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Heat Stress Effects on Culex pipiens Mosquitoes

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Abstract

Extreme weather events such as heat waves are becoming more frequent. This is a concern for both humans and other organisms like insects. *Culex pipiens f. molestus* mosquitoes are primary vectors of diseases including equine encephalitis, West Nile virus, Rift valley fever, and St. Louis encephalitis. With *Cx. pipiens f. molestus* being the primary vector of West Nile virus in North America, it is important to understand how more frequent heat waves affect their population dynamics. In this study, I compared the thermal tolerance of larval *Cx. pipiens f. molestus* that were exposed to prior heat stress to those that were not. I also compared the wing size of adult *Cx. pipiens f. molestus* when reared in three temperatures, low (16 °C), medium (22 °C), and high (28 °C). No significant differences were found in the survival between individuals that went through the simulated prior heat wave and those that did not. However, significant results showed an inverse relationship between temperature and wing size. These results suggest potential population growth issues within the *Cx. pipiens* due to smaller wing size signaling lower fecundity.

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Heat Stress Effects on *Culex pipiens* Mosquitoes

by

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Introduction

Recent climate change is attributable to an increase of Earth's global average temperature. For example, between 2002 and 2011, the average global surface temperature increased 0.77 ºC-0.80 ºC compared to the pre-industrial mean (Rocha et al., 2017). It is predicted that the average global temperature will continue increasing, and be 1.1 - 6.4 ºC warmer than current temperatures by the year 2100 (Rocha et al., 2017, Vohees et al., 2012). Models have been used to better understand the effects of climate change, predict global warming temperatures, and improve our understanding of extreme weather events (Rocha et al., 2017). Such research has shown global warming is likely causing longer and more frequent heat waves (Lassandro and Turi, 2019, Schliep et al., 2019, Bezirtzoglou, 2011). Such heat waves have been observed over the world including in China from 2011-2014, in Russia in 2010, in Europe in 2003, and many places in North America such as the midwest United States in 1995, New York in 2003, California 2006, central United States in 2011 and again in 2012, and various places in both the United States and Canada in 2018, (Orlando, et al., 2021, Dong, et al., 2023). These extreme heat waves raise concerns for human health and economic security. They may also have important ecological consequences such as changes in population density (Schliep, et al., 2019, Orlando, et al., 2021).

Heat waves can affect ectotherms both behaviorally and developmentally, as well as affect their survival (Rocha, et al., 2017). For example, Asian corn borers (*Ostrinia furnacalis*) that experienced a simulated heat wave had decreased survival and lower rates of egg hatching (Mason, et al., 2022). They also experienced an increase in developmental time at extreme high temperatures. Rocha et al. describe the effects of heat waves on the pine processionary moth (*Thaumetopoea pityocampa*) focusing on the effect on egg survival of three phenologies: one that lays its eggs in the summer, another that lays its eggs during the spring, and the final that lay its eggs in the fall. After a simulated three-day heat wave using both constant heat treatments and temperatures ranging from 30 °C to 42 °C, they found that at the highest experimental temperature, none of the embryos survived. They also found that the developmental time of each embryo increased with the increase in temperature (Rocha, et al., 2017). In bumblebees, it has been found that when exposed to a heat wave (32 °C), their cognitive ability to form an association with a colored light and a reward was hindered. This could cause issues with bumblebees remembering and differentiating between flowers that are a good nectar source and those that are not. This could also prevent bumblebees from remembering landmarks to make their way back to the colony (Gérard, et al., 2022).

Other studies have been conducted exposing insects to extreme thermal environments, both in cold temperatures (Rako & Hoffmann, 2006) and hot temperatures (Krebs and Volker, 1996; Stazion, et al., 2019). Resistance to changes in environmental temperature in insects is an important topic with regards to climate change and the increase in temperatures. Researchers Stazione, et al., conducted an experiment in which they investigated induced thermal stress and its effect on mating success, specifically looking at the differences between *Drosophila melanogaster* placed under thermal stress with and without heat hardening (exposure to sub-lethal thermal stress at the beginning of a species' life). They found, due to a thermal resistance trait found in some *D. melanogaster*, when exposed to warmer temperatures, *D. melanogaster* respond more successfully when they have received heat-hardening pre-treatment; this concept can be applied to insects to predict their responses to climate change. This information can also be used to begin research for insect control during warmer conditions (Stazion, et al., 2019). Other studies showed an increase in physiological functions such as cardiac performance of *D. melanogaster* (Rodriguez, et al., 2021). Rodríguez and team found that under sub-lethal levels of heat stress, the flies with a heat resistant gene had hearts that performed better (Rodriguez, et al., 2021). Heat stress plays an important role in many insects.

