Temperature drives transmission of mosquito-borne pathogens:
improving entomological estimates for *Aedes aegypti*-borne virus transmission risk

By

OLIVIA CHASE WINOKUR
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Approved:

Christopher M. Barker, Chair

Geoffrey Attardo

Lark Coffey

Committee in Charge

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ABSTRACT

The mosquito *Aedes aegypti* is the primary vector of many viruses that cause a major burden on human health worldwide, including dengue, Zika, yellow fever, and chikungunya viruses. Currently, a widely adopted vaccine is available only for yellow fever virus, and thus mitigating the burden of diseases caused by these viruses predominantly relies on avoiding mosquito bites and controlling mosquito populations. Mosquito-borne virus transmission risk models can help mosquito control decision-makers efficiently use limited resources and reduce the use of chemical insecticides that can lead to resistance and therefore less effective control. As the Zika virus (ZIKV) pandemic emerged in 2016, estimates for ZIKV transmission risk were based on proxy evidence from closely related dengue virus. To improve risk estimates, we first studied how temperature affects ZIKV extrinsic incubation period (EIP). We concluded that, in agreement with findings for other mosquito-borne viruses, ZIKV EIP decreased as temperature increased and ZIKV EIP was relatively shorter than for dengue virus across temperatures. We then sought to further improve ZIKV risk estimates by studying thermal preferences of *Ae. aegypti* mosquitoes in the laboratory and in the field. Current mosquito-borne pathogen risk models primarily use temperatures from weather stations or thermal imagery as a proxy for the temperatures mosquitoes experience; however, such approaches do not account for the range of local environments or microclimates available to adult mosquitoes nor the microhabitats mosquitoes select and therefore may lead to an inaccurate estimate of risk. In the lab, *Ae. aegypti* generally preferred the coldest temperatures available (<20°C) and avoided the hottest temperatures (>31°C) on a gradient ranging from 17.5°C to 36.5°C. However, mosquitoes reared at cooler temperatures (22°C) were larger and rested at warmer temperatures compared to mosquitoes reared at warm temperatures (26°C and 30°C). In the field, female *Ae. aegypti* were

found resting at temperatures that were increasingly cooler than ambient as ambient air temperature increased. Accordingly bias in air-temperature-based models of Zika virus transmission risk is expected to be greatest at the hottest temperatures, and overall, accounting for *Ae. aegypti* thermal preferences yielded lower estimates for Zika virus transmission risk compared to models based on air temperatures alone. Taken together, the results of these studies can be used to improve prediction of mosquito-borne pathogen risk and inform mosquito control decisions.

INTRODUCTION

Mosquito-borne pathogens cause major burden on human health worldwide. Control of mosquito-borne pathogens relies on mosquito control as (1) vaccines and therapeutics are not widely available and (2) there are and will be emerging and re-emerging pathogens for which therapeutics and vaccines are not available and development of therapeutics and vaccines is an expensive and lengthy process. A prime example of the need for mosquito control to mitigate an emerging pathogen is the 2015-16 Zika virus (ZIKV) pandemic. ZIKV is a flavivirus first isolated in 1947 from a sentinel rhesus macaque in the Zika Forest of Uganda [1] that caused occasional human cases and mild disease in Africa and Asia. The first isolated human disease outbreaks occurred in 2007 and 2013 in the South Pacific [2–4], and beginning in 2015, ZIKV spread to susceptible populations in the Americas and quickly became a pandemic that spread to 48 countries in North and South America in 2016, including local transmission in the continental United States in Florida and Texas [5–9]. This was the first time that severe disease outcomes including microcephaly and Guillain-Barré syndrome were linked to ZIKV infection, and consequently therapeutics, vaccines, and quality diagnostic tools were not available at the time [10,11]. There was an urgent need to develop diagnostics and therapeutics, but also an immediate need to suppress ZIKV transmission through mosquito control.

The ability to anticipate areas of likely spread of ZIKV and to implement effective mosquito control to mitigate pathogen transmission risk relies on mosquito and pathogen surveillance and accurate predictive risk models to effectively target mosquito control. Mosquito control is economically costly, and excessive chemical control can lead to insecticide resistance that limits future efficacy. To make evidence-based mosquito control decisions to allocate

limited resources and limit insecticide resistance, it is imperative to be able to accurately predict transmission dynamics of mosquito-borne pathogens.

***Aedes aegypti*-borne viruses**

The mosquito *Aedes aegypti* is the primary vector of a range of viruses that cause a major burden on human health worldwide, including dengue virus (DENV), Zika virus (ZIKV), chikungunya virus (CHIKV), and yellow fever virus (YFV) [12–15]. DENV infects an estimated 400 million people annually [16,17] and can cause a range of clinical manifestations from subclinical asymptomatic cases to severe febrile disease, and less commonly to severe dengue and fatal dengue shock syndrome [16,17]. ZIKV emerged as a global health threat in 2015 and started a pandemic in 2016. The majority of ZIKV infections are asymptomatic or manifest with mild febrile disease including headache and general malaise, however infection can cause congenital Zika syndrome in fetuses and neonates and Guillain-Barré syndrome in adults. Congenital Zika syndrome can lead to spontaneous abortion and stillbirth and comprises a range of birth defects and disabilities including severe microcephaly, decreased brain tissue and brain damage, damage to the back of the eye, congenital contractures, and hypertonia, which restricts body movements shortly after birth [18,19]). Guillain-Barré syndrome causes the immune system to attack the nervous system and causes muscle weakness and sometimes paralysis, which can lead to life altering disabilities [20]. CHIKV has spread rapidly since 2004 and outbreaks have continued to occur worldwide in recent decades. CHIKV infection is usually mild, but can cause fever, often accompanied by joint pain. Joint pain is often debilitating and can lead to arthritis for days, weeks, months, and even years [21]. YFV is often clinically mild or asymptomatic but can cause severe febrile disease, and a small percentage of infections lead to jaundice, dark urine, abdominal pain with vomiting, and bleeding from the mouth, nose, eyes or

stomach; half of infections that reach this phase cause death within 10 days [15]. Of the four viruses primarily transmitted by *Ae. aegypti,* YFV is the only one for which a widely adopted and FDA-approved vaccine is available.

***Urban Aedes aegypti* ecology and geographic range**

Ae. aegypti mosquitoes are particularly efficient vectors of human pathogens, as they have evolved alongside humans for approximately 500 years [22,23], feed primarily on humans [24–27], feed during daylight hours when humans are active, and can feed more than once per gonotrophic cycle [28,29], which leads to more potential infectious bites.

Ae. aegypti are container-breeding mosquitoes; females lay their eggs in natural and artificial containers in and near human habitation (flower pots, tires, buckets, dog bowls, etc.) [30–34]. Females use skip oviposition, meaning they oviposit eggs from the same gonotrophic cycle in multiple containers [35–37]. The eggs are desiccation-resistant, and they can survive in containers without water for months, then hatch when the containers are flooded with water [38–40]. This means the eggs can survive for extended periods through unfavorable seasons or in containers that are moved to new locations. While adult flight ranges are short [41,42], eggs are easily transported on dry substrate or in dry containers such as potted plants, which increases the potential for establishment in non-native habitats.

The range of *Ae. aegypti* mosquitoes and the burden of disease has increased in the last few decades and continues to expand as a consequence of climate change leading to increased suitable habitat, and increased global trade and travel [16,43–45]. *Ae. aegypti* originated in the tropics, however they have able to adapt to environments with wide variation in temperature and humidity, such as in the Mediterranean climate of coastal and central California or the more arid deserts of southeastern California and Arizona. *Ae. aegypti* was first detected in California in

2013 and has since been identified in 23 countries and more than 330 cities or census designated places [46].

Estimating transmission dynamics of mosquito-borne viruses

Pathogen transmission dynamics can be estimated using mathematical models that can inform control decisions. Vectorial capacity is an entomological metric that estimates intensity of pathogen transmission as the number of infectious mosquito bites that would arise from the mosquitoes that bite an infectious person daily [47]. Vectorial capacity, *C*, is expressed as:

$$C = \frac{ma^2bp^n}{-\ln(p)}$$

where *m* is the mosquito density in relation to the host; *a* is the biting rate, which is raised to the second power because a mosquito needs to bite twice to transmit a pathogen; *b* is vector competence, or probability that a mosquito will be able to transmit the pathogen after ingesting an infectious blood meal; *p* is the probability of daily survival of the mosquito; *n* is the extrinsic incubation period (EIP), or the number of days it takes from pathogen ingestion to transmission in the mosquito; and 1/-ln(*p*) is the expected lifespan of the vector.

Vectorial capacity represents the entomological components of R_0, the basic reproductive number that quantifies the potential of an infectious pathogen to spread in a completely susceptible population [48]. R_0 is expressed as:

$$R_0 = \frac{ma^2bcp^n}{-\ln(p)r}$$

Where *c* is host competence and *r* is recovery rate of hosts (1/infectious period).

Effect of temperature on mosquito-borne pathogen transmission

Temperature drives mosquito-borne pathogen transmission via its nonlinear effects on the parameters of vectorial capacity, promoting transmission at some optimal temperature range, and suppressing it above and below [49–52]. Temperature alters mosquito population dynamics and by altering mosquito development rates, larval and adult survival, and fecundity [53–56]. Further, temperature affects pathogen transmission specifically by altering mosquito biting rates [57,58] and pathogen extrinsic incubation periods [59–63].

Current pathogen transmission models primarily use temperature data from weather stations or remotely sensed data as a proxy for the temperatures mosquitoes experience [50,64–68]. However, temperatures from these sources have been shown to be an inadequate representation of the temperatures of local environments or microclimates available to adult mosquitoes, especially in subtropical and temperate environments [69,70]. A key knowledge gap concerns microhabitat availability and mosquito thermal preferences and how these alter transmission risk estimates [50].

Behavioral thermoregulation in ectotherms

Behavioral thermoregulation, also called thermal preference or thermotaxis, is the primary method that ectotherms use to avoid temperature extremes and remain at physiologically suitable ambient temperatures. Behavioral thermoregulation has been documented widely in several ectotherms, including flies in the *Drosophila* genera [71,72], butterflies[73], nematodes [74], and lizards [75]. A small number of studies have examined mosquito behavioral thermoregulation in the lab, and have concluded that mosquitoes generally avoid extreme hot

temperatures when cooler or more mild temperatures are available. This dissertation includes the first study on *Ae. aegypti* thermal preferences in the field, and the first study examining how incorporating thermal preferences alters transmission risk compared to a model that uses ambient temperature as a proxy for the temperatures mosquitoes experience.

