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A comparison of egg desiccation tolerance and development under different temperatures for three common *Aedes* mosquitoes

By

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Abstract

With the rise in global temperatures, invasive mosquito species like *Aedes albopictus* may be able to reach regions that were previously inhospitable to these species. Therefore, if *A. albopictus* were to potentially reach Puerto Rico, it would come into contact with the species of *Aedes aegypti* and *Aedes mediovittatus* in local containers, and it is unclear how the tropical temperatures and precipitation patterns would affect the co-occurrence of these three species. Egg desiccation and temperature dependent growth among the species were measured to test how these species hatched and developed during different humidity and temperature. It was hypothesized that there would be variable desiccation and growth among the different species of *Aedes*, with *A. albopictus* faring better at higher temperatures and a higher degree of humidity and *A. aegypti* faring better at lower temperatures and a lower degree of humidity. The experiment was split into two parts: 1) egg desiccation, where eggs were placed in envelopes in incubators set to three different percentages of humidity (25%, 50%, and 80%) and left for two or four weeks before being induced to hatch and emerged larvae from hatching being counted; and 2) temperature, where newly hatched first instar larvae from a different treatment were placed in incubators of varying temperatures (20 ºC, 30 ºC, and 40 ºC) and checked daily for growth until the adult phase, at which point they were frozen, thawed, and weighed. The temperature data showed that development was fastest for all species at the highest temperatures but much slower at the lowest temperatures, with *A. mediovittatus* having the lowest survivability at 40 ºC. The humidity data showed that egg mortality was approximately equivalent among *A. aegypti* and *A. albopictus* (higher mortality at lower humidity and lower mortality at higher temperature), with *A. mediovittatus* progressing in the opposite direction of mortality. This part of the experiment was not completed due to the onset of COVID-19 causing a campus lockdown during data collection. It was concluded that, if *A. albopictus* were to invade Puerto Rico, it could exist alongside *A. aegypti* and *A. mediovittatus* due to Puerto Rico's relatively warm temperatures and high humidity contributing to low egg mortality and fast development times among *A. albopictus*.

Key Words: mosquito, biology, Puerto Rico, life history, humidity

Dedication

My parents Gayle and Kim and my brother Korey:

Thank you for all your support.

I could not have done this without you.

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L:D	Light: Dark
RO	Reverse Osmosis

Introduction

Mosquitoes are found all over the world and on every continent except Antarctica (Knudsen, 1995). They have adapted to various climates including the tropics, temperate locations, urban areas, and even some tundra regions (Knudsen, 1995; Honogh et al., 2012; Li et al., 2014). The advent of rapid climate change and urbanization across the world could allow mosquitoes to spread to areas that were once inhospitable to them (Reiter, 2001; Samy et al., 2016). For this study, two different tests were performed to assess how three species of tropical/subtropical mosquitoes in the genus *Aedes* compare in their growth, mass, and overall survival across different temperatures and how mosquito eggs from these three species withstand desiccation under different humidity levels.

Three species of *Aedes* were used in this experiment: *Aedes albopictus*, the Asian tiger mosquito; *Aedes aegypti*, the yellow fever mosquito; and *Aedes mediovittatus*, the Caribbean treehole mosquito. *Aedes albopictus* is a native species of southeast Asia but is highly invasive across the world thanks to its transport via tire importation. It is also a known vector of several important arboviruses like dengue (Knudsen, 1995; Benedict et al., 2007; Li et al., 2014). It is currently absent from Puerto Rico but co-exists in southern Florida with *A. aegypti*. *Aedes aegypti* is native to North Africa but is considered a historic invasive species in America (including Puerto Rico), although it has been recently outcompeted in its range by *A. albopictus* (Juliano & Lounibos, 2005). *Aedes aegypti* is the main vector of yellow fever virus worldwide. Finally, *A. mediovittatus* is native to the Caribbean but is absent from the mainland United States. It is considered a vector of dengue fever virus in Puerto Rico, although is it likely of minor importance compared to *A. aegypti* and *A. albopictus* (Little et al., 2011; Barrera et al., 2012).

Two hypotheses were crafted for this experiment, one for the humidity test, and one for the temperature test. The first hypothesis was that eggs of three species of *Aedes* will desiccate at different rates. It was thought that the highest humidity would favor egg hatching of *A. albopictus* as mortality for this species tends to be relatively low in wetter conditions (Costanzo et al., 2005; Urbanski et al., 2010). Lower humidity may not favor any of the species, although *A. aegypti* has been shown to be more desiccation tolerant than *A. albopictus* under drying conditions (Costanzo et al., 2005). At present, there are no data on egg desiccation in *A. mediovittatus*, although this species of mosquitoes is not found in the hot and dry areas of San Juan, Puerto Rico but is found in the cooler and wetter rural areas in southern Puerto Rico (Little et al., 2011).