There is scientific interest in mosquitoes and how they are affected by incidence of heat waves. Mosquitoes are responsible for the transmission of pathogens that have led to many human deaths. Understanding and predicting the density of mosquitoes is crucial to understand the spread of diseases such as Zika, malaria, and dengue (Moustaid and Johnson, 2019). *Culex pipiens*, the northern house mosquito, transmits several diseases such as equine encephalitis, West Nile virus, Rift valley fever, and St. Louis encephalitis, making it an important disease vector (Azarm, et al., 2021). These arboviruses are transmitted through the bite of infected mosquitoes. In the United States, West Nile virus is the largest mosquito-borne illness (Holcomb, 2023). While about 80% of people infected with West Nile virus are asymptomatic, 20% of infected people develop West Nile fever which can cause fever, headache, fatigue, nausea, vomiting, swollen lymph nodes, body aches, and a skin rash (WHO, 2017). Less than 1% of people infected with West Nile virus result in West Nile neuroinvasive disease which can cause encephalitis, meningitis, and/or acute flaccid paralysis (Holcomb, 2023). *Cx. pipiens* feed on infected birds and pass on the virus through their salivary glands during their next blood meal, which could be humans or other animals (WHO, 2017).

Cx. pipiens is geographically widespread. They are mainly found in temperate rural areas in North America, South America, Europe, Asia, Australia, and Africa (Arich, et al., 2022, Azarm, et al., 2021). There are many different subspecies and forms of *Cx. pipiens*. They utilize a wide variety of aquatic habitats for laying eggs. These habitats could be above ground such as any stagnant water outside human habitation or subterranean such as using a stormwater drainage system (Arich, et al., 2022, Azarm, et al., 2021, Mackay, et al., 2021). Some subspecies and forms also vary in meals. While many subspecies and forms require a blood meal to be able to lay eggs, there are some forms, such as the *molestus* form, where the females are able to lay their first eggs without a blood meal. After this first meal, the *molestus* form's primary blood meal is humans (Arich, et al., 2022). Blood feeding in female mosquitoes, as well as dispersal and mating, are impacted by variation in body size, all of which impact survival and fitness. Shin et al., states that female mosquitoes that are larger tend to have greater fecundity and a greater life span (Shin, et al., 2012). However, little is understood about how heat waves influence the survival and the development of *Cx. pipiens* which in turn will affect their population dynamics. This study is intended to test for survival of *Cx. pipiens f. molestus* during multiple, simulated heat waves and to determine any differences in morphological development due to that heat stress. I designed an experiment to determine if exposing *Cx. pipiens* to a heat wave early in the larval stage will impact later larval survival when exposed to a second heat wave. I also examined the influences of temperature on adult wing length. My hypothesis was that *Cx. pipiens* mosquitoes that are exposed to simulated heat waves at the larval stage will lead to higher survival when exposed to a subsequent heat wave. Additionally, adult mosquitoes will be smaller due to more rapid development when exposed to high temperatures during the larval stage.

Materials and Methods

Early Heat Wave Simulation

I designed this experiment using *Cx. pipiens* f. *molestus* mosquitoes. I first collected eggs, by placing two oviposition trays filled with 400 mL of dechlorinated (DI) water in two different colony maintenance cages for 48 hours. Using a wooden chopstick, I removed and isolated ten egg rafts and placed them in ten different 100 mL glass jars with 50 mL of DI water. I then placed these in a TriTech™ Research Digitherm® 38 1 Heating/Cooling Incubator at a temperature of 23° C for 24 hours to hatch. Twenty-four hours after hatching, I isolated 20 larvae from each family, then separated these into two groups (‘control’ & ‘experimental’). Each group consisted of 10 larvae each. Both groups for each family were placed in separate 100 mL glass jars with 50 mL of DI water and labeled by family and group. I created a food solution using a mixture of 2.00 g of crushed fish flakes suspended in 200 mL of DI water. Each jar was given 2000 µL of this food solution. I then placed both groups of each family in the control incubator with a temperature of 23 °C for 48 hours.

After 48 hours passed, I fed the larvae a third time using the same amount of food solution as before. I then placed the control groups into the control incubator and placed the experimental groups into an experimental incubator set for 35 °C, to simulate a high temperature environment. The experimental groups remained in the experimental incubator for 48 hours. Following this 48-hour period, both groups were taken out of their incubators, fed, and all groups were placed into the control incubator for a final 48 hours.

