study conducted by Benoit and Denlinger (2007) showed that female *Culex pipiens* were more susceptible to water loss than males of that species. *Culex pipiens* and *Ae. aegypti* have different mechanisms to avoid dehydration. *Culex pipiens*, or the northern house mosquito, has evolved a larger body size compared to *Ae. aegypti*. Their size decreases the surface area to volume ratio thus decreasing water diffusion rates through the cuticle (Benoit & Denlinger, 2007). This also allows these mosquitoes to expand to more northern latitudes (Ruybal *et al*., 2016), and experience colder temperatures than *Ae. Aegypti,* which is a (sub)tropical species (Kraemer *et al*., 2015). Similar to other species, *Ae. aegypti* was shown to utilize larval energy reserves and behavioral changes to find a more suitable microclimate, often cooler temperatures with higher humidity levels, mosquitoes will rest and decrease respiration to conserve water, allowing the mosquito to survive longer (Kessler & Guerin, 2008).

Upshur *et al.* (2019) also found that survival rates increased significantly for both male and female mosquitoes when they had access to sugar. Sugar meals have a major impact on mosquito physiology, reproduction, survival, and overall fitness (Foster, 1995). Carbohydrates obtained from sugar meals provide the mosquito with energy to carry out essential functions such as flight (Foster, 1995.; Nayar & Van Handel, 1971). In the current study we found that (in the correct conditions, optimal to lower temperatures with higher humidity) both sexes could survive significantly longer after a sugar meal than starved mosquitoes (control). Carbohydrates provided essential nutrients that allowed several individuals to live up to 30 days.

Our data showed that humidity had a more significant effect on survival than temperature for both males and females. Mosquitoes were able to live longer under high humidity conditions compared to the groups maintained under low humidity, regardless of temperature. Humidity has also been shown to greatly impact the survival and oviposition of eggs in *Ae. aegypti* (Costa *et al*., 2010)**.** This species' eggs require a period of desiccation before hatching while *Culex* mosquitoes lay eggs that are not resistant to desiccation and thus need to hatch within hours after oviposition.

Cooler temperatures did not have a significant effect on survival for female mosquitoes. This is supported by the findings of Upshur *et al*. (2019), showing that mosquitoes can easily survive seven days in a cooler environment (20°C). This suggests that female *Ae. aegypti* can easily adapt to cooler temperatures by reducing their overall activity. In our experiments, males placed under colder temperatures (21-22°C) did not live as long as those in optimal temperatures (26-27°C). The difference can be explained by a difference in nutrients available as well as the composition of the fat bodies between sexes. Fat bodies play an essential role in energy storage for insects. Females typically have more fat bodies, which can use the stored energy for diapause, and prolonged flight (Arrese & Soulages, 2010). Female *Cx. pipiens* use energy storage from fat bodies to overwinter or diapause as adults (Arrese & Soulages, 2010). It is worth mentioning that while females are overwintering, they must also ensure to balance water levels to avoid the risk of desiccation (Arrese & Soulages, 2010). Mechanisms such as fat body hypertrophy, where all stored lipids are metabolized, will provide the mosquito energy that can be used to find sugar meals (Koenraadt et al., 2019).

Overall, this study brings insights to understand the effects of temperature and humidity on sugar fed *Ae. aegypti.* Future research will consist in testing the effects of a higher temperature on mosquito survival (31-32℃) with low and high humidity. Testing higher temperatures is relevant in the context of climate change and the fact that *Ae. aegypti* is gradually expanding its geographic range. Thus, determining how temperature and humidity are affecting invasive species is crucial for understanding future population dynamics. It would also be interesting to assess the effects of temperature and humidity on the mosquito metabolism by conducting respirometry experiments as well as testing more extreme temperatures which might be experienced by mosquitoes in the future due to global warming.

Funding

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Chapter 2: Development of an Attractive Toxic Sugar Bait to Target *Aedes j. japonicus.*

Lauren Fryzlewicz, Ashlynn VanWinkle, Chloé Lahondère

In preparation for *Journal of Medical Entomology*

Abstract:

Mosquitoes transmit multiple pathogens killing about a million people every year. Both males and females, must consume sugar meals to obtain carbohydrates used for energy. This behavior has recently been identified as a possible mosquito control target, as the World Health Organization has urged for the development of integrated vector management (IVM). This is needed as many medically important mosquito species are developing insecticide resistance, resulting in current control strategies becoming non effective. Additionally, the traditional use of insecticides is harmful to many beneficial insects such as pollinators. The main goal of the present study was to develop an attractive toxic sugar bait (ATSB) to limit the populations of a local invasive mosquito, *Aedes j. japonicus.* An ATSB is an insect lure bait that is composed of an attractant odorant, a toxic component, and sugar that the mosquitoes can feed on. ATSBs are cost effective, sustainable, environmentally friendly, and can be species specific. To develop our ATSB we used field caught mosquitoes to conduct our assays. Female and male mosquitoes were isolated into cages and each group had access to either a toxic sugar solution (containing boric acid) or a control solution and their survivability was monitored for 96 hours. We tested multiple fruits such as mango, peach, blueberries, and blackberries, as well as a soda and grape juice. We observed 100% mosquito mortality of those who fed on toxic solutions indicating that boric acid is an effective oral toxin against *Ae. j. japonicus*. Further experiments will be conducted in the field to determine these ATSBs efficacy and to monitor potential effects on the surrounding environment and off target species.

<u>Keywords</u>: mosquito, invasive species, ATSB, mosquito control, *Aedes*, *Aedes j. japonicus*

1. Introduction

Mosquitoes are responsible for the death of millions of people each year because of the pathogens (*e.g.*, *Plasmodium sp.*, dengue virus) females can transmit while biting (WHO 2019). As treatments and vaccines remain unavailable for many of these diseases, mosquito control is one of the main strategies to limit populations and consequently pathogen transmission. Management techniques such as mechanical (*e.g.*, breeding habitat removal), biological (*e.g.* introduction of predators, microorganisms) and chemical (*e.g.* insecticides, attractive traps) ones, are essential to reduce mosquito burden on human populations worldwide. In particular, integration of pesticides/insecticides in the 1940s greatly reduced the spread of many mosquito vectored pathogens worldwide. Unfortunately, after many years of overuse of these chemicals, several mosquito species (*e.g.*, *Aedes aegypti, Anopheles gambiae, Culex pipiens*) have developed resistance. Additionally, the delivery of these insecticides (*i.e.*, spraying on ground and by aircraft) have had negative impacts on many off target species that are beneficial to the environment including pollinators such as bees (Brittain and Potts 2011; Geiger *et al*. 2010; Johansen 1977). The World Health Organization has thus urged for the development of novel control strategies that are cost-effective, sustainable and environmentally friendly (WHO, 2019).