The second hypothesis was that larvae of three *Aedes* species would show variable growth, size as adults, and survival across temperatures. Specifically, it was predicted that *A. aegypti* would grow faster than *A. albopictus* and *A. mediovittatus* at lower temperatures due to being found at higher latitudes, while warmer temperatures would benefit *A. albopictus* (Alto & Juliano, 2001). It was also predicted that all three species would grow equally well at higher temperatures and higher percentages of humidity as all species can be found in tropical areas, although *A. albopictus* struggles at lower temperatures (Juliano et al., 2002).

Literature Review

Mosquitoes are a type of fly that belong in the family Culicidae within the order Diptera (Rey, 2011). They are mainly known for their blood-sucking characteristics (present in females but not males), their three pairs of long legs, and their elongated mouthparts (Rey, 2011). There are approximately 3,500 species of mosquitoes known to science, with many of them acting as vectors for disease, including malaria, yellow fever, and dengue fever (Rey, 2011).

Mosquito Life Cycle. The mosquito life cycle consists of four stages: the egg, the larva, the pupa, and the adult (Crans, 2004). The eggs are laid by females in association with water. Species of *Aedes* differ from other mosquito genera like *Culex* by laying single eggs above the water, instead of laying large rafts of eggs on the surface of the water (Allan & Kline, 1998). Eggs of *Aedes* can remain dry for long periods of time and hatch under the right conditions of temperature, humidity, and pH after being inundated with water (Juliano et al., 2002). Larvae After hatching, larvae feed on algae, detritus, and microorganisms in the water and pass through four instars, each separated by a molt (Crans, 2004). After the larvae pupate, a stage at which they can swim but are unable to feed, they eclose into adults (Ramasamy et al., 2011). After emerging, adult will live for several days, during which time males and females feed and reproduce (Crans, 2004). Adult females require a blood meal that they use as a source for iron and protein for producing eggs, whereas both males and females feed on nectar to meet their energy needs (Peach & Gries, 2019).

Mosquito Habitats. Mosquitoes are usually found in habitats with stagnant water, as this is where mosquitoes lay their eggs and where the aquatic larvae grow and feed (Crans et al., 2004). Standing water habitats that benefit mosquitoes include larger, more permanent bodies of water, like ponds and lakes, and smaller, less permanent bodies of water, like puddles and swamps

(Adeleke et al., 2008). Mosquitoes do not need a lot of space to lay eggs and for larvae to thrive, as they can easily spread to containers with water in them, making pest control difficult (Li et al., 2014).

Mosquito Competition. Larvae of mosquitoes, including *Aedes*, compete with each other for space and resources (Barrera, 1996). They also compete for oxygen, food, and space in a container of water or other environments of limited space or permanence (Barrera, 1996). Survivability and mortality of eggs are part of that competition, as it will create more room or less room for larvae in small areas, like containers (Juliano et al., 2002). This is important, as *A. albopictus* and *A. aegypti* are known to compete against one other in the United States, with *A. albopictus* having the best survivability in cooler, wetter environments, and *A. aegypti* having the best survivability in warmer, drier environments (Juliano et al., 2002), although there is no data about competitive interactions between either of these species and *A. mediovittatus*. Thus, it is unclear at this time what the competitive effects would be if *A. albopictus* were to reach Puerto Rico and compete alongside *A. aegypti* and *A. mediovittatus*.

Florida offers an opportunity to test how competition among *Aedes* species may occur on the island of Puerto Rico. The species *A. albopictus* has spread throughout Florida, displacing *A. aegypti* in some regions, namely in warmer and more rural regions (O'Meara, 1995). *Aedes aegypti* still exists in hotter, drier areas and in urban regions, where it coexists with *A. albopictus* (O'Meara, 1995). *Aedes albopictus* has also displaced *A. triseriatus*, a native mosquito, in tire containers in Florida, as the invasive mosquito's eggs hatch more rapidly than the native mosquito's eggs (Lounibos et al., 2001).

In Puerto Rico, two species of *Aedes* mosquitoes currently reside and compete in local containers: the invasive *A. aegypti* and the native *A. mediovittatus*. Both species are found in local containers, but *A. aegypti* is more prominent in urban areas (Little et al., 2011). In contrast, *A. mediovittatus* is more prominent in areas with high densities of trees (Little et al., 2011). A reason for concern in Puerto Rico regarding a potential invasion of *A. albopictus* is that Puerto Rico is an island, which is isolated and represents a more fragile ecosystem. Also, *A. mediovittatus* is a species that has not been studied as well as other mosquito species and may potentially be displaced if competition with *A. albopictus* and *A. aegypti* intensifies (Gubler et al., 1985).