Thermal Assay

To test potential induction of increased thermal tolerance in these, I used an acute thermal assay. After the final 48-hour period (see above), I took out all the jars from the incubators and placed each individual larva in a separate 15 mL Falcon tube with 2 mL of DI water. I labeled each tube by family, group, and thermal assay group (i.e. 15 m, 30 m, 45 m, or 60 m). The thermal assay groups were assigned sequentially per family and group. I transferred all the

Falcon tubes to a controlled 28 °C water bath for 30 minutes. After 30 minutes, I transferred all tubes to the experimental 38 °C water bath. The tubes were removed at 15 minute intervals depending on their thermal assay group and placed back into the control water bath. Once I removed the final group and placed them in the control water bath, all larvae remained there for an additional 30 minutes. Finally, I placed all Falcon tubes into the control incubator for 24 hours before examining for survival. Survival was assayed by gently tapping the bottom of the tube 4-6 times and noting whether the larva displayed directed movements (i.e. swam).

Morphological Development

I also wanted to examine the effect of thermal stress on mosquito development. To do this, *Cx. pipiens* f. *molestus* larvae from 11 different families were isolated using the same methods as described above. The larvae in each family were divided into three groups, and placed in incubators set to 16 °C, 22 °C, or 28 °C respectively. These larvae were allowed to develop to adulthood at which time they were killed at -4 °C. At a later time, one wing from each adult mosquito was measured under a Nikon stereoscopic microscope (model C-PS) at 40× magnification using a metric miniscale to the nearest 0.1 mm.

Analytical Methods

I analyzed the experimental trial survivor data using the Chi Square Goodness of Fit test. To do this, I ran the analysis using all four thermal assay groups to determine if there was a difference between the survivorship of the observed versus expected data. The expected value was found by multiplying the total number of individuals in the experimental group by the percent survivors calculated from the control group. My null hypothesis was that there would be no significant difference between the observed surviving mosquitoes and the expected surviving mosquitoes in each thermal assay group. Using the control and experimental data of each thermal assay group separately, I also used the Chi Square Test of Independence to determine if the control and experimental groups were dependent on each other. My null expectation for this analysis was there would be no relationship between the survival of the mosquitoes who experienced a simulated heat wave as a larva and the control mosquitoes who did not experience a simulated heat wave throughout larval development. To control for the family variable between the control and experimental treatments, I additionally calculated the average mortality rate for both treatments and conducted separate paired t-test analyses on all four thermal assay groups. My null expectation for this analysis would be that there would be no significant difference between the average mortality rate of either treatment in all four groups. Finally, I visualized the wing length and temperature data by creating a box and whisker plot showing the average wing length for each temperature group. I analyzed these data using a linear mixed model with temperature and wing length as the fixed effects and family as a random effect.

Results

Heat Stress and Survival

The Chi Square Goodness of Fit test was used to determine whether the actual survival of mosquitoes at the end of each thermal assay trail differed from the expected survival of mosquitoes. No significant difference was shown between the observed and expected survival (χ^2 = 3, N=52, 2.953, P = 0.399). The Chi Square Test of Independence was used to look for a relationship between the control and experimental groups in each thermal assay trial. This statistical analysis was calculated with the expectation that there would be no significant difference between the survival of the control mosquitoes and the experimental mosquitoes. In each thermal assay trial, no significant difference was shown between the control and experimental groups on 15 m (χ^2 = 3, N=52, 0.843, p = 0.8392), 30 m (χ^2 = 3, N=52, 0.371, p = 0.9462), 45 m (χ^2 = 3, N=52, 3.295, p = 0.3483) and 60 m (χ^2 = 3, N=52, 1.413, p = 0.7026). A paired t-test analysis allowed for the comparison between the average mortality of the control and experimental groups in each thermal assay trial while taking into account the genetic relatedness between the two groups. In each thermal assay trial, there was no significant difference between the average mortality rate of either treatment in all four groups 15 m (t_{25} = 0.19866, p = 0.8441), 30 m (t_{25} = 0.27753, p = 0.7837), 45 m (t_{25} = 1.5875, p = 0.125), and 60 m (t_{25} = 1.305, p = 0.2038).

Table 1. Average survival rates and standard deviation for control and experimental groups at all 4 thermal assay time points.