Dissertation outline:

When ZIKV emerged as a pathogen of global concern in 2015-2016, estimates for DENV EIP from previous studies were used to understand transmission dynamics of ZIKV. However, more precise estimates of ZIKV EIP were needed. For chapter 1, we conducted a laboratory study to test the hypotheses that ZIKV EIP is temperature-dependent and follows a similar trend to the EIPs of other mosquito-borne flaviviruses with warmer temperature resulting in shorter EIP and is most similar to other flaviviruses transmitted by *Ae. aegypti.* To test these hypotheses, we presented *Ae. aegypti* mosquitoes from California to mice infected with a 2015 South American outbreak strain of ZIKV, held the mosquitoes in incubators at a range of temperatures, and tested rates of infection, dissemination, and transmission over time. We then fitted a logistic regression model to the data to get estimates of ZIKV EIP and compared these estimates to those of other arboviral EIPs.

At present, most pathogen transmission models use ambient temperature from weather stations as a proxy for the temperatures mosquitoes experience. However, mosquitoes are ectotherms, and weather station temperatures do not represent the varied microhabitats available in a given environment. Therefore, following chapter 1, we sought to further improve transmission risk estimates of *Ae. aegypti*-borne viruses by studying thermal preferences of *Ae. aegypti* in the lab and in the field in California's Central Valley.

In chapter 2, we conducted a laboratory study aimed to determine if *Ae. aegypti* mosquitoes exhibit thermal preferences toward a temperature range that optimizes their fitness. To test this hypothesis, we built an experimental arena consisting of an aluminum thermal gradient bar connecting two hot/cold plates with a Plexiglas enclosure on top. We released batches of mosquitoes in a gradient of field-relevant temperatures and tested the effects of rearing temperature, age, gonotrophic status, and laboratory colonization on thermal preference.

Finally, in chapter 3, we conducted a field study to characterize the microhabitats available in central California and assess the thermal preferences of *Ae. aegypti* in the field during the late-summer season of peak abundance. To do this, we placed thirty resting boxes spanning a variety of microhabitats in the backyards of homes for 6 weeks in Madera, California. Each box was equipped with an iButton temperature sensor and aspirated the boxes two times a day, once in the early morning before the morning activity peak, and once in the afternoon before the late afternoon activity peak. We then investigated how *Ae. aegypti* thermal preferences affect R_0 relative to a model that uses ambient temperature as a proxy for mosquito temperature.

Taken together, these studies can improve transmission risk models to better inform mosquito control decisions, especially as *Ae. aegypti* continues to expand into subtropical and temperate environments.

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CHAPTER 1:

Impact of temperature on the extrinsic incubation period of Zika virus in *Aedes aegypti*

Olivia C. Winokur, Bradley J. Main, Jay Nicholson, Christopher M. Barker

Abstract

Since Zika virus (ZIKV) emerged as a global human health threat, numerous studies have pointed to *Aedes aegypti* as the primary vector due to its high competence and propensity to feed on humans. The majority of vector competence studies have been conducted between 26–28°C, but arboviral extrinsic incubation periods (EIPs), and therefore transmission efficiency, are known to be affected strongly by temperature. To better understand the relationship between ZIKV EIPs and temperature, we evaluated the effect of adult mosquito exposure temperature on ZIKV infection, dissemination, and transmission in *Ae*. *aegypti* at four temperatures: 18°C, 21°C, 26°C, and 30°C. Mosquitoes were exposed to viremic mice infected with a 2015 Puerto Rican ZIKV strain, and engorged mosquitoes were sorted into the four temperatures with 80% RH and constant access to 10% sucrose. ZIKV infection, dissemination, and transmission rates were assessed via RT-qPCR from individual mosquito bodies, legs and wings, and saliva, respectively, at three to five time points per temperature from three to 31 days, based on expectations from other flavivirus EIPs. The median time from ZIKV ingestion to transmission (median EIP, EIP50) at each temperature was estimated by fitting a generalized linear mixed model for each temperature. EIP50 ranged from 5.1 days at 30°C to 24.2 days at 21°C. At 26°C, EIP50 was 9.6 days. At 18°C, only 15% transmitted by day 31 so EIP50 could not be estimated. This is among the first studies to characterize the effects of temperature on ZIKV EIP in *Ae*. *aegypti*, and the first to do so based on feeding of mosquitoes on a live, viremic host. This information is critical for modeling ZIKV transmission dynamics to understand geographic and seasonal limits of

ZIKV risk; it is especially relevant for determining risk in subtropical regions with established *Ae*. *aegypti* populations and relatively high rates of return travel from the tropics (e.g. California or Florida), as these regions typically experience cooler temperature ranges than tropical regions.

Introduction

Zika virus (ZIKV) is a primarily mosquito-borne flavivirus that was first isolated in 1947 from a sentinel rhesus macaque in the Zika Forest of Uganda[1]. The virus has since spread beyond Africa, causing human outbreaks in the South Pacific in 2007 and 2013 [2,3]. In 2015, local transmission of ZIKV was first detected in Brazil [4]. ZIKV subsequently spread through much of the Americas, where transmission was detected in 48 countries in North and South America in 2016, including in the continental United States in Florida and Texas (CDC, PAHO). Symptomatic Zika disease typically involves fever, muscle and joint pain, conjunctivitis, and rash. At least 20% of infections are symptomatic, although estimates vary by study and subpopulation [2,5]. As ZIKV spread throughout the Americas, more severe manifestations were noted, including Guillain-Barre syndrome and microcephaly in neonates [6–8].

Aedes aegypti is the primary vector of ZIKV globally [9–11]. At present, the majority of ZIKV vector competence work in *Ae*. *aegypti* has been conducted at temperatures between 26–28°C [11–16]. Only one study has assessed vector competence outside of this range [17], even though *Ae*. *aegypti* has well-established populations that extend into subtropical regions such as California and Florida. Further, it is known that the arboviral extrinsic incubation period (EIP), the time from ingestion of virus until a mosquito is able to transmit, is strongly affected by temperature [17–20]. Most mechanistic models for Zika virus transmission use estimated EIP from other related viruses, most notably dengue, as the effect of temperature on ZIKV EIP has not been characterized until recently [17].

In this study, we conducted an experiment to quantify the relationship between ZIKV EIP and temperature, with the expectation that ZIKV transmission would accelerate at warmer temperatures. We were then interested in contrasting the EIP-temperature relationship for ZIKV with that of other flaviviruses of tropical and temperate origins. Our laboratory experiments were conducted with *Ae*. *aegypti* at four constant temperatures spanning the relevant range to which *Ae*. *aegypti* mosquitoes are likely to be exposed in nature. These results will be broadly applicable as *Ae*. *aegypti* is the most important vector of ZIKV globally. Results can be compared to the limited work on ZIKV EIP and can be incorporated into statistical models in order to understand geographical and seasonal ZIKV disease burden [17]. These results are of particular importance to subtropical regions such as Mexico, Florida, California, and Texas, where *Ae*. *aegypti* populations are already established and travelers frequently arrive from tropical locations that experienced Zika outbreaks.

Materials and methods

Ethics statement

This study was carried out in strict accordance with the UC Davis Institutional Animal Care and Use Committee (IACUC) Protocol #19404 that was reviewed and approved on June 29, 2017. The UC Davis IACUC adheres to the Office of Laboratory Animal Welfare Health Research Extension Act of 1985 (Public Law 99–158) as well as the United State Department of Agriculture's Animal Welfare Act. UC Davis is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC) and has an Animal Welfare Assurance (number A3433-01) on file with the Office of Laboratory Animal Welfare (OLAW).

Mosquitoes, virus, and mice

Ae. aegypti mosquitoes colonized in 2016 from field-collected eggs in Clovis, California were used. The F4 generation used in this study was reared under standard, controlled conditions at 26°C, 80% RH, 12:12 L:D cycle with 200 larvae in one liter DI H20 and one pinch fish food (c.a. 0.5 g, Tetramin) every other day until pupation. Pupated mosquitoes were transferred into a 30x30x30 cm mesh cage (BugDorm, MegaView Science Co., Taiwan) to emerge. Upon emergence, adult mosquitoes had constant access to 10% sucrose until 24 hrs before experimental use when sucrose was removed.

An Asian-lineage outbreak strain of ZIKV from Puerto Rico was used. The virus was first isolated from human serum during the outbreak in 2015 (PR15, PRVABC59), passaged four times in Vero cells, and sequenced. The coding sequence for the complete genome was identical to GenBank accession number KX601168. PR15 was obtained from Aaron Brault at the U.S. Centers for Disease Control and Prevention in Fort Collins, Colorado.

Female interferon-deficient (IFN-α/βR−/−; C57BL/6) mice aged five weeks (B6.129S2-Ifnar1tm1Agt/Mmjax, The Jackson Laboratory) were used.

Mouse infections

Five five-week-old mice were inoculated with 105 Vero plaque forming units (PFU) of ZIKV PR15 via subcutaneous injection, and mosquitoes were allowed to feed on the anesthetized mice two days post-inoculation, at peak viremia [13,21] (Fig 1). Blood was collected immediately prior to the mosquito feed via each mouse's submandibular vein (i.e., cheek punch) and centrifuged for five minutes at 6.6 RPM to separate serum from whole blood and stored at -80°C. Blood serum from each mouse was thawed once for Vero cell plaque assay to determine ZIKV viremia. Briefly, cell monolayers were inoculated with 15–30 μL of undilute mouse serum from individual mice mixed with DMEM containing 2% (vol/vol) FBS, and 1% (vol/vol)

penicillin/streptomycin. After a one- hour incubation period to allow for viral attachment to cells, an overlay of 0.8% agarose/DMEM was added to cover the cells. Culture plates were incubated at 37°C in 5% CO2 for eight days. The cells were then fixed with 4% formaldehyde and stained with 0.05% crystal violet. Plaques were visualized as holes in the Vero cell monolayer and counted to determine PFU values. Two technical replicates were performed for each sample.