Mosquito Environmental Effects. With rising temperatures around the world due to global warming, invasive mosquito species like *A. albopictus* have spread to regions that previously had no records of these species (Reiter, 2001; Samy et al., 2016). These mosquitoes carry the increased risk of transmitting disease agents that may not be as prevalent in native mosquitoes (Knudsen, 1995; Benedict et al., 2007; Li et al., 2014). Thus, it is important to measure the conditions that are best for eggs and larvae, as this knowledge can potentially be used to quell the spread of mosquitoes and the diseases that they can carry in areas that may be hardest hit by these invasions. Furthermore, if *A. albopictus* were to eventually reach the island of Puerto Rico, it would interact with *A. aegypti* and *A. mediovittatus* in local containers, and at present, we do not know how the tropical temperatures and precipitation patterns would affect the co-occurrence of these three species. This project had the goal of assessing the role of temperature and humidity in affecting the co-occurrence of these species of *Aedes*.

In previous experiments, it was found that mosquitoes tended to grow faster at higher compared to lower temperatures (Rueda et al., 1990). Mosquitoes that live in higher temperature tend to have smaller body sizes than those that grow at lower temperatures (Rueda et al., 1990).

When it comes to humidity levels, higher levels benefit *Aedes* egg survivability compared to drier conditions (Juliano et al., 2002).

Mosquito Importance. Mosquitoes occupy multiple niches over their life span. Larvae may eat algae, microorganisms, and detritus and provide a food source for fish and amphibians (Godfray, 2013). Adult mosquitoes pollinate some flowers, provide food for birds and bats, and even shift the paths of migrating caribou in the Arctic tundra (Rey, 2011). Most predators of mosquitoes are non-specific to mosquitoes, with some predators such as mosquitofish becoming extinct due to lack of a primary food source (Rey, 2011).

Several hundred species of mosquitoes are vectors for human disease (Rey, 2011), including malaria, yellow fever, dengue fever, Zika virus, and West Nile fever (Godfray, 2013). Malaria alone kills approximately one million people every year, with other diseases responsible for additional thousands of deaths (Godfray, 2013). Otherwise, mosquitoes are commonly seen as a pest and a nuisance in places where these diseases are not as prevalent (Rey, 2011). Control of mosquitoes is difficult due to their adaptiveness to a variety of habitats, natural and artificial, and resistance to many insecticides (Juliano et al., 2002; Weil et al., 2003).

Materials & Methods

Colonies of all *Aedes* species were started using field collected larvae or eggs from southern Mississippi (*A. albopictus*) and Puerto Rico (*A. aegypti* and *A. mediovittatus*). Larvae were fed with dog food (Purina Puppy Chow) and held in 1-L pans kept inside incubators set at a constant temperature of 22 °C. When larvae were found to have pupated, they were collected with pipettes and placed in water within species-specific colony cages where they were to eclose into adults. Cages consisted of 20-L plastic containers that have screens on the sides and top and a sleeve to allow for easy access. All cages were kept in a colony room between 22 and 25 °C with a photoperiod of 16:8 hours light to dark phases (L:D) and 1.5-hour dawn and dusk phases. Adults were given a 10% sucrose solution to feed on. They were also supplemented with blood feeding from *Coturnix japonica* (IACUC protocol #17101203) once a week to elicit egg production.

Single black cups (400 ml) lined with a wet piece of seed emergence paper were placed in each cage to provide a place for mosquitoes to lay their eggs. Egg papers were then stored in a sealed plastic container in an incubator kept at 22 °C until experiments began. The eggs were used to conduct two separate experiments: a) hatched larvae were subjected to three rearing temperatures where development time (d), mass (mg), and survival were measured, and b) a humidity test to determine egg desiccation tolerance across different humidity levels.

For the humidity experiment, egg papers from each species were cut into six strips per humidity level, resulting in 18 different strips per species. Strips were divided into pairs and placed into standard mailing envelopes, with three envelopes stored in three levels of humidity: 25%, 50%, and 80%, respectively. Humidity was maintained within individual incubators (Percival, Inc.) and verified using humidity gauges (Veanic, Inc.). Eggs were left in the incubators for two or four weeks and were hatched by placing individual strips in shell vials filled with a nutrient solution comprised of 0.33 g of nutrient broth (Difco Industries) in 0.75 liters water. The number of eggs hatched based on emerging larvae was recorded. Differences in hatching rate among species and across humidity levels were analyzed using logistic regression.