Attractive-toxic sugar baits (ATSB) are a recently developed trapping technology that can be species specific, sustainable, and inexpensive to produce (WHO, 2019, Beier *et al*., 2012; Fiorenzano *et al*., 2017; Müller *et al*., 2010; Qualls *et al*., 2014, 2015; Revay *et al*., 2014; Sissoko *et al*., 2019). As both female and male mosquitoes must consume carbohydrates as their primary energy source (Foster 1995.; Nayar and Van Handel 1971), these baits appear as an interesting control strategy by exploiting sugar feeding behaviors to lure and kill the insects after they ingest a sugar coated oral toxin. ATSBs are usually composed of three parts: an attractive odorant, sugar, and a toxic chemical. They can be deployed in multiple ways including traps, residual and oral toxin soaked materials, or can be actively sprayed on vegetation around highly populated areas (Fiorenzano *et al.*, 2017; Fulcher *et al*., 2014; Ller *et al*., 2010.; Müller *et al*., 2010). Recent studies also showed that impacts on nontarget arthropods can be reduced by this control strategy (Revay *et al*. 2014, Ller *et al*. 2010). Previous studies have shown their efficacy in multiple different mosquito species, including invasive species and major disease vectors such as *Ae. aegypti* and *Aedes albopictus,* but a trap and a bait for *Aedes j. japonicus* mosquito populations has yet to be implemented.

Aedes j. japonicus mosquito is an invasive species to North America and Europe originating from Japan and Korea. Increased trade and travel lead to this species introduction to many new areas around the world (ten countries in Europe and in multiple states in the US, including Hawaii) (ECDC, 2020). This species was first located in the US in 1998 and has rapidly expanded to much of the east and midwestern US as well as some parts of Canada. *Ae. j. japonicus* is a cold tolerant species (Kaufman *et al.* 2014, Schaffner *et al*. 2004) which presents a high degree of ecological plasticity (*i.e.*, host preference, developmental niches) (Kaufman *et al.* 2014, Schaffner *et al*. 2004). In particular, its ability to reproduce for longer periods ranging from early spring to late fall (temperature range: 10°C to 35°C) has contributed to its invasive success (Kaufman *et al*. 2014, Schaffner *et al* 2004). Females feed primarily on large mammals, birds, and humans and are known to be competent for viral pathogens including La Crosse virus, Japanese encephalitis and West Nile virus (Kaufman *et al*., 2014). As current control strategies against this species are limited (Kaufman *et al*. 2014, Schaffner *et al*.,2004), it is essential to explore new avenues to limit *Ae. j. japonicus* populations.

In this context, the present study aims at developing an ATSB targeting *Ae. j. japonicus*. We tested the efficacy of several fruit solutions using an array of exotic and native fruits, juices, and a soda paired with boric acid as the toxic component. Recent studies have shown that boric acid has successfully limited populations of other invasive and pest arthropods by insects consuming the water soluble crystal which interrupts nutrient absorption and metabolism (Bhami & Das, 2015, 2015; Naranjo *et al*., 2013a, 2013b; Rust *et a*l., 1991; Xue *et al*., 2006). Boric acid is reported low in toxicity on off target species including most fish species, birds, and honey bees (Revay *et al*., 2014; Valdovinos-Flores *et al*., 2016). Survival rates after ingestion and bait attraction were monitored to determine the most effective ATSB solution for this species that could then be deployed and tested in the field as a control tool to reduce populations.

2. Methods and Materials

2.1 Insects

Mosquito larvae were collected from two field sites (37.375654° -80.522140° and 37.209962° -80.435831°) located in the New River Valley of Southwest Virginia. Larvae were then reared in the laboratory in 26 x 35 x 4 cm covered trays filled with deionized water 24°C and 60% relative humidity and a 12:12 light:dark regime. The larvae were fed Hikari Tropic First Bites (Petco, San Diego, CA, USA) fish food until developed into pupae. The pupae were placed into mosquito breeding containers (BioQuip, Rancho Dominguez, CA, USA) until emergence. After emergence mosquitoes were identified using an identification key based on morphological traits (REF Darise and Ward). For experiments *Ae. j. japonicus* mosquitoes between 2 to 15 days post emergence were selected and starved for 24 hours before experiments were performed.

2.2 ATSB Solution Preparation

Control and toxic solutions were produced using organic frozen fruits: mango, peach, blueberry and blackberry (Kroger, Simple Truth Organic), a cola soda, and grape juice (Santa Cruz). Frozen fruits were slightly heated on a hot plate in a glass dish for 20 minutes to ensure maximum juice extraction after thawing. The softened fruits were mechanically pressed to a puree, filtered using a fine mesh strainer to remove the pulp. The juice was then collected into clean 50 mL falcon tubes (Heathrow Scientific, #HEA4427R). A 1:1 g/mL ratio of fruit juice to sucrose (Sigma Aldrich, CAS #57-50-1) was added to the falcon tube to create a fruit concentrate. The concentrates were then diluted to a 1:4 ratio using deionized water to produce the final control solution that contained a 20% sugar concentration. Toxic solutions were produced by adding a 1% of w/v of boric acid (Sigma-Aldrich, CAS #10043-35-3). No sugar was added to the soda solution as sugar concentration was already over 20%. Stock grape juice was packaged at 15% sugar, 2.5 g of sucrose was added to 50 mL of juice to increase the concentration to 20%.

2.3 Cage assays

In order to determine whether mosquitoes would show a preference for either the control or the toxic solution and to test the possibility of the toxic solution to have a repellent effect on the mosquitoes, males and females (10 < n > 20 individuals per cage) were released into mesh cages (20 in x 20 in x 20 in) (Restcloud, CAT#B074ZKRVZZ) (Fig. 5A) . The cages were placed into a climate chamber (Percival, USA) under the same climatic conditions as previously described. Six conditions were tested in which mosquitoes were provided with either two controls, one toxic and one control, or two toxic cotton balls imbibed with sucrose or the peach solutions as previously described. The cotton balls were replaced every 24 hours over a 96-hour period. Survival was monitored every 24 hours and dead mosquitoes were removed from the cage.

2.4 Cup Assays

To assess mosquito feeding on the different solutions, male and female mosquitoes (n = 6 / cup) were separated into paper cups (8 oz) (WebstaurantStore, CAT #760SOUP16WPA) covered with a mesh netting (Fig. 5B). The cups were then placed in a climate chamber at 24°C and 60% relative humidity and a 12:12 light:dark regime. A control or toxic sugar solution-soaked cotton ball was provided to the mosquitoes and changed every 24 hours over a 96-hour period. Survival was monitored and recorded on a daily basis. For each solution tested, at least 10 replicates were performed (10 < n > 60 per condition). Survival rates were compared between the control and the toxic solution groups.