Mosquito infection, dissemination, and transmission

Mice were anesthetized prior to mosquito exposure with a ketamine (VETone Zetamine CIII, 75 mg/kg), xylazine (AnaSed, 10 mg/kg), and acepromazine (AceproJect, 1 mg/kg) solution administered intraperitoneally. Viremic mice were presented at once to >1,200 3–5 day old adult female Ae. aegypti in a one ft3 mesh cage (BugDorm) for 45–60 minutes (Fig 1). Engorged females were randomly sorted via vacuum aspiration into four half-gallon ice cream cartons with 175–190 mosquitoes per carton. Cartons were sorted into four reach-in environmental chambers (Binder KBF 115, Binder, Tuttlingen, Germany); or Darwin IN034, Darwin Chambers, St. Louis, Missouri, USA), each held at a different mean temperature (18°C, 21°C, 26°C, or 30°C), 70–80% relative humidity, and 12:12 hour light:dark cycle. Temperature and humidity in the chambers were measured using HOBO UX100 data loggers (Onset Computer Corporation, Bourne, MA); Binder chambers experienced small fluctuations of up to 2°C around the setpoint mean temperatures, while Darwin chambers held constant temperatures. All mosquitoes had constant access to 10% sucrose after blood-feeding for the remainder of the experiment. Following the infectious bloodmeals, at days chosen based on published extrinsic incubation studies of other flaviviruses [18–20], mosquitoes were cold-anesthetized in cohorts of 20 females at -20°C for five minutes and then legs and wings were removed with forceps while immobilized on ice. Saliva was collected by inserting the proboscis into a capillary tube

containing fetal bovine serum (FBS, GenClone, San Diego, CA, USA) for 20 minutes (Fig 1) [22]. Individual bodies, legs/wings, and the saliva sample from each mosquito were stored separately in 2-mL tubes containing 250 μL Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 1% penicillin/streptomycin and 10% FBS and either a 5-mm glass bead (bodies, legs/wings) or a 5-mm metal bead (saliva, Qiagen). All samples were stored at -80°C until further processing.

Mosquito tissues and glass capillary tubes containing saliva samples were thawed and homogenized in DMEM by shaking for two to four minutes at 30 shakes/second using a Tissuelyser (Qiagen, Hilden, Germany) and immediately centrifuged at 6.6 RPM for two to three minutes. Viral RNA was extracted using the MagMax Viral RNA Extraction Kit (ThermoFisher, Waltham, MA). A total of 50 μL of homogenate for mosquito tissue and 100 μL of saliva samples were extracted. All RNA extracts were eluted in 50 μL of elution buffer (Buffer EB, Qiagen) and stored at -80°C until further testing.

ZIKV viral RNA (vRNA) titers were determined for each body, legs/wings, and saliva sample using the Taqman Fast Virus 1-Step Master Mix (ThermoFisher) reverse transcription RT-qPCR kit with a previously described ZIKV-specific assay (primers: ZIKV 1086 and ZIKV 1162c, probe: ZIKV 1107-FAM [23]). Two technical replicates were processed for all samples. Samples for which least one technical replicate with a cycle threshold (Ct) value of 38 or below were considered positive for ZIKV vRNA. This limit of detection was determined from prior testing of serially diluted samples of known ZIKV vRNA concentrations with the same extraction and RT-qPCR reagents and protocols and equipment [24]. Ct values were converted to genome copies using standards of known concentration.

The infection rate at each temperature and time point combination was calculated as the number of mosquito bodies positive for ZIKV vRNA by RT-qPCR out of the total number of tested mosquitoes that ingested a bloodmeal. Dissemination and transmission rates were calculated similarly as the number of mosquito leg/wing samples and saliva samples that were vRNA-positive out of the total tested, respectively.

Statistical analyses

To characterize ZIKV vRNA transmission as a function of time and temperature, a logistic regression model was fitted. The outcome of interest was the proportion of mosquitoes that transmitted ZIKV vRNA, and temperature and days post feeding (dpf) were explanatory variables, with an interaction term included in the model. Temperature and dpf were centered at 26°C and 7dpf prior to model fitting. EIP50 was estimated from the fitted curves. To determine whether dissemination titer was predictive of ZIKV vRNA transmission for each temperature, we fitted a logistic regression model for proportion transmitting as a function of dissemination titer (log10(genomes)/tissue) with temperature as a categorical covariate. All analyses were done using R version 3.5 [25].

Results

Mouse viremias

ZIKV titers in the five mice ranged from 5.3–5.7 log10 PFU/mL ZIKV as determined via Vero cell plaque assay (individual mouse titers: 5.3, 5.5, 5.6, 5.6, 5.7 log10 PFU/mL). As further verification of the infectious dose, a single blood-fed mosquito collected immediately after feeding imbibed 5.9 log10 genomes ZIKV as determined by RT-qPCR.

Effect of temperature and time on Zika virus infection, dissemination, and transmission

In total, 320 blood-fed mosquitoes survived the duration of the study and were tested for infection, dissemination, and transmission of ZIKV vRNA. In a few samples, a high Ct value >38 or no Ct value was determined in one of two technical replicates where the other technical replicate was ≤38. These samples were considered positive and at the limit of detection by this measurement [19].

Infection, dissemination, and transmission rates increased over time for each temperature, except at 18°C where overall dissemination and transmission was low and inconsistent (Table 1, Fig 2). For all temperatures, the infection rate reached 100% at the first or second time point tested. Dissemination rates reached 100% at 21, 26, and 30°C at the second or third time-point tested. Dissemination rates at 18°C reached a maximum of 55%, which was observed at the last time point of this study (31 dpf). Transmission was detected at the first time point for each temperature: 10% transmitted at 25 dpf at 18°C, 5% transmitted at 10 dpf at 21°C, 30% transmitted at five dpf at 26°C, and 30% transmitted at three dpf at 30°C. Due to low and inconsistent transmission at 18°C, data from the 18°C treatment were omitted from the remainder of the analysis.

Transmission as a function of time and temperature

The combined effect of time and temperature, represented by the interaction term, was positively associated with the probability of transmission (β3 = 0.027, P = 0.002). The coefficients from the logistic regression model (Table 2) describe the cumulative probability of transmission over time for any given temperature, according to the formula:

$$ln\left(\frac{p}{1-p}\right) = -0.667 + 0.299D + 0.378T + 0.027D \times T$$

where p is the probability of transmission, and D and T are the centered time and temperature covariates, D = DPF-7 and T = Temperature-26, respectively.

The median time from ZIKV ingestion to transmission of vRNA (median EIP, EIP50) was derived from the fitted regression function for each temperature tested and ranged from 5.1 days at 30°C to 24.2 days at 21°C. At 26°C, EIP_{50} was 9.6 days (Fig 3).

Relationship between disseminated Zika virus titer and probability of transmission

At 21°C, there was no significant relationship between transmission and dissemination titer (log10(genomes)/tissue) ($P = 0.34$, Fig 4A, S1 Table). Dissemination titer was associated with increased probability of transmitting ZIKV vRNA at 26°C and 30°C (Fig 4A, S1 Table). The fitted relationship between transmission and dissemination titer was not significantly different between 26°C and 30°C ($P = 0.86$).

Because there was no significant difference observed between the 26 and 30°C curves, data were pooled for these temperatures, dissemination titer was binned to the nearest .5 log10(genomes), and a linear regression model was fitted (Fig 4B). The linear model fit the data well ($R^2 = 0.71$). Dissemination titer was a significant predictor of proportion transmitting, such that for each 10-fold increase in dissemination titer, proportion transmitting increases by 13% ($P = 0.008$, S2 Table).

Discussion

Understanding environmental effects on transmission efficiency of vector-borne pathogens over the seasonal and geographical ranges of their vectors is critical for understanding transmission dynamics and risk. As climate and land use change and invasive mosquitoes are able to establish populations in locations where previously not possible, it is increasingly important to understand this relationship in order to take appropriate measures to protect public health [26–28]. Warmer temperatures are known to shorten the extrinsic incubation of a wide range of mosquito-borne pathogens, but with one recent exception [17], this is not well-

characterized for ZIKV outside of the small range from 26–28°C. Other published studies aiming to estimate ZIKV risk have borrowed estimates for other flaviviruses, most notably closely related dengue virus, to mechanistically estimate risk [29,30]. In this study, we determined the effect of a range of constant temperatures on vector competence of Ae. aegypti from California for a ZIKV strain from Puerto Rico. We demonstrate that increasing temperature shortens EIP and we provide estimates of the combined effects of temperature and time post-feeding on ZIKV transmission potential. These parameters can inform mechanistic models to better understand ZIKV transmission dynamics. Further, we demonstrate that dissemination titer is highly correlated with probability of transmission at warm temperatures, but the relationship is less consistent at the cooler temperatures tested.

In line with results from other mosquito-borne flaviviruses, including dengue virus in Ae. aegypti and West Nile virus in Culex tarsalis, we demonstrate that warmer temperatures shorten the time from an infectious blood meal to transmission within the range tested [17–20,31]. This characterization is especially important where temperatures geographically and seasonally deviate from the 26–28°C range, such as in California and Florida. Infection was high at all temperatures, however dissemination was considerably lower at 18°C, and transmission was rare at 18°C when compared to the other temperatures tested (21°C, 26°C, 30°C). This limitation to midgut escape, dissemination, and overall transmission at 18°C could be due to the effects of low temperature on mosquito immunity [32,33], mosquito physiology[34], and/or viral structure and binding in the mosquito [34,35].

One potential limitation to this study is the use of RT-qPCR to quantify viral RNA. Though it is a common method among vector competence studies, RT-qPCR is more sensitive than other methods such as plaque assay because RT-qPCR quantifies viral RNA, all of which is

not necessarily derived from infectious viral particles and may include noninfectious virions. In this study, the use of RT-qPCR across temperatures and timepoints allows for consistent comparison of infection, dissemination, and transmission rates. It is possible that our results underestimate EIP50, but we expect any effect would be very slight because two recent studies in our laboratory showed that 71 and 75% of mosquito saliva samples that were positive for ZIKV and WNV, respectively, by RT-qPCR were confirmed positive for infectious virus by plaque assay [13,19].

Origins of the mosquitoes and viral strains used for vector competence and extrinsic incubation studies can affect results [14,16,36]. In this study, we provide estimates for median Zika virus extrinsic incubation periods at the temperatures tested as well as the logistic formula to calculate probability of transmission at any temperature. These estimates and model are solely the result of data from the interactions of one mosquito population and one viral strain assessed in one laboratory. Potential variation in these estimates will become more clearly resolved as more studies are conducted using different mosquito populations and viral strains.