For the temperature experiment, eggs were hatched at the same temperature (25 ºC). This batch of eggs was treated separately from the humidity batch of eggs, and newly hatched first instar larvae were added to treatment level replicates. Sixty larvae were divided among six 250-ml Tri-pour beakers (Fisherbrand) (10 larvae per beaker, two beakers for each species) containing 200 ml of Reverse Osmosis (RO) water. One milliliter of tire inoculum was collected from local tires and mixed with 0.3 g of dried cricket to use as food source for development. Two replicates for each species were placed into incubators set at three different temperatures: 20 ºC, 30 ºC, and 40 ºC. Larvae were checked daily and any pupae that emerged were placed into 0.25-dram shell vials until adults emerged. Newly emerged adults were sexed and then frozen at 0 ºC after first removing any water with a pipette. Dead adults were placed in a 50 ºC drying oven before being weighed on an Ultra Micro balance (Mettler Toledo) to the nearest 0.001 mg. Survival was calculated as the percentage of larvae that survived to an adult. Data (mass of males and females, development time in days) were analyzed using separate analysis of variance (ANOVA) for each species. As each species has inherent differences in life history (size), separate analyses were conducted. Survival was analyzed using a single two-way ANOVA to compare differences between species and among temperatures. As survival did not meet the homogeneous variance assumption, the data was arcsine (square root (x)) transformed. To determine differences of significant effects, Tukey tests were used. All analyses were conducted in SAS (2004).

Results

Experiment 1. Larvae Development Across Different Temperatures. This phase of the experiment was performed to assess the length of time it takes for mosquito larvae to develop at different temperatures. Analysis revealed significant differences in female and male development times for all three species across the different temperatures, but no difference in mass (Table 1). For *A. albopictus*, both male and female development times at 20 ºC were much longer (15 d) than at higher temperatures (7 d), which were roughly equivalent in length (Fig. 1). For *A. aegypti*, both male and female development times were shorter at 30 ºC and 40 ºC (6 d and 7 d, respectively) than at 20 ºC (14 d) (Fig. 2). Finally, for *A. mediovittatus*, both male and female development times were much longer at 20 ºC (17 d) than at higher temperatures (5-7 d) (Fig. 3).

Table 1. Results from two-way ANOVA testing the relationships between male and female mass and development times in days of mosquitoes of different species across different temperatures. To control for comparison-wide error rate the p-value was adjusted for multiple tests. Adjusted P-value = 0.0125 for significance. Significant effects are shown in bold font.

	Female development Time (days)			**Female mass (mg)**			**Male development time (days)**			**Male mass (mg)**		
Species	Df	F-value	P-value	Df	F-value	P-value	Df	F-value	P-value	df	F-value	P-value
Aedes albopictus	2, 3	48.20	**0.005**	2, 3	14.90	0.028	2, 3	241.00	**0.005**	2, 3	2.50	0.230
Aedes aegypti	2, 3	217.00	< **0.001**	2, 3	2.15	0.264	2, 3	57.00	**0.004**	2, 3	1.49	0.356
Aedes mediovittatus	2, 3	231.50	< **0.001**	2, 3	0.94	0.482	2, 3	453.0	< **0.001**	2, 3	0.10	0.907