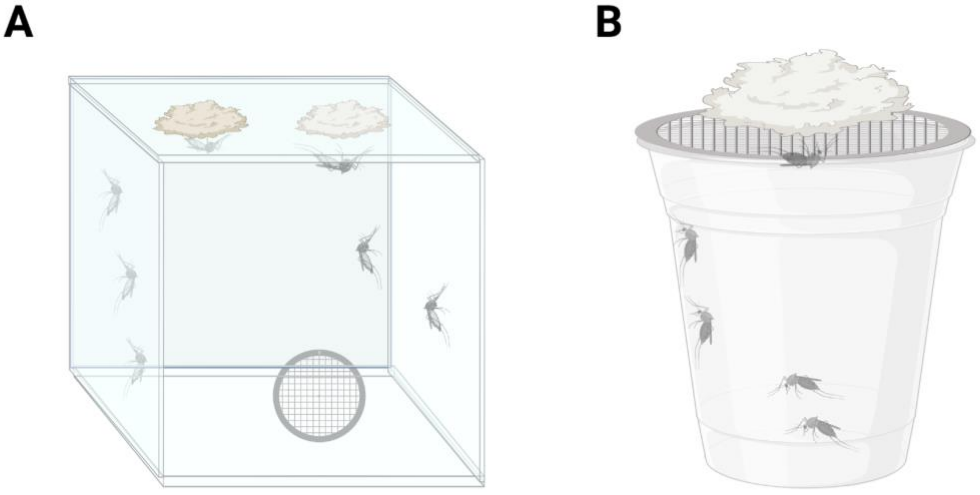


Figure 5: Schematic representation of cage **(A)** and cup **(B)** assays. Mosquitoes were isolated into each container, 20 mosquitoes per cage and 6 mosquitoes per cup. Each container was provided with a new cotton ball (saturated with desired solution) every 24 hours. Mosquito survival was monitored every 24 hours for a 96-hour period. (Schematic created with Biorender).

2.5 Data Analysis

We then performed Log-rank tests (Harrington, 1982) comparing survival curves built on our Kaplan–Meier estimates of the survival probability (Kaplan and Meier, 1958). All analyzes were performed using the programing language R (R Core Team, 2019).

3. Results

3.1 Cage Assay

In mosquitoes provided with a non-toxic sucrose solution, we observed a survival of 94 ± 10.6% and 96 ± 8.8% for females (n = 60) and males (n = 40) respectively, whereas 100% of individuals in both groups provided with the toxic sucrose solution died within 48 hrs (Log Rank test, $p < 0.001$) (Fig. 6 A, B). Similar results were obtained when mosquitoes were presented with a choice between a non-toxic and a toxic sucrose solution (males: 10 ± 17.3%, n = 23); females: 11 ± 14%, n = 77) (Log Rank tests, $p < 0.001$). The addition of the peach solution led to the same levels of mortality as when the mosquitoes were provided with sucrose only (Log Rank tests, $p < 0.001$ for all comparisons) (Fig. 6 C, D). No significant difference in mortality was noted between the sucrose and peach groups indicating that mosquitoes were equally feeding on both sucrose and peach solutions (Log Rank tests, control-control: $p = 0.6$; control-toxic: $p = 0.6$; sucrose toxic: $p = 0.9$). Interestingly, we noted a significant difference in mortality when mosquitoes were provided a choice between a control solution and a toxic solution for both the sucrose groups and the peach groups (i.e., mosquitoes took longer to die) (Log Rank tests, $p < 0.001$ for both comparisons) (Fig. 7).