	Cont. (15min)	Exp. (15min)	Cont. (30min)	Exp. (30min)	Cont. (45min)	Exp. (45min)	Cont. (60min)	Exp. (60min)
Avg.	0.88	0.89	0.89	0.87	0.81	0.62	0.72	0.87
SD	0.27	0.24	0.21	0.30	0.35	0.48	0.38	0.33

Temperature and Wing Size

Using a box and whisker plot, 11 families were used to compare the wing size when exposed to 16°C (low), 22°C (med), and 28°C (high) (Figure 1). The average wing size for low, med, and high were 4.26 mm, 3.88 mm, and 3.36 mm respectively. The linear mixed model analysis was used to determine the relationship between temperature (independent variable) and wing size (dependent variable) with family as a random effect. This analysis was conducted with the expectation that there would be a relationship between temperature and wing size. Significant results were found when comparing temperature and wing size ($p < 0.0001$). Covariance parameter estimates were family = 0.02 and residual = 0.09.

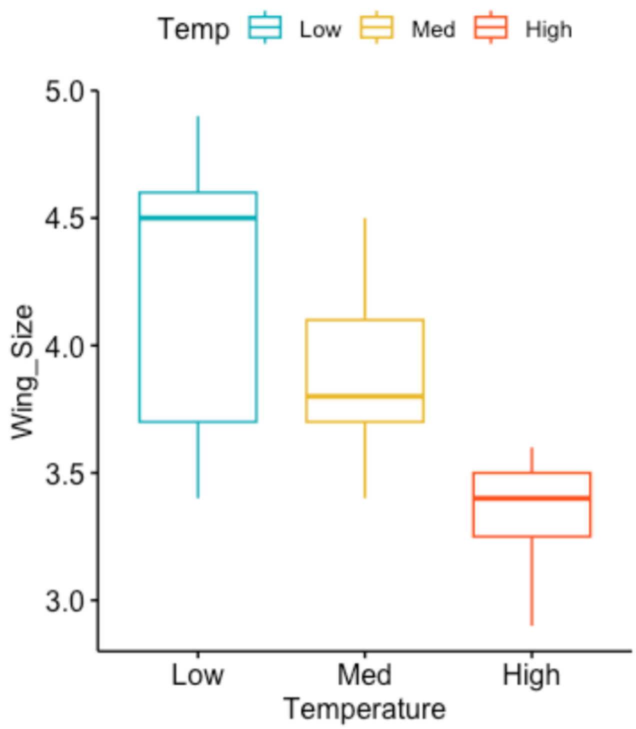

Figure 1. Developmental Temperature and Wing Size. Wing size of individual mosquitoes when exposed to 16°C (Low), 22°C (Med), and 28°C (High) during larval development.

Discussion

This study was designed to understand the effects of heat stress of *Cx. pipiens* mosquitoes during the larval stage and how that would affect survival and morphological development as an adult. The expectation for the first experiment was that earlier exposure to heat stress would prime mosquitoes to better withstand a subsequent heat stress. The results for the first experiment showed there were no significant differences between individuals that went through the simulated prior heat wave and the control individuals indicating the prior heat stress had no effect, positive or negative, on survival. These inconclusive results can be attributed to exposure to heat stress being insufficient to induce prolonged increase in tolerance to heat stress. A follow-up study should be conducted following a similar procedure comparing individuals exposed to prior heat stress to those not exposed. In this experiment, individuals exposed to prior heat stress should be exposed for longer periods of time, anywhere from a week to maturation. Mosquito thermal tolerance could also be assayed during the adult stage. This study would help clarify if prior heat stress during the larval stage has any effect on survival during a heat stress in adulthood.

The wing length experiment showed a significant relationship between rearing temperature and wing size. These results suggest temperature has a negative effect on wing development in *Cx. pipiens* mosquitoes. This inverse relationship was expected as this supports the common phenomenon, the temperature-size rule, observed in insects where developmental temperature affects body size (Machida, et al., 2022). In *D. melanogaster*, this phenomenon can be observed where high temperatures also result in smaller mass and wing size. This can be attributed to high temperatures signaling metamorphosis which causes an end to larval growth (Machida, et al., 2022). Furthermore, these results also indicate potential population growth issues within the *Cx. pipiens*. The size of *Culex* females has been found to have a direct correlation with fecundity where larger females were observed to lay higher amounts of eggs (Epstein, et al., 2021). Populations of females experiencing prolonged heat stress during larval development risk having smaller population sizes.

Understanding the effect heat stress has on *Cx. pipiens* is helpful in understanding population size. The results of this study indicate exposure to higher temperatures will produce individuals with smaller wing size which in turn could lead to lower eggs laid per year potentially decreasing population size. Conversely, studies done on *Aedes aegypti* (*Ae. aegypti*) in Brazil show that as temperature increases, developmental time increases as well (Marinho et al., 2015). Increased developmental time allows for quicker maturation of mosquitoes which in turn could lead to more generations per year providing an increase in population size. More research is needed to better understand how rising temperatures and increases in longer and more frequent heat waves will affect *Cx. pipiens*.

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