The current standard for detecting transmission by individual mosquitoes is the capillary-tube method, which requires inserting the mosquito proboscis into a small capillary tube with media and waiting >20 minutes for the mosquito to expectorate into the tube [22]. This method is time and resource-consuming and the additional processing required is a key constraint on the potential sample size for vector competence studies. In this study, we determined that dissemination titer is associated with increased probability of transmission of ZIKV vRNA at 26°C and 30°C, but not at 21°C (Fig 4A, S1 Table). These results suggest that dissemination titer quantified by RT-qPCR in controlled laboratory experiments performed under standard, consistent rearing conditions may be a reasonable proxy that is predictive of ZIKV vRNA

transmission. Dissemination titer from legs and wings was considered instead of body titer because this ensures that the virus has overcome the midgut escape barrier and because body titer was high at the first time point tested for each temperature and plateaued quickly compared to dissemination titer (S1 Fig). The lack of correlation at 21°C could be a result of the time it takes the virus to reach the salivary glands via hemolymph-mediated viral circulation at this cooler temperature being faster than binding and replication in the hemolymph or other secondary organs, however this was not been tested in the current study. Based on the data, these findings do not extend to field applications but could guide optimization of a laboratory method for assessing vector competence around the 26–30°C range tested without the need for relying on the capillary tube method.

The results of this study encompassed the inherent biological variation in the large number of bloodfed Ae. aegypti females that were sourced from a low-generation (F4) colony and fed on five viremic mice. Temperature treatments were not replicated because temperature is an abiotic variable that was verified by both the built-in chamber thermometer and secondary data loggers placed inside the chambers. Due to differences in the chambers available for our study, two of our chambers varied slightly (up to 2°C) around the mean temperatures of 18 and 21°C, whereas the other two chambers maintained constant temperatures of 26 and 30°C. We do not believe this caused any bias in our findings because a previous study with another flavivirus in our lab showed that small to moderate diurnal temperature ranges–larger than those in this study–did not alter extrinsic incubation periods compared to constant-temperature treatments [37]. Also, an earlier study on infection and dissemination of dengue virus in Ae. aegypti found that even large daily temperature fluctuations of 20°C affected only the proportion of mosquitoes with midgut infections but did not alter dissemination compared to constant temperatures [38]. In

our study, infection rates were consistently high across all temperature treatments, giving further confidence that chamber assignments did not alter our findings.

The relationship between temperature and ZIKV EIP in Ae. aegypti reported in this study was compared to published EIPs of other mosquito-borne viruses using linear regression (Fig 5). West Nile virus data in Cx. tarsalis from two previous studies in our laboratory using the same methods were used [18,19]. Data on dengue virus from a meta-analysis of EIP studies in Ae. aegypti was used [20]. Finally, we compared estimates for ZIKV EIP from the current study using a 2015 ZIKV outbreak strain from Puerto Rico (ZIKV–PR) to recently published data using a 2016 ZIKV outbreak strain from Tapachula, Chiapas, Mexico (ZIKV–MX) and a sympatric Ae. aegypti population [17]. EIP50 was calculated at timepoints spanning 18 to 30°C using the fitted models reported in the respective studies, including the current study. We fitted linear models to extrinsic incubation rates (1/EIP) for each virus (Fig 5).

The regression curves (Fig 5) suggest that WNV in Culex tarsalis has a broader range of temperatures at which transmission is feasible when compared to ZIKV and DENV, however at higher temperatures ZIKV and DENV transmission are faster than WNV. Both ZIKV EIP curves have a similar shape to DENV, however the curves suggest ZIKV EIP is shorter across temperatures when compared to DENV in Ae aegypti. Finally, the EIP curve determined in this study was remarkably similar in shape to another published curve for ZIKV, although our study's median EIP was 0.7–1.3 d longer for temperatures from 30 to 26°C where transmission was most efficient and approximately 4.0 d longer at 22°C. It is not possible to disentangle the possible explanations for the differences because our study differed from [17] in viral strain, mosquito strain, method of mosquito infection (viremic mouse vs. artificial membrane feeder), method of ZIKV detection (RT-qPCR vs. plaque assay), and calculation of EIP (transmission out

of total vs. transmission out of infected). Overall, it is interesting to note that both ZIKV studies (ours and [17]) have shorter EIPs across temperatures when compared to DENV, which may partially explain the rapid ZIKV spread throughout the Americas. It is important to note that both ZIKV EIP studies have used outbreak strains from the 2015–2016 Latin American Zika epidemic. We do not conclude that ZIKV EIP will always be shorter than DENV and it is possible that non-outbreak Zika virus strains or populations of Ae. aegypti from outside of the Americas will yield different results. The effect of temperature on ZIKV EIP will be better resolved as more data is collected on using different strains of ZIKV and populations of Ae. aegypti.

In summary, this study determined the effects of temperature and time on infection, dissemination, transmission, and EIP of a Puerto Rican strain of ZIKV in Ae. aegypti from central California. This combination has relevance to the potential for ZIKV to be transmitted within the continental U.S. We fitted a logistic regression model to determine ZIKV vRNA transmission as a function of time and temperature and estimated EIP50 from the fitted curves; we report that as temperature increases, EIP decreases within the temperature bounds tested. Further, we demonstrate that dissemination titer is correlated with probability of transmission at high temperatures at standard rearing conditions, but not at 21°C. This method could be optimized for use a laboratory method to estimate relative transmission rates without using the capillary tube method. Finally, our data, in addition to data from a similar study, suggest that ZIKV EIP may be accelerated compared to dengue virus over the range of temperatures tested. This information is critical for modeling ZIKV transmission dynamics to more accurately understand geographic and seasonal limits of ZIKV transmission risk.

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Figures and Tables

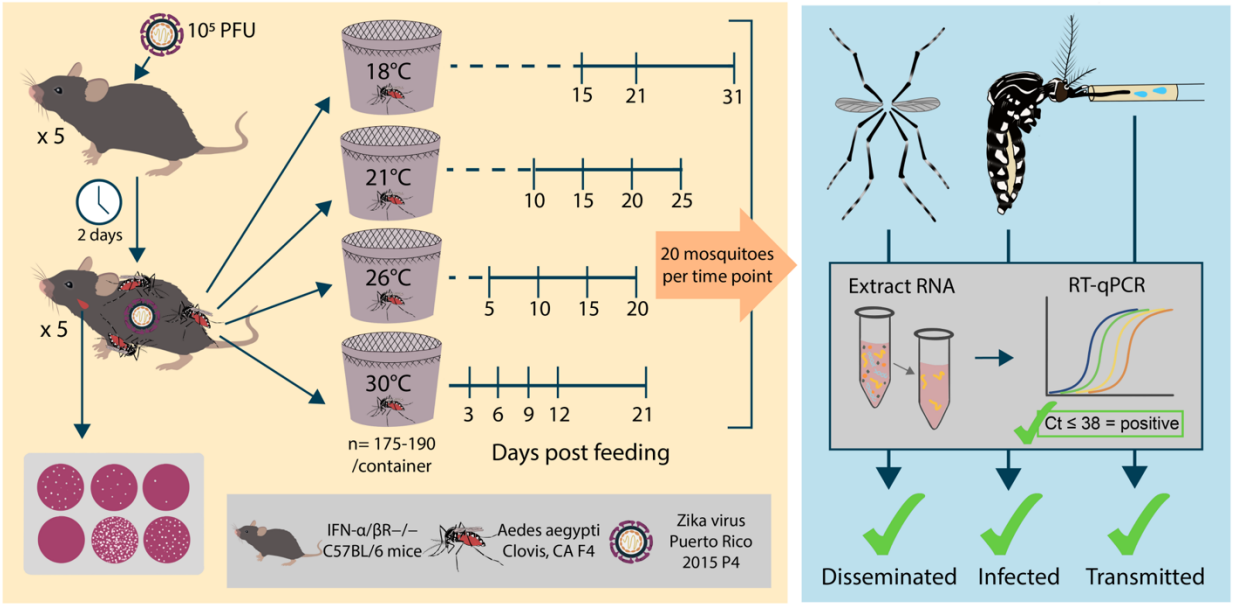


Fig 1. Study design workflow.
Five five-week-old interferon knockout mice were inoculated with 10^5 Vero PFU of ZIKV PR15 via subcutaneous injection and held for two days to reach peak viremia. After two days, mice were anesthetized, blood was collected via each mouse's submandibular vein (i.e., cheek punch) and blood serum ZIKV titer was assessed using Vero cell plaque assay, and 1,200 female *Aedes aegypti* mosquitoes aged 3–5 days were exposed to all five mice. Bloodfed mosquitoes were randomly sorted into four half-gallon cartons held at one of four constant temperatures: 18, 21, 26, or 30°C. At days chosen based on published extrinsic incubation studies of other flaviviruses, mosquitoes were cold-anesthetized in cohorts of 20 females and then legs and wings were removed and saliva was collected by inserting the proboscis into a capillary tube containing fetal bovine serum for 20 minutes. Individual bodies, legs/wings, and the saliva sample from each mosquito were placed separately in tubes containing cell culture media and homogenized. Viral RNA was extracted and ZIKV viral RNA (vRNA) titers were determined for each body, legs/wings, and saliva sample using RT-qPCR. A cycle threshold (Ct) value ≤ 38 was considered positive for ZIKV RNA. Positive bodies indicated ZIKV infection, positive legs and wings indicated ZIKV dissemination, and positive saliva indicated ZIKV transmission.

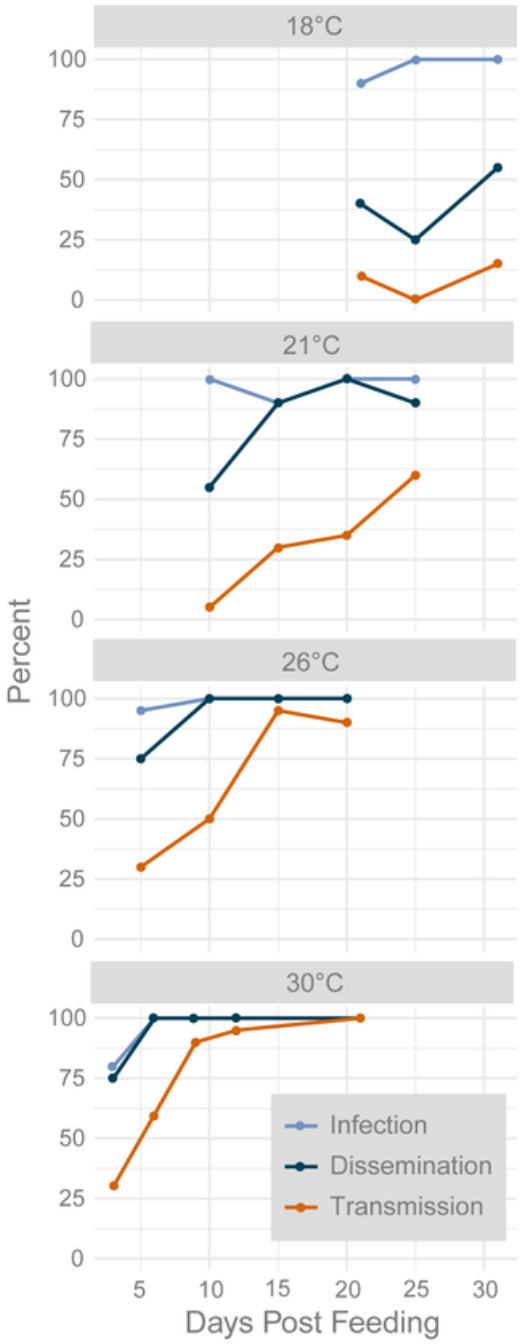


Fig 2. Percent of mosquitoes with infected, disseminated, and transmitted ZIKV vRNA of 20 tested at each temperature and time point combination tested.
Samples were processed in duplicate by RT-qPCR. Ct values < 38 in at least one of the duplicates indicated positive vRNA.