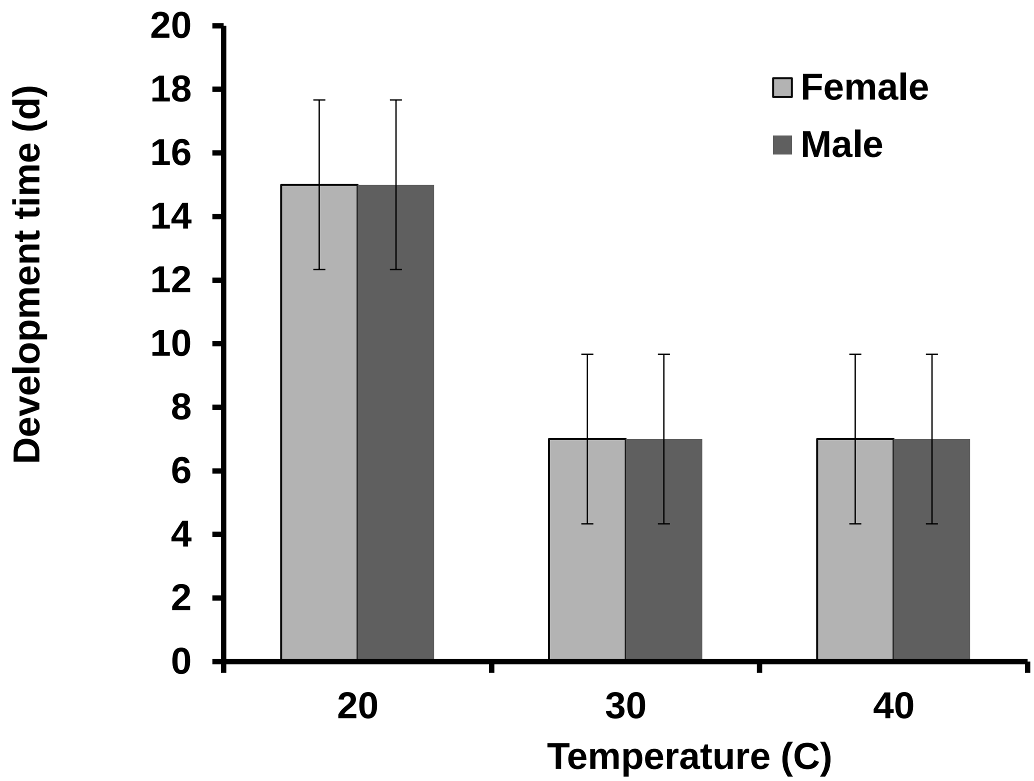

Figure 1. Mean ± standard error of female and male development time across different temperatures for *Aedes albopictus.*

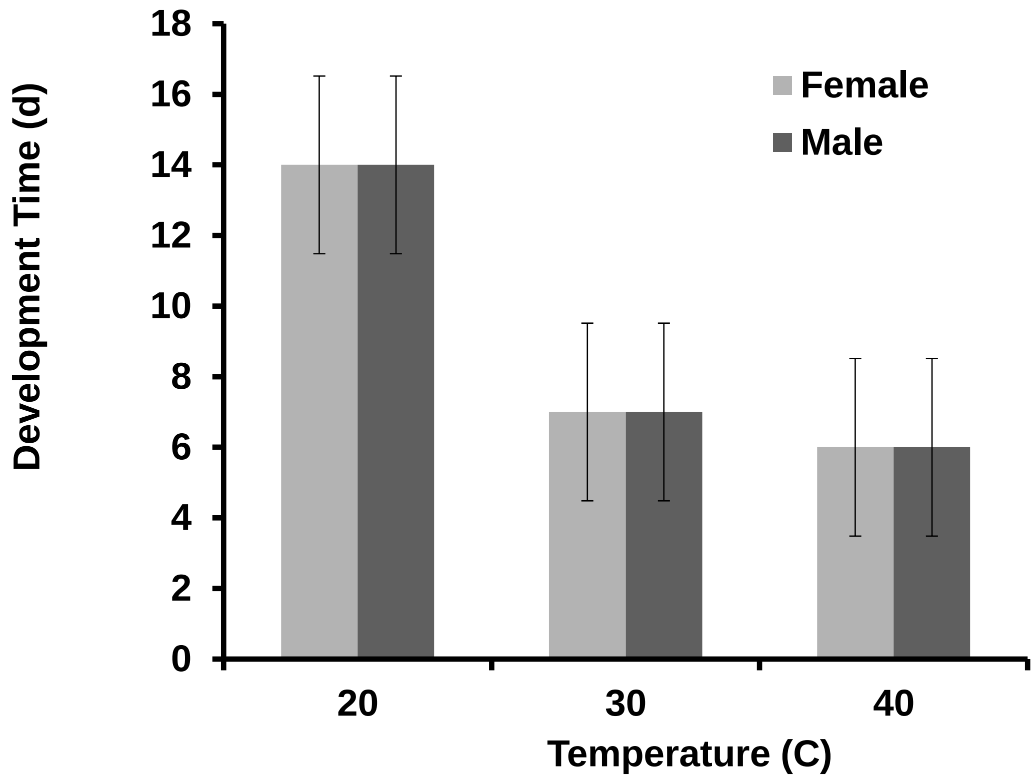

Figure 2. Mean +/- standard error of female and male development time across different temperatures for *A. aegypti*.