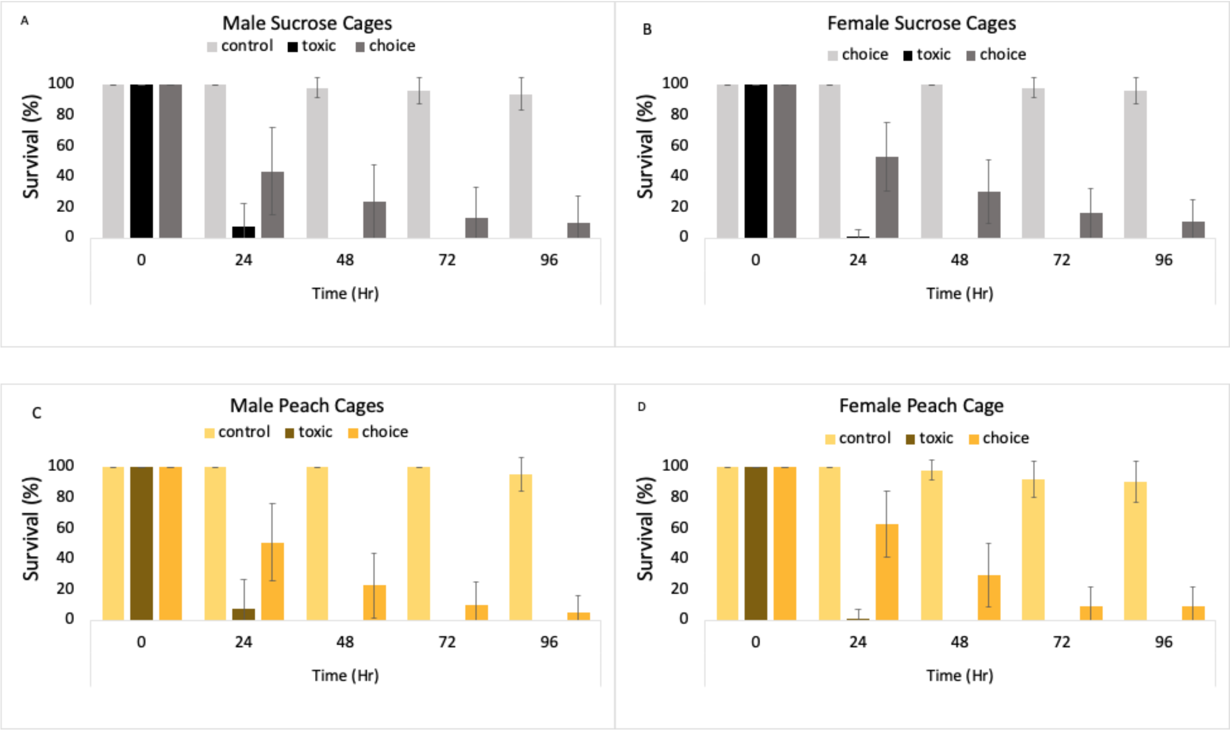
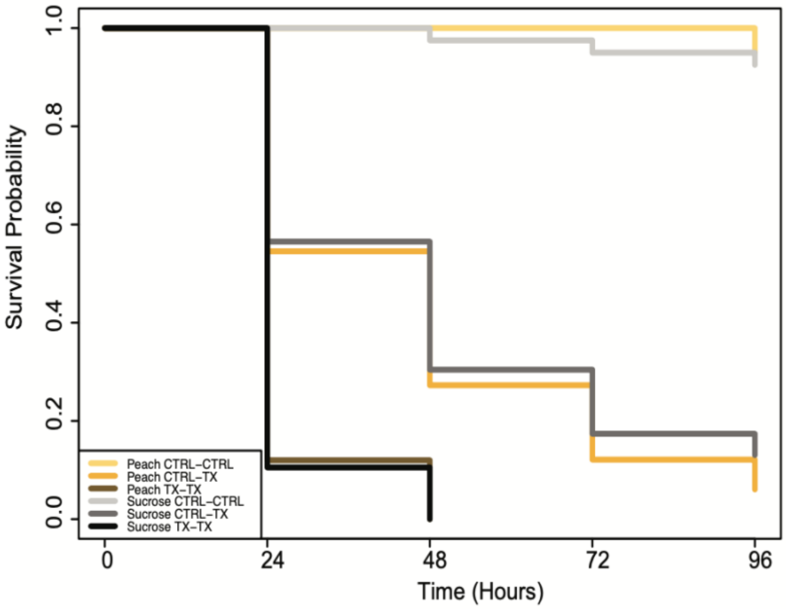
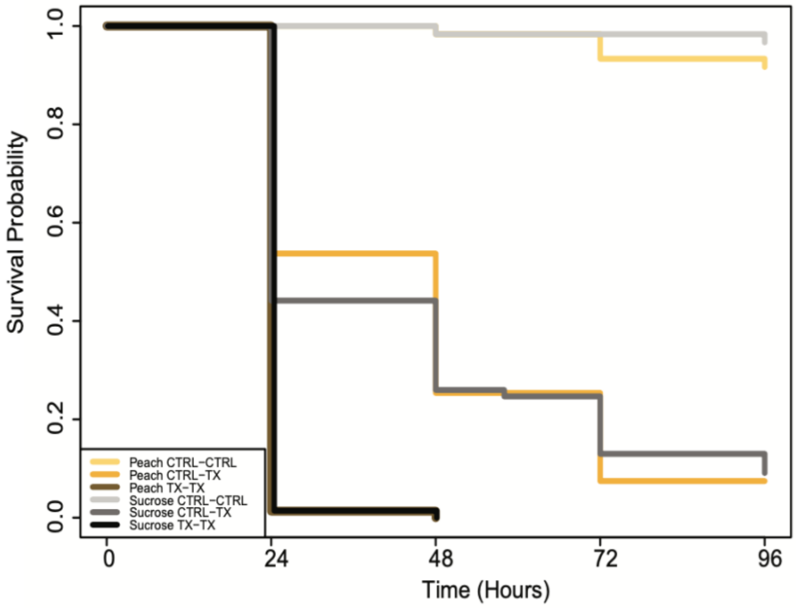


Figure 6: Average (± standard error of the mean) survival rates (%) of male and female *Aedes j. japonicus* mosquitoes provided with non-toxic (i.e., control), toxic, or a choice between the two (i.e., choice) over a 96-hour period. **A.** Male sucrose groups (control, n = 40; toxic, n = 19; choice, n = 23). **B.** Female sucrose groups (control, n=60; toxic, n = 70; choice, n = 77). **C.** Male peach groups (control, n = 40; toxic, n = 25; choice, n = 33). **D.** Female peach groups (control, n = 60; toxic, n = 75; choice, n = 67).