Temp (°C)	Days Post Feeding (5.3–5.7 $\log_{10}$ PFU/mL)	Infected (%)	Disseminated (%)	Transmitted (%)
18	21	18/20 (90)	8/20 (40)	2/20 (10)
18	25	20/20 (100)	5/20 (25)	0/20 (0)
18	31	20/20 (100)	11/20 (55)	3/20 (15)
21	10	20/20 (100)	11/20 (55)	1/20 (5)
21	15	18/20 (90)	18/20 (90)	6/20 (30)
21	20	20/20(100)	20/20 (100)	7/20 (35)
21	25	20/20 (100)	18/20 (90)	12/20 (60)
26	5	19/20 (95)	15/20 (75)	6/20 (30)
26	10	20/20 (100)	20/20 (100)	10/20 (50)
26	15	20/20 (100)	20/20 (100)	19/20 (95)
26	20	20/20 (100)	20/20 (100)	18/20 (90)
30	3	16/20 (80)	15/20 (75)	6/20 (30)
30	6	20/20 (100)	20/20 (100)	12/20 (60)
30	9	20/20 (100)	20/20 (100)	18/20 (90)
30	12	20/20 (100)	20/20 (100)	19/20 (95)
30	21	20/20 (100)	20/20 (100)	20/20 (100)

Table 1. ZIKV vRNA infection, dissemination, and transmission rates by temperature and time point. Samples were processed in duplicate by RT-qPCR. Ct values < 38 in at least one of the duplicates indicated positive vRNA.

Variable	Coefficient (95% CI)	*P-value*
Intercept	-0.667 (-1.16,-0.21)	0.006
DPF	0.299 (0.22,0.39)	<0.001
Temperature	0.378 (0.24,0.53)	<0.001
DPF x Temperature	0.027 (0.01,0.05)	0.002

Table 2. Coefficients from the logistic model for the probability of ZIKV transmission as a function of time and temperature

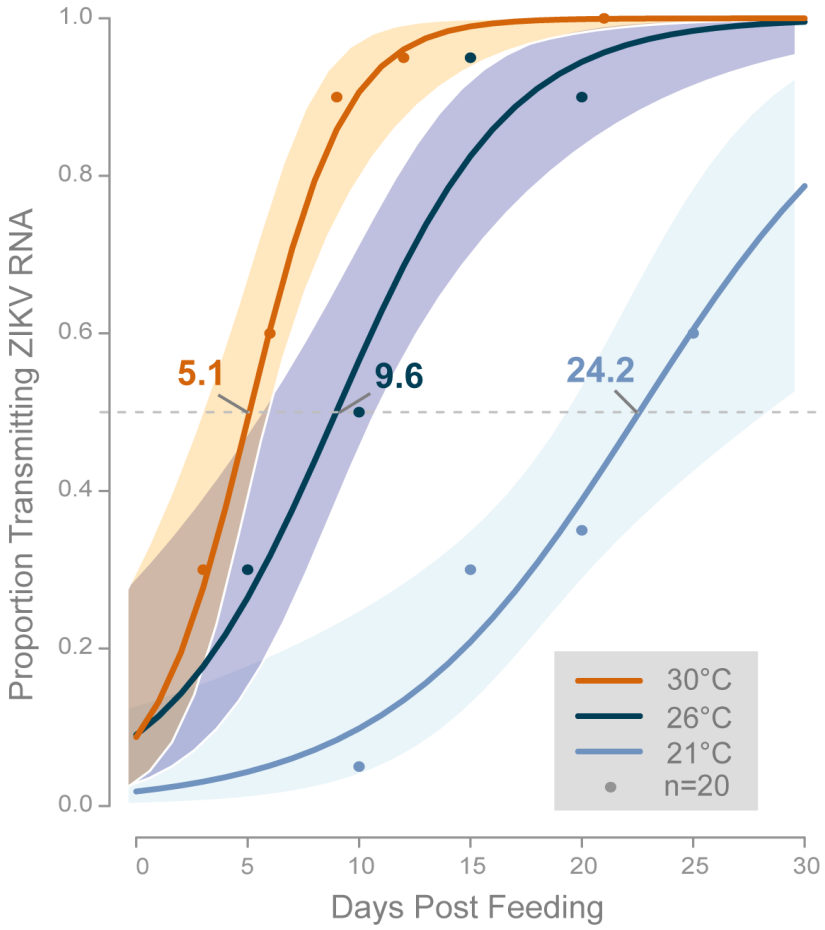


Fig 3. Fitted logistic curves showing the proportions of *Ae. aegypti* transmitting ZIKV vRNA over time by temperature.

Each point represents the observed proportion of mosquitoes (of 20 tested) that transmitted at each temperature and time-point. The estimated EIP_{50} is indicated for each temperature.

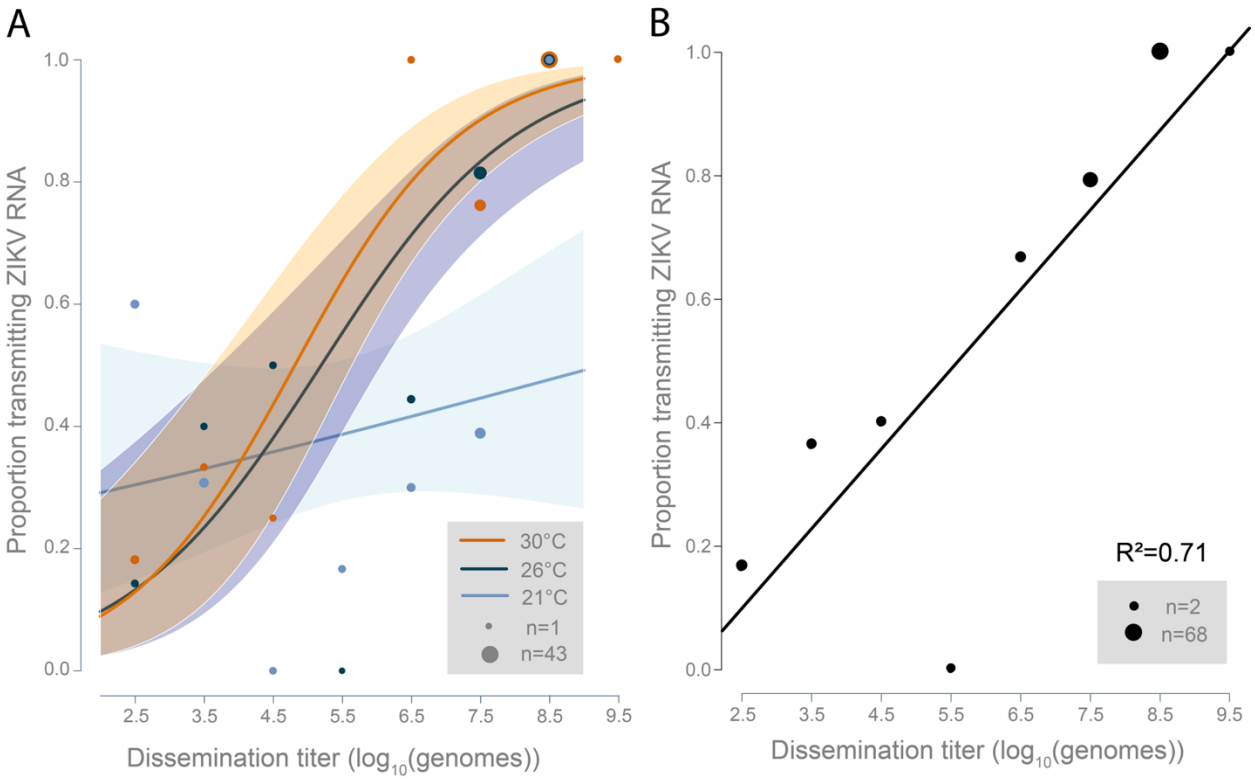


Fig 4.

(A) Fitted logistic curves showing the relationship between dissemination titer (log_{10}(genomes)) and proportion transmitting ZIKV RNA for each temperature. Dot size indicates the number of mosquitoes within ±.5 log_{10}(genomes) of plotted number. (B) Fitted line showing the relationship between dissemination titer (log_{10}(genomes)) and proportion transmitting ZIKV RNA for 26 and 30°C combined. Dot size indicates the number of mosquitoes within ±.5 log_{10}(genomes) of plotted number.