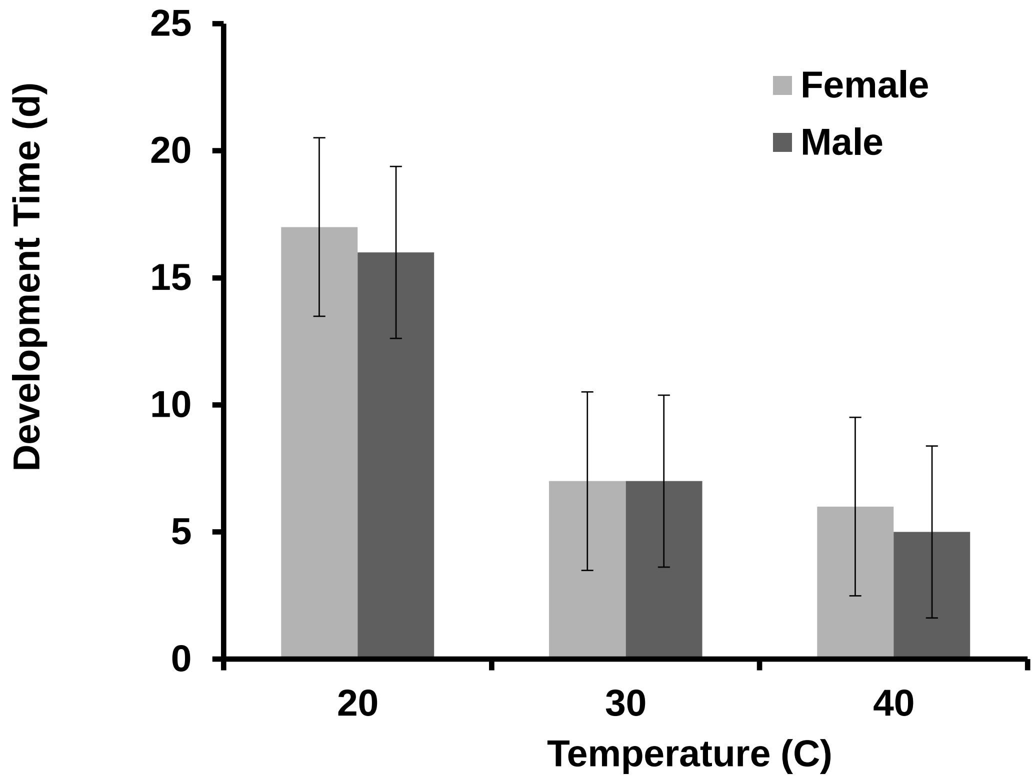

Figure 3. Mean +/- standard error of female and male development times across different temperatures for *A. mediovittatus*.

Survival data for *A. albopictus*, *A. aegypti*, and *A. mediovittatus* was transformed using an arcsine (square root (x)) to meet assumptions**.** There were significant differences in survival among temperatures (F-value = 23.44, P = 0.0003), among species (F value = 6.53, P = 0.0177), and the interaction between the species and temperature (F value = 8.36, P = 0.0042). Specifically, survival was near 100% for mosquitoes of all species at 20 ºC and 30 ºC, however survival for *A. mediovittatus* was significantly lower at 40 ºC compared to that of other species (Fig. 4).

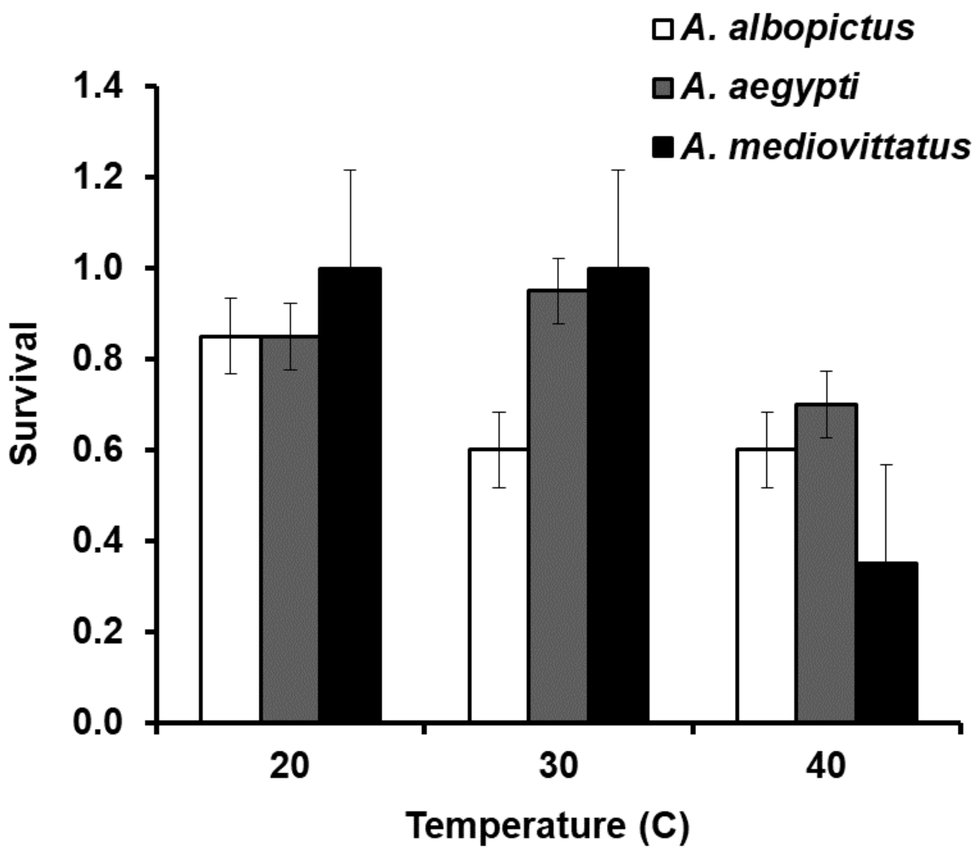

Figure 4. Mean +/- standard error of survival across different temperatures for different species of *Aedes* mosquitoes.