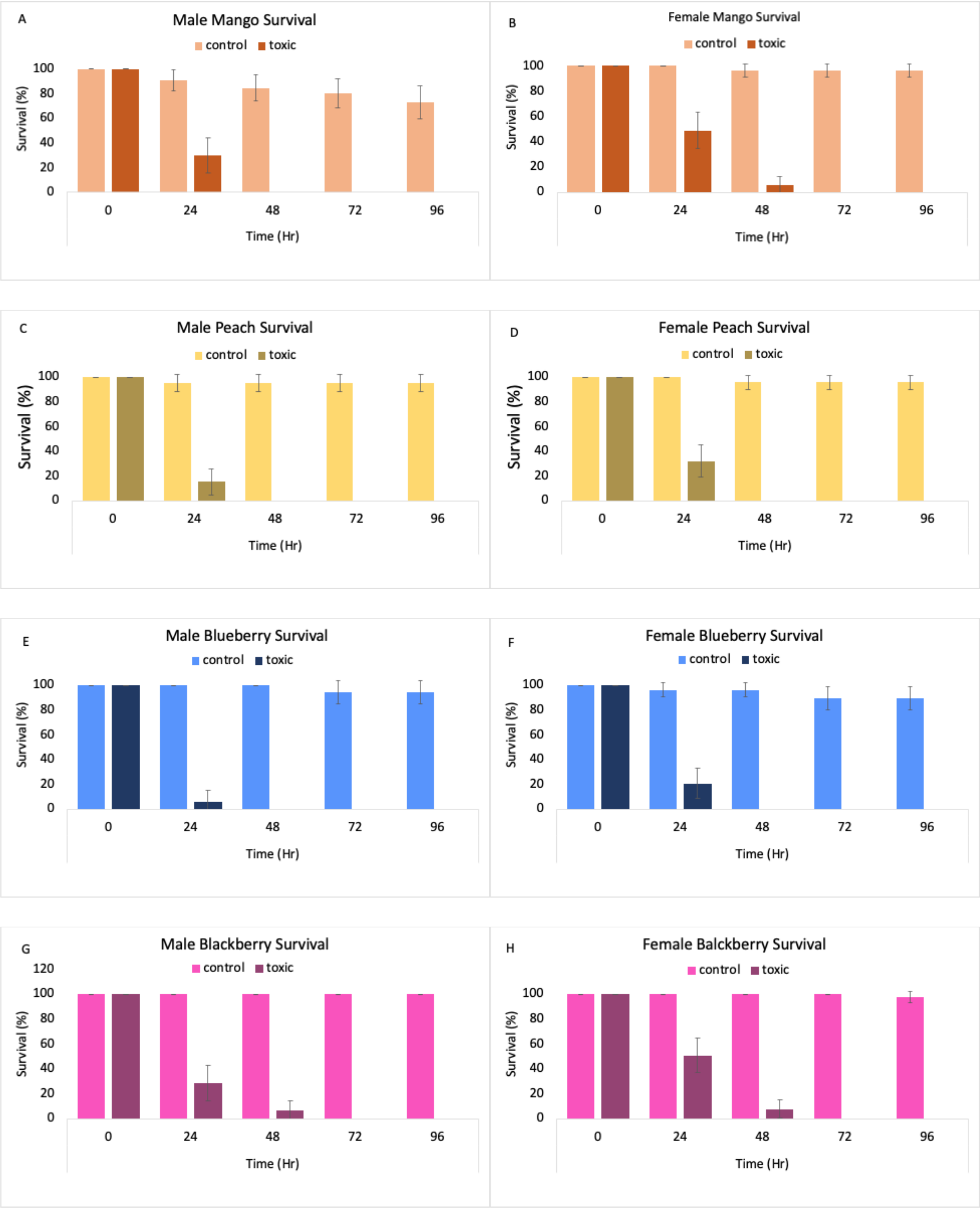




<u>Figure 7</u>: Raw (Kaplan-Meier) survival curves throughout the 96-hour period for the different conditions tested in males (top) and females (bottom)

3.2 Cup Assay

Significant differences in survival were found when comparing mosquitoes fed with toxic solutions to those fed control solutions (Log-rank tests, $p < 0.001$ for all comparisons) (Table 2) (Fig. 8; Fig. 9). When provided with toxic sugar solutions, we observed 100% mortality regardless of the fruit or juice tested, while control groups had survival rates above 90% after 96 hrs. Interestingly, when provided with grape juice, we noted a lower survival rate in both females and males compared to other control solutions (Females: blackberry: $p < 0.001$; blueberry: $p = 0.01$; mango: $p = 0.003$; peach: $p < 0.001$; sucrose: $p < 0.001$, soda: $p < 0.001$); Males: blackberry: $p = 0.03$; peach: $p = 0.01$; sucrose: $p = 0.06$ - no significant difference for blueberry: $p = 0.1$ and mango: $p = 0.7$). Mosquitoes provided with toxic solutions only nearly reached 100% mortality by 24 hours, with only 1% (sucrose) and 1.25% (peach) of females and 7.5% of males (sucrose and peach) remaining alive (Fig. 8).


A
Male Mango Survival
control
toxic
Survival (%)
100
80
60
40
20
0
0
24
48
72
96
Time (Hr)
B
Female Mango Survival
control
toxic
Survival (%)
100
80
60
40
20
0
0
24
48
72
96
Time (Hr)
C
Male Peach Survival
control
toxic
Survival (%)
100
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D
Female Peach Survival
control
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Male Blueberry Survival
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Survival (%)
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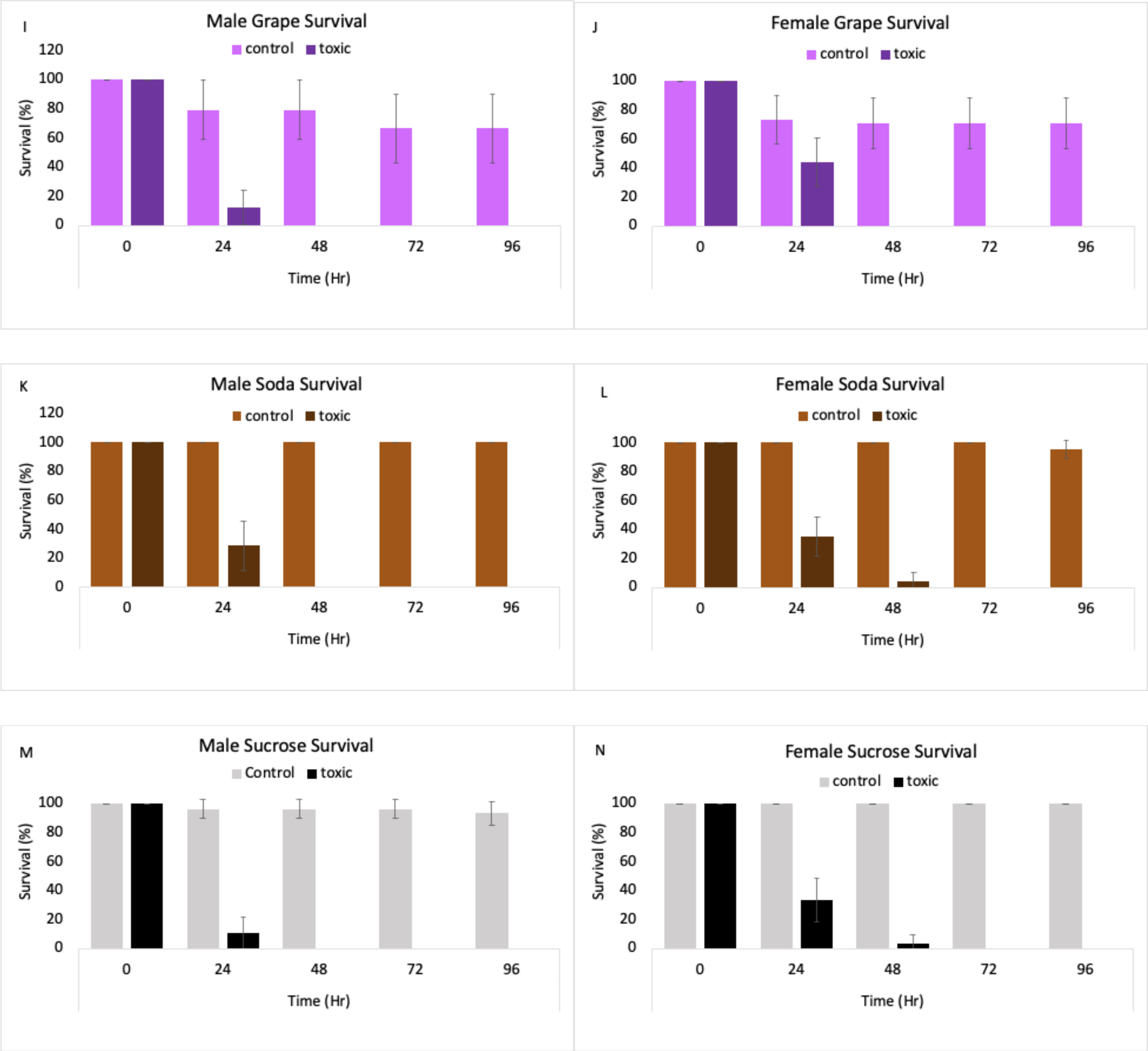
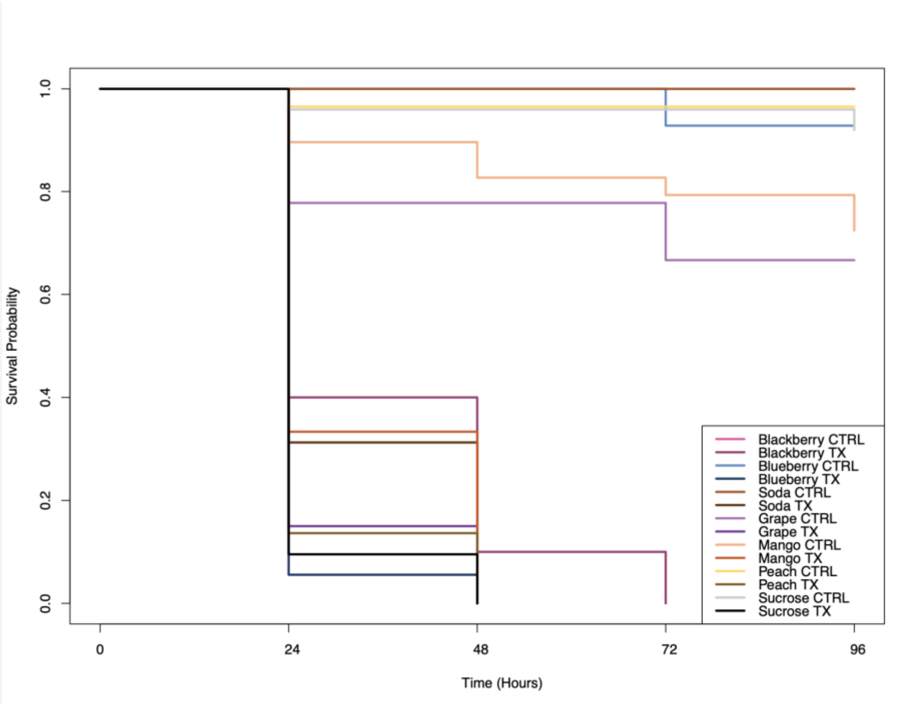