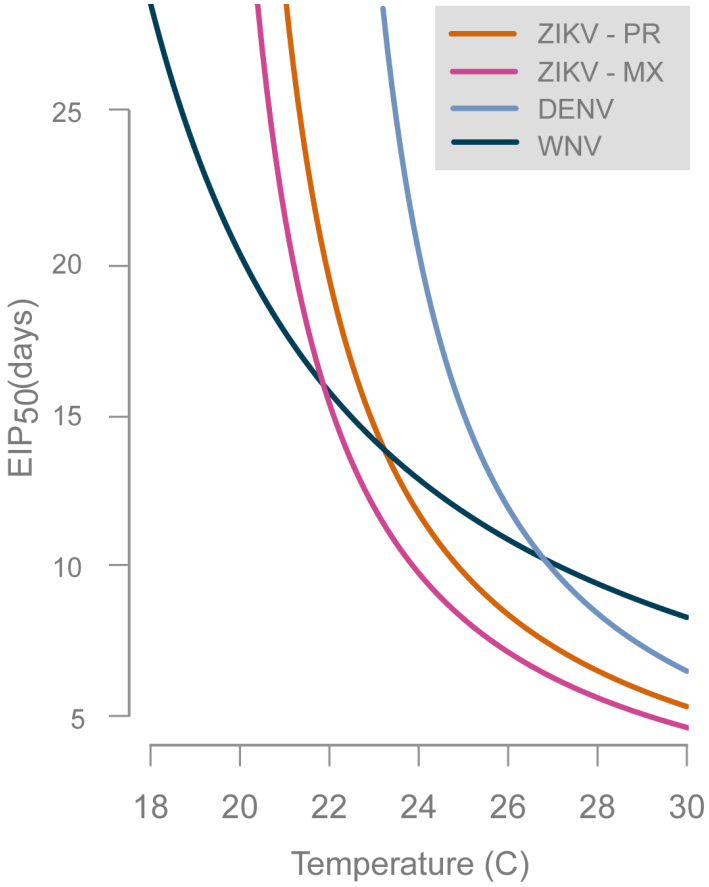
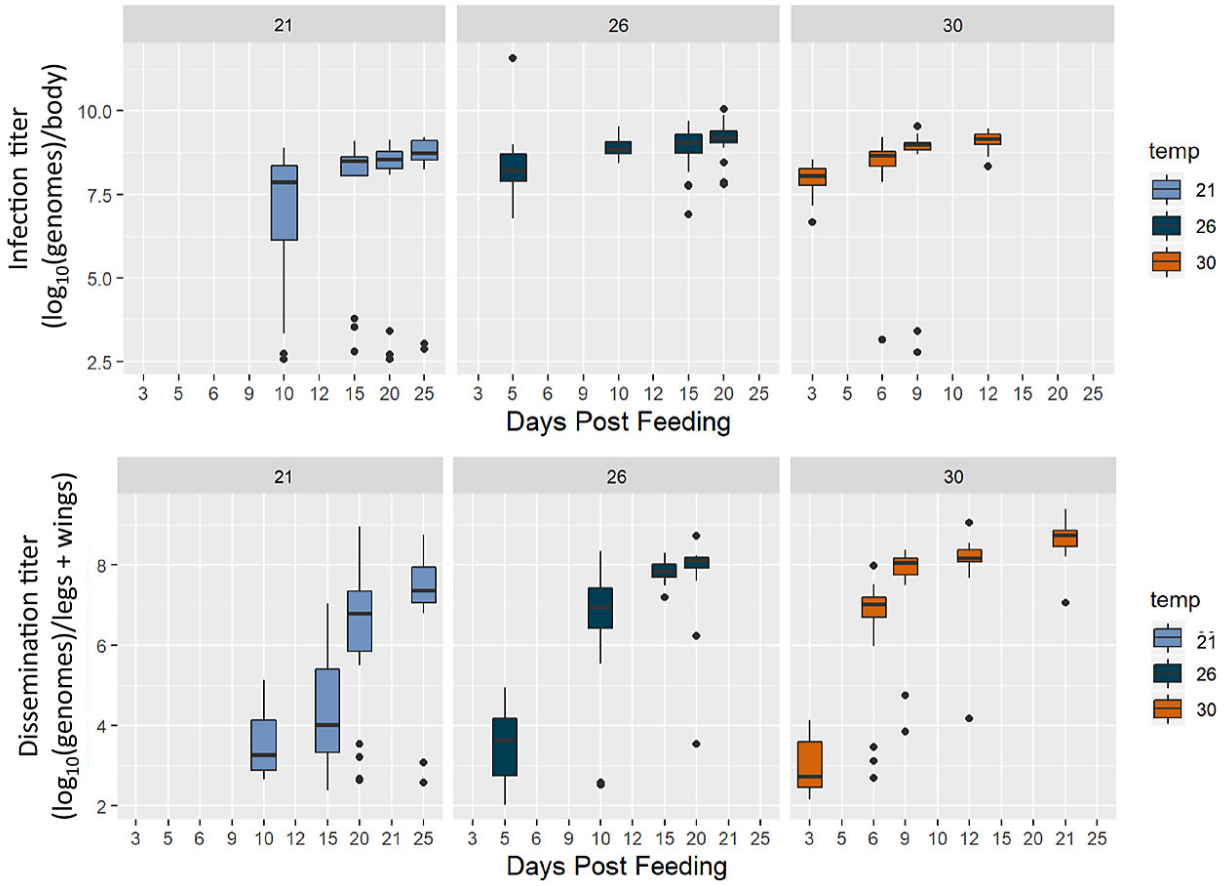


Fig 5. Fitted curves showing the median EIP of the flaviviruses ZIKV, dengue virus (DENV), and West Nile virus (WNV) over a range of temperatures.

ZIKV–PR is the Puerto Rico strain used in this study, whereas ZIKV–MX is a Mexican strain used by Tesla et al. 2018.



S1 Fig. ZIKV infection titer (top panel) and dissemination titer (bottom panel) over time for each temperature.

Variable	Coefficient (95% CI)	*P*-value
Intercept	-1.13 (-2.62, 0.36)	0.136
Dissemination Titer	0.12 (-0.13, 0.37)	0.340
Temperature (26°C)	-2.50 (-5.10, 0.10)	0.059
Temperature (30°C)	-2.84 (-5.28, -0.40)	0.022
Dissemination Titer x Temperature (26°C)	0.58 (0.17, 0.98)	0.006
Dissemination Titer x Temperature (30°C)	0.70 (0.31, 1.10)	<0.001

S1 Table: Coefficients from the logistic regression model for the probability of ZIKV transmission as a function of dissemination titer and temperature. 21°C was the referent group. Coefficients are on the log odds (logit) scale.

Variable	Coefficient (95% CI)	*P-value*
Intercept	-0.23 (-0.75,0.30)	0.332
Dissemination titer	0.13 (0.05, 0.21)	0.008

S2 Table: Coefficients from the linear model for the probability of ZIKV transmission as a function of dissemination titer. Due to no significant difference in the logistic curves for 26 and 30°C, data were pooled and dissemination titer was binned to the nearest 0.5 log(genomes).

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CHAPTER 2:

Thermal Preferences of temperate *Aedes aegypti* mosquitoes across a thermal gradient

Olivia C. Winokur, Claire M. L. Chapman, Christopher M. Barker

Abstract:

Behavioral thermoregulation is the main process used by ectotherms, organisms whose body temperature relies on environmental temperature, to remain at biologically suitable temperatures and avoid death from temperature extremes. Behavioral thermoregulation, also called thermal preference, is well documented in many ectotherms, however, the literature on mosquito behavioral thermoregulation is sparse. Temperature is not only important to mosquito fitness and survival; temperature also drives transmission of mosquito-borne pathogens. Here we examine *Ae. aegypti* thermal preferences using a thermal gradient arena in the laboratory and provide the first evidence of how age, gonotrophic status, rearing temperature, and generation number from wild affect thermal preferences in mosquitoes. Nulliparous mosquitoes reared at 26°C tolerated the coldest temperatures on a gradient spanning 17.5°C to 36.5°C, had a gradual decrease in resting density as gradient temperature increased, and strongly avoided high temperatures above 31°C, regardless of age and generation from wild (F1 vs F3). Mosquitoes reared at cooler temperatures were larger and rested at warmer temperatures compared to mosquitoes reared at warm temperatures. Gonotrophic status 1 day post blood feeding and beyond did not alter preferences meaningfully compared to age matched nulliparous females. These conclusions, along with data from future field studies on thermal preferences can be incorporated into pathogen transmission models to understand mosquito-borne pathogen risk more accurately and improve mosquito control decisions.

Introduction:

Temperature is a crucial abiotic factor for all living organisms, as it can alter physiologic and metabolic processes and fatally hinder them altogether at thermal extremes. Temperature particularly affects ectotherms, organisms with minimal internal ability to regulate body temperature and therefore must depend heavily on environmental temperature. Behavioral thermoregulation, also called thermal preference or thermotaxis, is the primary method that ectotherms use to avoid temperature extremes and remain at physiologically suitable ambient temperatures. Behavioral thermoregulation has been documented widely in several ectotherms, including flies in the *Drosophila* genera [1,2], butterflies[3], nematodes [4], and lizards [5].

Temperature drives transmission of mosquito-borne pathogens, increasing transmission within an optional range, and suppressing it above and below [6]. Temperature has nonlinear effects on mosquito traits such as fecundity, biting frequency, and lifespan of the ectothermic mosquito vector [6–9], as well as effects on mosquito-pathogen interactions including the time from ingestion of a pathogen to transmission, known as the extrinsic incubation period [10–12]. However, with the exception of host-seeking, mosquito behavioral thermoregulation is not well-resolved. Current pathogen transmission models primarily use air or land-surface temperatures from weather stations or thermal imagery as a proxy for the temperatures mosquitoes experience. However, temperatures from these sources are an inadequate representation of the temperatures of local environments or microclimates available to adult mosquitoes and therefore may lead to inaccurate estimates of transmission risk [13].

A small number of studies have examined behavioral thermoregulation of nulliparous female mosquitoes. In one study, the malaria vector, *Anopheles stephensi,* avoided temperature

extremes at the edges of a thermal gradient ranging from 14°C to 38°C, irrespective of malaria parasite infection status [14]. Vectors of West Nile virus and St. Louis encephalitis virus, *Culex tarsalis and Cx. quinquefasciatus,* and related *Cx. territans,* exhibited thermal preference in the lab when exposed to two gradients (15-35°C and 25-50°C); *Cx. quinquefasciatus* females sought the coolest locations available on both low and high gradients, whereas *Cx. tarsalis* and *Cx. territans* females sought temperatures toward the middle of both gradients [15]. Additionally, *Cx. fatigans* females, now recognized as *Cx. quinquefasciatus*, showed no marked preference on 5°C gradients between 5°C and 25°C, but strongly avoided the warmer end in a 25°C to 30°C gradient [16]. *Aedes aegypti,* the primary vector of Zika, dengue, and yellow fever viruses, and *Aedes japonicus,* a vector of Japanese encephalitis virus, preferred cooler temperatures down to 20°C in most gradients and avoided high temperatures (above 31°C for *Ae. aegypti* and 33°C for *Ae. japonicus*) when exposed to a higher temperature gradient [17].