Experiment 2. Egg Desiccation at Different Levels of Humidity. This phase of the experiment was performed to assess what the hatching success of the three species of mosquitoes was at different levels of humidity. There was a significant difference in egg desiccation across species ($\chi^2 = 25.89$, $P < 0.001$), but no significant difference across the different percentages of humidity ($\chi^2 = 2.093$, $P = 0.148$); however there was a significant interaction between species and humidity ($\chi^2 = 35.83$, $P < 0.001$). At 25% humidity, *A. aegypti* and *A. albopictus* hatched at nearly equivalent rates of approximately 50%, whereas *A. mediovitattus* hatched at a significantly higher rate of approximately 75%. At 50% humidity, all three species hatched at a rate of approximately 50%, showing an intermediate range of hatching among the humidity levels for the three species. At 80% humidity, none of the *A. mediovittatus* eggs hatched, while *A. aegypti* and *A. albopictus* hatched at their highest rates of approximately 60% and 75%, respectively. The failure of eggs of *A. mediovittatus* to hatch at high humidity may have been due to algae that had affected that sample. Note that data could not be collected during the second time period (4 weeks) due to the lockdown of the campus for coronavirus precautions.

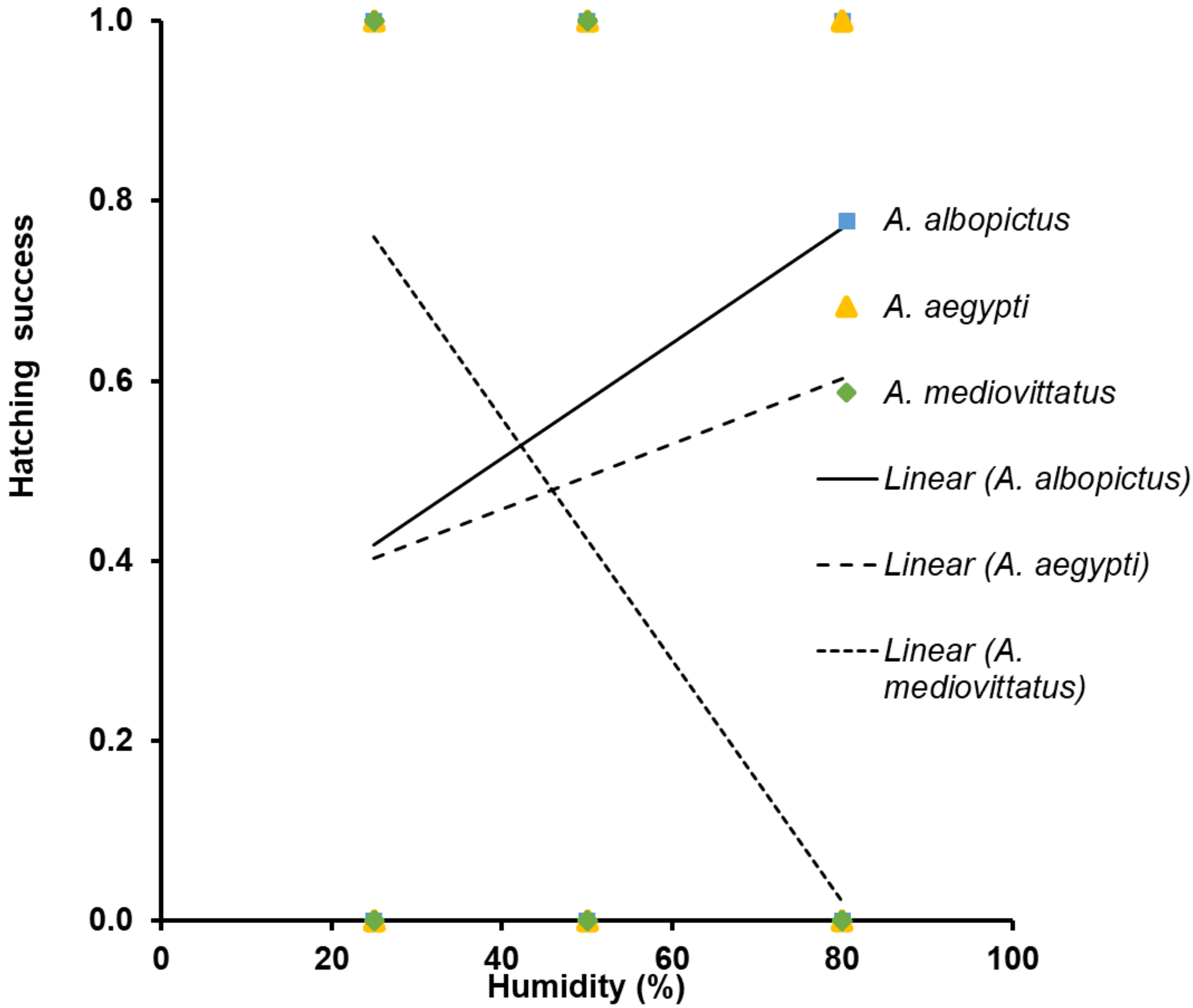

Fig. 5. Egg survival of species of *Aedes* across different degrees of humidity based on logistic regression.

Discussion

This study was performed to compare the egg hatching and temperature responses of *A. albopictus, A. aegypti*, and *A. mediovittatus*.

With the first phase of the experiment, it was hypothesized that there would be variable growth, variable size, and variable development times among the three species under different temperatures. This was supported by the experiment, as the growth and development times varied due to temperature effects. In general, temperature variation induced mosquitoes to grow faster at the highest temperature and slower at the lowest temperature. Reinhold et al. (2018) found that *A. albopictus* and *A. aegypti* could survive and grow at temperatures lower than 20 ºC. In this experiment, both species grew more slowly at 20 ºC than at higher temperatures but still went through their full development cycle. This indicates that colder temperatures slow development for mosquitoes, at least in the *Aedes* genus.

With the second phase of the experiment, I hypothesized that the eggs of the three species of *Aedes* would survive at different rates, with the highest humidity favoring hatching rate of the three species and *Aedes aegypti* hatching more in the lowest humidity. *Aedes albopictus* had the highest hatching success under 80% humidity. However, the eggs of all three species desiccated at different rates, with *A. mediovittatus* eggs having 0% hatching success under the highest humidity. *Aedes albopictus* had the highest number of eggs hatch at 80% humidity, which had been observed in previous tests showing that egg mortality in this species was lowest in wetter environments (Urbanski et al., 2010). Under drier conditions, *A. aegypti* had lower egg mortality compared to *A. albopictus*. It is still unknown what humidity promotes *A. mediovittatus* egg survival as the algae that were present in the vial in 80% humidity may have kept the eggs from hatching.