Figure 8: Average (± standard error) survival rates of male and female *Aedes j. japonicus* mosquitoes that fed on various control and toxic fruit solutions for a 96-hour period. **A.** Male, mango groups (control, n=29; toxic, n=24). **B.** Female, mango groups (control, n=36; toxic, n=42) **C.** Male, peach groups (control, n=29; toxic, n=22). **D.** Female, peach groups (control, n=51; toxic, n=58). E- Male, blueberry groups (control, n=14; toxic, n=18). **F.** Female, blueberry groups (control, n=42; toxic, n=51). **G.** Male, blackberry groups (control, n= 13; toxic, n=20). **H**. Female, blackberry solution (control, n=45; toxic, n=56). **I.** Male, grape group (control, n=9 ; toxic, n=20). **J.** Female, grape groups (control, n=23; toxic, n=32). **K.** Male, soda group (control, n=14; toxic, n=16) **L.** Female, soda groups (control, n=57; toxic, n=57) **M.** Male, sucrose group (control, n=25; toxic, n=21) **N.** Female, sucrose group (control, n=32; toxic, n=33)

Table 3: Average percent survival (± SEM) of male and female *Ae. j. japonicus* 96 h post exposure to toxic and control solutions.

ATSB Solution	**Control Survival (%) (average ± SEM)**	**Toxic Survival (%) (average ± SEM)**	**Log rank test - *p* value**
Sucrose			
Female	100	0	$p < 0.001$
Male	93.5 ± 8.2	0	$p < 0.001$
Mango			
Female	96.7 ± 5.2	0	$p < 0.001$
Male	72.7 ± 13.4	0	$p < 0.001$
Peach			
Female	95.8 ± 5.5	0	$p < 0.001$
Male	95 ± 6.9	0	$p < 0.001$
Blueberry			
Female	89.6 ± 9.6	0	$p < 0.001$
Male	94.5 ± 9.3	0	$p < 0.001$
Blackberry			
Female	98 ± 4.4	0	$p < 0.001$
Male	100	0	$p < 0.001$
Soda			
Female	95.5 ± 6	0	$p < 0.001$
Male	100	0	$p < 0.001$
Grape			
Female	70.7 ± 17.2	0	$p < 0.001$
Male	66.7 ± 23.6	0	$p < 0.001$



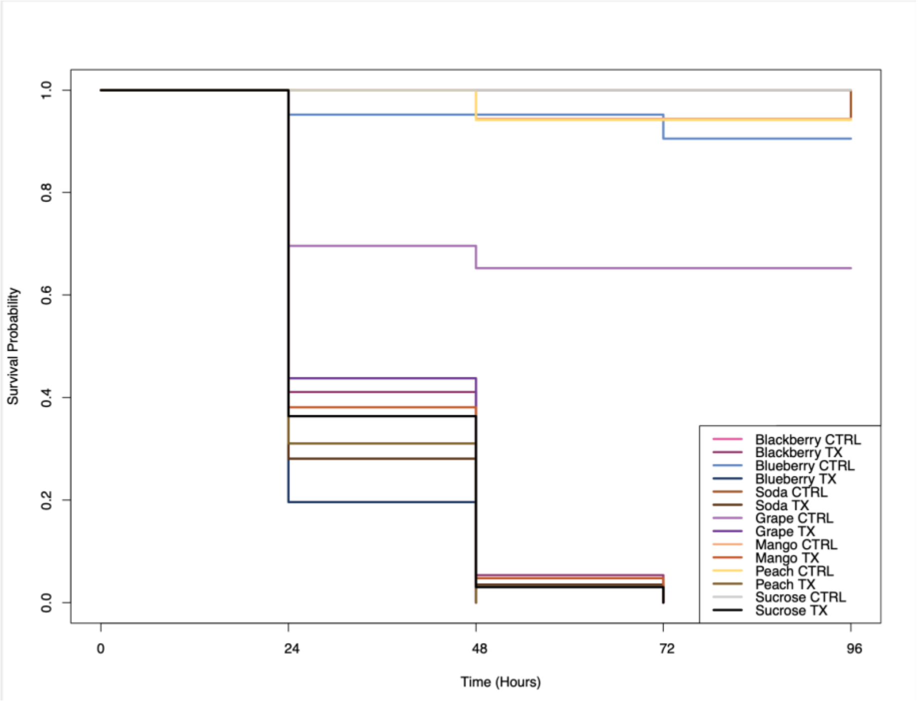


Figure 9: Raw (Kaplan-Meier) survival curves throughout the 96-hour period for the different conditions tested in females (bottom) and males (top). CTRL. Control TX. Toxic