Thermal preference from blood feeding through digestion is of particular interest for understanding transmission dynamics and risk of mosquito-borne pathogens, as resting temperatures during this period determine the extrinsic incubation period, or time from ingestion to transmission of a pathogen. *Ae. aegypti and Ae. japonicus* females one day post blood feeding showed the same trend as nulliparous females and avoided hot temperatures [17], however the effects gonotrophic status beyond one day have not been measured. Further, it is important to understand if life history traits such as age and rearing temperature and the resulting differences in physiology affect resting temperature to improve transmission risk estimates. This study expands on the limited data of *Ae. aegypti* thermal preferences and is the first study to examine how age, gonotrophic status, rearing temperature, and generation from wild affect thermal preferences in mosquitoes.

Materials and Methods:

Thermal gradient arena design

Thermal preference was tested using a thermal gradient arena consisting of an aluminum bar model TGB-5030 (Thermoelectric Cooling America Corp., Chicago, Illinois, USA) placed atop two Versatile Cold/Hot Plates AHP-1200CPV (Thermoelectric Cooling America Corp., Chicago, Illinois, USA) with a custom made 5-sided Plexiglas arena (6”x18”x1”) attached to the metal (TAP Plastics, Sacramento, California, USA) (Fig 1a). The Plexiglas had holes covered with mesh to allow for air flow (Fig 1b). The temperature gradients of the metal plate and Plexiglas sides were quantified by placing thermocouple sensors (Omega, Norwalk, Connecticut, USA) across the surfaces to determine exact temperature ranges on horizontal and vertical surfaces of the arena (S1 Fig). Calibration and experimental trials were conducted at least 30 minutes after the hot and cold plate temperatures were set to allow for establishment of a stable gradient. This set up was chosen out of three potential designs as it maximized the ease of measuring surface temperatures and therefore the number of mosquitoes for which temperature could be determined (S2 Fig).

Mosquitoes

Ae. aegypti mosquitoes used in this study originated from field-collected eggs in Madera, California in 2021. Adults were visually identified to species prior to experimental trials. The F1, F2, and F3 generations were used as specified in each section below. Mosquitoes were reared under controlled conditions at and 80% relative humidity, 12:12 light:dark cycle, 200 larvae in one liter DI H20, and one pinch fish food (ca. 0.5 g, Tetramin, Spectrum Brands Pet, Blacksburg, Virginia, USA) every other day or every three days until pupation. Mosquitoes were reared at 26°C, except in the rearing temperature trials, where mosquitoes were reared at either 22°C,

26°C, or 30°C. Upon emergence, adult mosquitoes were held at their rearing temperature and had constant access to 10% sucrose until transferred to the thermal preference arena.

Experimental trials

Trials were conducted in a temperature and humidity-controlled chamber (Darwin, St. Louis, Missouri, USA) at 23°C and 60% humidity and conducted between the hours 10:00-16:00. Temperatures in the arena were monitored continuously with thermocouple sensors (Omega, Norwalk, CT, USA) and the gradient direction was randomly switched (left to right, right to left, or no gradient control) every 3-4 trials. Gloves were worn when interacting with the arena to avoid interference of human scent and no humans were present in the chamber during the trials. In all gradient trials, the gradient temperature ranged from 17.5°C to 36.5°C (plates set to 10°C and 45°C) and the control trials had no temperature gradient with each plate set to 25°C. Mosquitoes were released from an aspirator into one of three release locations (left, middle, right; Fig1); release location is specified in each section below and depended on the variable being tested. Mosquitoes were monitored in a snapshot approach using time lapse photography with three c920 Logitech HD Pro Webcam (Logitech, Lausanne, Switzerland) and VideoVelocity software [18]. One camera was placed directly over the Plexiglas arena to capture mosquitoes on the metal gradient bar, and the two other cameras were placed above either long side of the gradient bar and angled to capture mosquitoes on the Plexiglas sides (Fig 1). Mosquitoes were left in the arena for 20 minutes in each trial as in Verhulst et al. [17] and preliminary studies performed in our apparatus using mosquitoes left for longer periods (up to 330 minutes) showed that locations didn't change meaningfully after 15 minutes. Mosquito locations on the metal and Plexiglas walls were determined from a single image after 20 minutes using ImageJ [19] and the Figure Calibration plugin Version 2009 [20].

Effect of entry position

Cohorts of 15 mosquitoes (3-6 days old, F2 generation) were added to the arena from varied locations (cold end, middle, hot end or left, middle, right in control trials) with the gradient already established and were monitored and locations recorded as described above. At the end of the study time, mosquitoes were anesthetized with CO_2 and discarded. 8 replicates were completed for each release location with the gradient established (n=120 mosquitoes / release location) and 4 replicates for each release location with a control of 25°C along the entire bar (n=60 mosquitoes / release location).

Effect of age and gonotrophic status

F3 mosquitoes were reared using the same protocol as in the entry position section above; 4 day old mosquitoes were bloodfed in the mesh cage using a Hemotek feeder (Hemotek Ltd, Lancashire, UK) and heparinized sheep blood (Hemostat Laboratories, Dixon, California, USA). Bloodfed females were visually identified and separated from unfed females and both groups were held at 26°C and 80% RH until tested. Batches of 15 bloodfed females were added to the thermal gradient arena from the middle position with the gradient already established on days 0, 1, 3, and 5 post feeding, and unfed age matched mosquitoes were tested at 4, 5, 7, 9, and 14 days old. Each batch of mosquitoes was only placed in the arena once and then was anesthetized with CO_2 and discarded. After trials on day 3 post feeding, a cup of water lined with seed germination paper was added to the cage to allow for oviposition, therefore mosquitoes on day 5 post feeding were no longer carrying eggs. 6 replicates were completed for blood fed females for each dpf with the gradient established (n=90 mosquitoes / dpf) and 6 replicates for each age matched unfed female with the gradient established (n=90 mosquitoes / age match day).

Effect of rearing temperature

F1 mosquitoes were reared at either 22°C, 26°C, or 30°C. Cohorts of 15 mosquitoes (3-6 days old) were added to the arena from the middle location with the gradient already established and were monitored and locations recorded as described in the previous section. At the end of the study time, mosquitoes were anesthetized with CO_2 and placed in the freezer. Wing lengths of 20 mosquitoes per rearing temperature were measured as a proxy for body size. 8 replicates were completed for each rearing temperature with the gradient established (n= 120 mosquitoes/ rearing temperature) and 8 control replicates (25°C along the entire bar) were completed for each rearing temperature (n= 120 mosquitoes/ rearing temperature), except at 30°C where 7 control replicates were completed (n=105).

Effect of generation number from wild

Trials from the previously listed variables that were age matched, nulliparous, reared at 26°C, and released in the middle of the arena were used to assess the effect of three generations of laboratory colonization (F1, F2, F3; denoted by asterisks in table 1).

Statistical analyses

Analyses was performed using R Software version 4.1.3 [21]. Thermal gradient models of the arena were fitted using linear regression to create a heatmap to calculate temperature of any location in the arena. A series of spatial point-pattern Poisson models were fitted to all no-gradient control data to compare spatial landing preferences in an otherwise neutral arena using the 'spatstat'[22], 'raster' [23], 'maptools' [24], and 'splines'[25] packages. Models were fitted with one coefficient for edge (lumping edge of metal + walls together) or with separate coefficients for edge and walls. Non-edge metal (here-on referred to as middle) was the referent category in all models. One and two-term models were fitted with various definitions of edge (on the metal plate within 2", 1" 0.5", or 0.25" of the walls). To assess thermal preferences for each

variable tested, polynomial point-pattern Poisson generalized additive models (GAM) were fitted. For the GAMs, we required knot points to be at least 3.5°C from either end of the temperature distribution (17.5°C to 36.5°C, knot points ≥21°C and ≤33°C) to ensure enough data points to inform each fitted segment; knot points were selected by assessing all combinations of 2 knot points within this range to avoid overfitting the model (S1 Table). Models were compared using Akaike information criterion (AIC) and the model with the lowest AIC was chosen. GAMs were plotted between 18°C and 34.5°C to exclude edge temperatures that represented less than 4% of the arena surface (S3 Table), which were subject to extreme model uncertainties. Wing lengths were compared using one way ANOVA and Tukey's honest significance test and assumptions of normality and residuals were tested using the Shapiro-Wilk test. Statistical significance was specified as $P < 0.05$.

Results

Edge effect in control trials

In control trials with a constant temperature of 25°C (i.e., without a temperature gradient), mosquitoes preferred resting along the edges of the metal plate near the Plexiglas walls of the arena. No mosquitoes were recorded upside-down on the top of the Plexiglas. The best fitting model describing this preference was a two-term spatial point-pattern model with edge defined as the metal within 0.5" of Plexiglas walls (AIC=2163) (S3 Table). With no gradient, mosquitoes had different preferences for middle, edge, and wall, with the strongest preference for resting within 0.5" of the edge on the metal (rate ratio (RR)=5.58 vs. middle, *P*<.001*)*, followed by the Plexiglas walls (RR=3.63 vs. middle, *P*<.001) (S3 Table). Coefficients for edge within 0.5" and Plexiglas walls were included in all gradient models as a statistical adjustment for these spatial resting preferences of the mosquitoes within the arena that were not attributable to temperature.

Thermal preferences of nulliparous females across variables

For all gradient trials with nulliparous female mosquitoes reared at 26°C combined, mosquito resting density was the highest was at the coldest temperatures and decreased as temperature increased with a relatively stable resting density between 23°C and 30.5°C (Fig 2, Fig 3). Rate ratios Resting densities were 43% higher at 22°C compared to 26°C (RR=1.43; 95%CI: 1.16-1.70), 15% lower at 30°C compared to 26°C (RR=0.85; 95%CI: 0.66-1.04), and 57% lower at 34°C compared to 26°C (RR=0.43; 95%CI: 0.26-0.60) (Fig 3, Table 1).

Effect of entry position

Release temperature (hot end vs. cold end) had a small effect on thermal preference curves. All release locations had a high resting density at the coldest temperatures available. Mosquitoes released on the cold and in the middle end increasingly avoided temperatures above 31°C and 28°C, respectively. Mosquitoes released on the hot end had wider range of temperature tolerance with a general gradual decrease as temperature increased, but never significantly differed compared to the resting density at 26°C (Fig 3, Table 1).