Costanzo et al. (2005) performed an experiment of condition-specific competition in *A. albopictus* and *A. aegypti* at adult, larval, and egg stages during treatments of drying and fluctuating amounts of water. Costanzo et al. (2005) found that *A. albopictus* had lower egg mortality in wetter environments compared to *A. aegypti*, and *A. aegypti* had lower egg mortality in drier environments compared to *A. albopictus*. The current experiment was similar in that *A. albopictus* and *A. aegypti* did have similar egg mortality rates dependent on the differing levels of humidity. Juliano et al. (2002) found that *A. albopictus* had higher egg mortality in drier environments, compared to *A. aegypti,* and that *A. albopictus* had a higher difference between egg mortalities in dry and wet environments. The current experiment found that a large difference between egg mortalities in dry and wet environments did exist for *A. albopictus*, but *A. albopictus* and *A. aegypti* had similar egg mortality rates across all humidity levels.

New studies could be performed with this same methodology, as part of the humidity experiment was not finished due to the outbreak of COVID-19, causing the campus to be locked down. The longer experiment would have given additional periods to collect egg desiccation data among all three species and may have produced more differences in the data for desiccation. The *A. mediovittatus* trial in 80% humidity had been contaminated by what appeared to be a form of algae. This contamination could be prevented in future tests by using more replicates so that other vials may not be contaminated or by more carefully handing egg papers before placing in envelopes.

In conclusion, the results suggest that temperature is an important factor in affecting development and growth in mosquitoes. Greater variation in temperatures could be used to have a better understanding of larval growth and development at a wider range of temperatures. This experiment can also be used to predict how mosquitoes grow and develop in their native habitats.

The mean temperature of San Juan, Puerto Rico, where only *A. aegypti* and *A. mediovittatus* are found, is 26 ºC, but these species grow faster at higher temperatures. The mean temperature of the Florida Keys, where *A. aegypti* and *A. albopictus* coexist, is 25 ºC, but they grow faster at higher temperatures and develop much more slowly at lower temperatures. If *A. albopictus*, an invasive species, were to arrive in Puerto Rico, it would interact with *A. aegypti* and *A. mediovittatus* in local containers, and the relatively warm temperatures in Puerto Rico and high humidity (mean humidity is 76.5% in San Juan) could cause fast development times and low egg mortality for the newly introduced species. Those factors could result in *A. albopictus* outcompeting the other two species of *Aedes* in the absence of strong competition in local containers.

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