4. Discussion

To address current challenges with mosquito control, including insecticide resistance, the WHO has urged for the development of novel pest management techniques that are inexpensive, sustainable, and environmentally friendly (WHO, 2019). Attractive toxic sugar baits have emerged as an efficient control strategy, capable of limiting populations of several mosquito species including major disease vectors such as *Ae. albopictus*, *Ae. aegypti*, *An. gambiae* and *Cx. pipiens* (Bhami *et al*., 2015; Ller *et al*, 2010; Muller *et al*, 2011; Revay *et al*. 2014.). Many of these baits contain active ingredients such as permethrin, spinosad, or boric acid. Allan *et al.* (2011) conducted experiments on the toxicity of oral toxins on adult mosquitoes and found that some were more efficient than others depending on the mosquito species. For example, *Culex quinquefasciatus* is less susceptible to permethrin, a widely used insecticide, than *Aedes* species. Applying the correct oral toxin is imperative as many ATSBs are limited due to their repulsive nature of active ingredients. Busvine (Busvine, 1964) documented the variance of mosquito behavior when exposed to dichloro-diphenyl-trichloroethane (DDT), where some mosquitoes were less repulsed than others. Shin *et al.* (2011) conducted bioassays to quantify the repulsive nature of different active insecticides in sucrose solutions, and showed that deltamethrin was the least repulsive chemical against *Cx. pipiens molestus* (Shin *et al*., 2011).

Trihydrooxidoboron, or boric acid, is a weak acid registered pesticide worldwide that has been shown to be effective against *Ae. albopictus* and *Ae. aegypti* (Bhami and Das *et al*. 2015 , Fulcher *et al*. 2014; Naranjo *et al*. 2013; Scott-Fiorenzano *et al*. 2017; Zue *et al*. 2006). In the present study, we demonstrated its efficacy in another invasive species, *Ae. j. japonicus*. Our results indicate that mosquitoes are either not repelled by the boric acid solution and / or cannot taste it demonstrating that this chemical is a good candidate to be incorporated in ATSB solutions for field tests.

Two types of sugar baits have been developed and used in the field: TSBs and ATSBs. Toxic sugar baits (TSBs) use phagostimulants (such as sucrose) to induce ingestion of an oral toxin, but sugar alone is not particularly attractive (Fiorenzano *et al.* 2017). Adding an attractive component to solutions (*i.e.,* ATSB) can increase the efficacy of solutions in the field. Indeed, mosquitoes rely, in part, on specific plant volatiles emitted by their food sources to locate them (Lahondère *et al.* 2019, Nyasembe and Torto 2014). These insects have also been documented feeding on damaged or rotting fruits (Foster *et al.* 1995). Multiple ATSB studies have shown that fruit solutions produce attractive volatiles to lure mosquitoes and are quite effective at reducing populations (Müller *et al.* 2011). For example multiple studies have used different tropical fruits such as mango, guava, prickly pear, and melon to produce sugary solutions that attract mosquitoes (*e.g.*, *Ae. albopictus, An. gambiae*) to the bait in both tropical and arid environments (Beier *et al.* 2012; Naranjo *et al.* 2013a). Some of these studies include volatile analysis using gas chromatography aiming at identifying specific attractive compounds from both plant hosts (Gouagna *et al.* 2010; Lahondère *et al.* 2019; G. Müller and Schlein 2006; Nyasembe and Torto 2014; Xue *et al.* 2006). For example, Meza *et al.* (2020) reported four detectable chemicals from a mango fruit ATSB using behavioral assays, showing that two of these chemicals were attractive (terpinolene and humulene), one was marginally attractive ((E)-caryophyllene), and one was repellent (myrcene) to *An. gambiae.* Interestingly, a synthetic blend composed of the three attractive chemicals was found as attractive as the whole mango solution (Meza *et al.* 2020). This indicates that using a synthetic blend or a whole fruit solution in an ATSB could be equally as effective. A benefit to using whole fruit solutions is that as fruits decay, carbon dioxide is produced as a byproduct of fermentation. Carbon dioxide is a highly attractive chemical to mosquitoes and is one of the main cues involved in host seeking behavior. However, to our knowledge, nothing is known about sugar feeding behavior and attractive plant and fruit volatiles in *Ae. j. japonicus*.

In the present study, we tested several fruit solutions that may be suitable for the development of an ATSB targeting *Ae. j. japonicus*. In our initial hypothesis we anticipated that these mosquitoes would present a preference for fruits that they may encounter in their natural habitats, in particular berries. However, we found that this mosquito species consumed all fruit solutions provided (*i.e.*, mango, peach, blueberry, and blackberry) suggesting that this species might feed on a wide range of fruits in the wild. We indeed found no difference in terms of mosquito survival between the four different types of fruits tested, indicating that a sustainable, easy to produce bait can be achieved to target this mosquito species. Moreover, we found similar results when testing a soda which requires less preparation (and might be easier to deploy in the field) as sugar is already present in high quantities in these drinks and can be easily found and stored. In contrast, grape juice, another premade sugar solution, led to a higher mortality in our control groups compared to the fresh fruit solutions and the soda, which might be due to high amounts of tartaric acid in grapes. This study provides the first insights for developing an efficient ATSB against *Ae. j. japonicus* which are critical before conducting field tests. We showed that boric acid is a suitable oral toxin to be used against this species. While some other active ingredients can repel mosquitoes, the cage assay data show that *Ae. j. japonicus* mosquitoes cannot detect / taste boric acid or that it is not repellent to them. The next step consists in designing a mosquito trap, to deploy and test our ATSB in the wild to better understand the efficacy of these solutions and possible off target species interactions.

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GENERAL DISCUSSION AND PERSPECTIVES

The work presented in **Chapter One** was conducted to build a better understanding of the effects of abiotic factors, such as temperature and humidity, on the survival of *Ae. aegypti.* Because mosquitoes cannot actively maintain a constant body temperature, it is crucial to understand how changing environmental factors will impact the behavior and physiology of mosquitoes and in particular of invasive species such as *Ae. aegypti*. We compared the survival of sugar fed male and female mosquitoes in different environmental conditions- optimal (26-27 ℃) and cold (21-22 ℃) temperatures with low (55-65%) and high (80-90%) humidity. Our data show that low humidity largely contributed to a decrease in survival while temperature did not have as significant of an impact.

In order to have a more complete understanding of the combined effects of temperature and humidity on *Ae. aegypti*, the next step consists in conducting experiments under warmer conditions (31-32 ℃). Conducting these experiments in which temperatures are at the higher extreme of this species active ranges can provide more information on how this species will cope with climate change.

To expand upon this data, it would be interesting to look at the effects of temperature and humidity on mosquito activity. The sugar solution used in these experiments is more closely related to what mosquitoes would consume in the wild, compared to 10% sucrose or just water. The change in monosaccharide composition and concentration could impact mosquito activity. Activity experiments could be conducted using an actometer as in Upshur *et al.* (2019). These experiments would be run in the same environmental conditions (*i.e.*, cold, optimal, hot temperatures; low and high humidity) to evaluate the effect of these factors on the activity of invasive species, such as *Ae. aegypti*, *Ae. albopictus* and *Ae. j. japonicus.*

Respirometry could also be used to understand how temperature and humidity affect the patterns of respiration in mosquitoes. A respirometer measures the exchange rate of oxygen and carbon dioxide, as well as water vapor release, of a single organism that is placed in a tube. Metabolic rate in *Drosophila* flies is directly related to carbon dioxide output (Yatsenko et al., 2014). By measuring and comparing the carbon dioxide output of starved, water fed, and sugar fed mosquitoes can help identify how access to food or different food sources can benefit mosquitoes by reducing metabolic costs under various climatic conditions. For example, As temperature, and more specifically extreme temperature, can be a stressor in insects, it was found that an insect's metabolic rate will increase when it is placed outside of its preferred temperature range (Acar et al., 2001). A more in depth understanding of the effect of abiotic factors on mosquito behavior and physiology can lead to the development of more informed and consequently effective control strategies.

In **Chapter Two**, we aimed to develop a suitable ATSB for the invasive mosquito, *Ae. j. japonicus.* This species is newly invasive to the United States and Europe and is quickly establishing dominant populations in suitable environments. As this mosquito is a competent vector for multiple diseases such as Japanese Encephalitis, West Nile virus, and La Crosse virus, it is imperative that these populations are controlled to limit the spread of disease (Kaufman & Fonseca, 2014). No control methods are currently available for this invasive species. Our data shows that boric acid was not repulsive and / or detectable to *Ae. j. japonicus*. This deemed boric acid a suitable oral toxin to use against this species. Fruit solutions were then tested as attractive components of the ATSB solutions. Interestingly, *Ae. j. japonicus* was able to consume all tested solutions, suggesting that they feed on a wide variety of sugar sources in the wild. Grape juice was found to be less effective and will not be incorporated into future experiments. More studies, such as the ones described below, must be conducted in order to optimize the effectiveness of our bait, before it can be used in highly populated areas.

Traditionally toxic sugar baits (TSBs) will only use sugar as a phagostimulant in order to enhance feeding on an oral toxin. In order to pass limitations of repellency of oral toxins, attractive sugar baits (ATSBs) were developed. ATSBs use attractive scents to lure both male and female mosquitoes to a toxic sugar source (Fiorenzano et al., 2017). Mosquitoes use olfactory cues, among others, to locate food sources (Lahondère *et al*. 2019, Nyasembe and Torto 2014). Moreover, scent profiles can be collected and analyzed from the fruit solutions used in the experiments conducted in Chapter Two. Scents can be collected to understand the change of the scent of the solutions over time. Four samples would be collected over a 96-hour period, one sample every 24 hours similar to Meza *et al*. (2020), where they observed that samples from 24-48 hours were the most attractive. Samples would be ten be analyzed using Gas Chromatography - Mass Spectrometry (GC-MS). Scent samples would be prepared with an internal standard of known concentration in order to better understand the volatile composition/concentration of the fruit solutions. Volatiles identified using this method could then be tested for detection using gas chromatography coupled with electroantennography (GC-EAGs) (Lahondère, 2021). This technique allows recording of the electrical activity of an insect's antenna when presented with a chemical. This method would inform us on which chemicals within a given fruit scent can be detected by the mosquito. It is worth mentioning that this method has yet to be conducted with *Aedes j. japonicus*.

Pairing methods above with behavioral experiments using a Y-maze olfactometer can show whether mosquitoes are attracted, repelled, or indifferent to specific detectable volatiles identified *via* GC-EAGs. Mosquitoes are released at one end of the maze, and can decide between the two arms of the Y-maze that are supplemented with a tested volatile or a control (Vinauger et al., 2018). If a mosquito chooses the volatile arm over the control, that would imply that the volatile is attractive. Understanding what specific volatiles attract this mosquito can be beneficial when developing baits. This method can also be used to understand if there is a preference for whole fruit solutions. Possibly, synthetic blends of attractive scents can then be synthesized to be deployed for long term attraction of a bait.

Once field season begins, we plan on conducting field observations and experiments with the ATSB solutions developed in this work. In order to test the efficacy of these solutions in the wild, we will develop a mosquito specific trap. Mosquitoes and pollinators, such as honey bees and butterflies, are not attracted to the same colors (Hoel et al., 2011; Menzel & Erber, 1978). We plan on designing a trap that will limit off target species interactions by limiting access to the solution so that larger insects such as butterflies and moths cannot enter it, as well as using visual preferences of mosquitoes. A black 3D printed trap will be placed at field sites and will be monitored every 96 hours.

In conjunction with testing our baits in the field, we also plan on observing sugar feeding behaviors of *Aedes j. japonicus* in both urban and rural areas. Mosquitoes will be collected using a backpack aspirator (Bioquip, USA). Specimens will be identified in the lab using morphological traits. Then, using the cold anthrone method (Van Handel, 1972), the amount of carbohydrates fed mosquitoes can be quantified. This method is a highly sensitive colorimetric assay that allows for the detection of fructose in mosquitoes. *Aedes j. japonicus* is a newly established invasive mosquito that is a competent vector of multiple diseases. Thus, a deeper understanding of its biology, ecology, and sugar feeding behaviors can optimize control strategies.

Overall, this work showed that sugar feeding greatly increased the survival of mosquitoes exposed to optimal conditions (optimal temperature and high humidity). Humidity conditions greatly impacted survival, as it was significantly lower in low relative humidity than in high humidity conditions, while temperature had a lesser effect on survival. Next steps will question whether these abiotic factors also affect metabolism and activity. In Chapter 2, we reported that boric acid was not detectable/repulsive to *Ae. j. japonicus* suggesting that this chemical is a suitable oral toxin to be implemented into our baits. It was also found that *Ae. j. japonicus* can consume a wide range of fruit solutions, but grape solutions seemed to be less effective. Further experiments will be conducted to expand the knowledge of *Ae. j. japonicus* biology, ecology, and behaviors in order to develop an effective bait. This work sheds light on the importance of abiotic factors and sugar feeding on mosquito survival and lays the groundwork for controlling an invasive mosquito species.

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