Effect of age and gonotrophic status

Thermal preferences of nulliparous females varied slightly with age (Fig 5A). Mosquitoes had a greater tolerance for the coldest temperatures on the gradient as they aged, regardless of having ingested blood or not. The trend on the warmer end of the gradient was less clear, however for all ages regardless of gonotrophic status, mosquitoes avoided the warmest temperatures on the gradient. Mosquitoes on the day of blood feeding (0dpf) had the same avoidance of warm temperatures as age matched nulliparous females, but also avoided cold temperatures where nulliparous mosquitoes did not. One day post blood feeding, mosquitoes regained their

preference for cold temperatures on the gradient. Nulliparous and gravid 7-day-old mosquitoes had very similar thermal preference curves, with a high resting density at the coldest temperatures on the gradient, a relatively stable resting density through moderate temperatures, and a strong avoidance of high temperatures on the gradient. At 9 days old, the avoidance of high temperatures was less strong, but this strong avoidance was seen again at 14 days old. 9-day-old mosquitoes that had blood fed and laid eggs followed a similar trend to 7-day-old gravid mosquitoes for the cooler half of the gradient, but had a spike in resting density between 30°C and 33°C (Table 1).

Effect of rearing temperature

For all rearing temperatures, mosquitoes reached the highest resting density at the coldest temperatures available on the gradient. Mosquitoes reared at 26°C and 30°C tended to rest at higher densities in the colder half of the arena (<26°C), and for both rearing temperatures, mosquitoes rested at lower densities at the hottest temperatures, although those reared at 26°C avoided a wider range of high temperatures compared to others reared at 30°C (Fig 5, Table 1). Mosquitoes reared at 22°C showed a drastically different thermal preference trend from the other warmer rearing temperatures; mosquitoes reared at 22°C still had a high resting density at the coldest end of the gradient below 20°C and a relatively stable resting density through the moderate temperatures on the gradient but showed a significant increase in resting density between 31.5C and 34°C (Fig 5).

Wing length was measured as a proxy for body size among our rearing temperature groups. We recorded significantly longer wing lengths for mosquitoes reared at 22°C (mean=3.0 mm; range: 2.8-3.3 mm) compared to 26°C (mean=2.8; range: 2.6-2.9 mm) or 30°C (mean=2.8;

range: 2.6-3.0 mm) (ANOVA and Tukey's HSD, $P < 0.001$), and no significant difference between 26°C and 30°C (S4 Fig).

Effect of generation number

Generation from wild did not have a strong effect on thermal preferences. All generations reached the highest resting density at the coldest temperatures available on the gradient; F3 mosquitoes had a stronger tolerance of the coldest temperatures in the arena. All generations had relatively stable resting densities through ~28.5°C before gradually decreasing through the warmest temperatures.

Landing in mesh holes

Across trials, no mosquitoes were recorded on the top of the Plexiglas. A total of 284/2,020 (14.1%) mosquitoes were recorded within the mesh holes (12.6% in gradient trials, 18.3% in control trials; S2 Table). Holes comprised 2.0% of the total landing surface area in the arena (excluding top of Plexiglas; 3.14 sq in./156 sq in.). In gradient trials combined, there were 3.31 times as many mosquitoes/ sq in. in holes compared to on the metal within 0.5" of Plexiglas (60.19 vs. 18.17). In control trials combined, there were 4.32 times as many mosquitoes/ sq in. in holes compared to on the metal within 0.5" of Plexiglas (30.25 vs. 7.00). Mosquitoes in holes were excluded from analysis as it was not possible to determine their temperature accurately.

Discussion

Understanding how temperature affects transmission dynamics of mosquito-borne pathogens is critical for determining risk and taking appropriate measures to protect public health. There are many studies that aim to understand how temperature alters mosquito life history and the traits that influence pathogen transmission dynamics [26], including biting frequency [27,28], fecundity [29,30], lifespan [29–31], vector competence [32,33], and extrinsic

incubation period of virus/vector combinations [10–12,34,35]. Further, there are studies that aim to incorporate these data into pathogen transmission models [6,36–40]. However, models that predict the dynamics of mosquito populations or pathogen transmission are only applicable if we understand and incorporate the temperatures mosquitoes experience in nature. Current models generally rely on weather station temperature as a proxy for mosquito temperatures, however, temperatures from these sources poorly represent local environmental temperature and microclimates available [13,41]. This study aimed to build on the limited data on *Ae. aegypti* thermal preferences. Mosquito thermal performance optima vary by life history trait; for example, biting rate is highest in *Ae. aegypti* and *Ae. albopictus* above 30°C, whereas lifespan is longest around 22°C [6]. There are tradeoffs between optimal temperatures for life history traits and therefore we would expect the optimal temperature range to be between these trait-specific optima.

Edge effect

In our study as well as in another recent laboratory study on *Ae. aegypti* and *Ae. japonicus* thermal preferences [17], mosquitoes preferred the edge of the arena. Interestingly, this edge preference was not noted in studies of thermal preference of other mosquito genera *Anopheles* [14] and *Culex* [15], which found that resting patterns did not differ significantly from a random distribution in no-gradient control trials. *Ae. aegypti* mosquitoes are anthropophilic and are well adapted to living in and around human homes; preference for the edge vs. middle in our arena followed by preference for the walls vs. middle could be due to avoiding exposed surfaces where they are less likely to be found and swatted by humans. In one study in Panama, female *Ae. aegypti* were frequently found on less exposed surfaces in homes [42], and in one study in

Sri Lanka, more female *Ae. aegypti* were found under or on furniture and wall hangings compared to on walls inside homes [43].

Thermal preferences of nulliparous females across variables

Generally, mosquitoes had the highest resting density in the coldest temperatures on the gradient and avoided temperatures at the hottest end of the gradient. This tolerance of cold temperatures on the edge of the gradient was also observed in Verhulst et al[17]. It is difficult to know if the cold tolerance is a true preference, or a limitation of thermal gradient laboratory studies. Potential explanations could be: (1) mosquitoes' preference for the edge overrides their true thermal preference, (2) a cold trap where mosquitoes' body temperatures drop and they lose locomotor activity, (3) temperature extremes represent a relatively small area in the arena such that a small number of mosquitoes landing skews the model, (4) the steepness of the gradient might not mimic natural gradients, and/or (5) convection within the arena that might induce vertical gradients experienced by mosquitoes in flight. A recent study video tracked lab colonized *Ae. aegypti* mosquitoes in a thermal gradient arena and concluded the preference for lower temperatures (down to 10°C) is not a result of a cold trap as mosquitoes were able to fly and walk in all temperature treatments including the coldest ones (down to 10°C), albeit movement was slowed [44]. Interestingly in Verhulst et al., they also noted no significant different in resting density in the warmest sector (27°C -30°C) compared to the middle sector (21°C- 24°C), whereas in our gradient that expanded beyond 30°C we recorded a significantly lower resting density in the warmer range (27°C- 30°C) compared to 21°C -24°C. This potentially points to a preference for the edge overriding true thermal preference in laboratory studies when the edge temperature is tolerable.

Effect of entry position

We studied how temperature at the release point affected thermal preferences in the thermal gradient arena to ensure mosquitoes do not just stay where they are released and actively move to avoid extreme temps. For all release points, mosquitoes had the lowest resting density at the hottest temperatures available on the gradient, supporting our general conclusion that mosquitoes reared at 26°C avoid the hot temperatures in our gradient arena. Mosquitoes released at hot and moderate temperatures tended to have slightly more moderate temperature preferences than those released on the cold end, but the same general trend of avoiding the hottest temperatures was seen for all release positions.

Effect of age and gonotrophic status

Thermal preferences of nulliparous females did not drastically vary as they aged, though resting density at the coolest end of the gradient increased with age. This may be more evidence for a cold trap at these temperatures; it is possible that as mosquitoes age, they have less ability to escape the extreme cold temperatures. Females immediately after blood feeding (0dpf) had a moderate temperature distribution, but many mosquitoes did not move from the release point. Mosquitoes in our study fed to repletion, and thus were heavy, which could affect their ability to move. Those that did move moved toward the cold end of the arena and strictly avoided the hot end. By day 1 post blood feeding, mosquitoes mimicked the same resting densities across temperatures as 4-day-old nulliparous females. *Ae. aegypti* often take partial blood meals nature [45]; we would expect females 0dpf that take partial bloodmeals to move more than those that feed to repletion, however we did not test this.

Effect of rearing temperature

Ae. aegypti is a container breeding mosquito that is capable of breeding in a small volume of water that is prone to large temperature shifts due to low volume. We studied the effects of

rearing at three field-relevant temperatures: 22°C, 26°C, and 30°C. Females from all rearing temperatures tolerated the very cold end of the temperature gradient. Beyond this, mosquitoes reared at cooler temperatures (22°C) preferred warmer temperatures on the gradient, females reared at a warmer temperature (30°C) preferred moderate to low temperatures, and mosquitoes reared at a moderate temperature (26°C) preferred moderate temperatures. Mosquitoes reared at 22°C had significantly longer wing lengths than those reared at 26°C or 30°C.

In line with our findings, adult *Drosophila subobscura* flies developed at cooler temperatures preferred warmer microclimates [46]. Smaller bodied *Ae. aegypti* were previously shown to be significantly more likely to become infected and to disseminate dengue virus than larger individuals [47]. If small-bodied mosquitoes prefer cooler temperatures this would decrease the period between pathogen ingestion and transmission [10–12,34] and increase lifespan [6] and thus could lead to an increase in transmission risk. Additionally, smaller *Ae. aegypti* fed more frequently on blood in Thailand, which could lead to more frequent transmission, however, this trend of increased feeding rate of small mosquitoes was not reported in Puerto Rico [27]. In contrast to our result, large adult black soldier flies (*Hermetia illucens* L) preferred significantly cooler temperatures than small black soldier flies [48]. Black soldier fly body size differences were produced from different larval densities, not rearing temperature. Future studies should assess mosquito body size resulting from other larval conditions such as competition and food density to determine whether this trend is solely due to body size or due to possible changes in physiology resulting from rearing temperature, which could lead to a better understanding of how body size affects pathogen transmission efficiency and risk.

Effect of generation number

Generation from wild (F1 vs F3) did not have a strong effect on thermal preferences. Our results using nulliparous females follow the same thermal preference trend seen in [17] that used a laboratory colony originally stemming from another institution. This suggests that, in the laboratory, colonization does not have a strong effect on thermal preferences.

Across many of the variables tested, our models showed a much higher resting density at the coldest temperatures with wide confidence intervals (Table 1). This could be due to a few mosquitoes resting in temperatures that make up a relatively small area of the arena (S4 Table), which could skew our models and lead to high uncertainty. This was rarely seen at the warm end where the warmest temperatures also make up a relatively small area of the arena, which supports our general conclusion of a preference or tolerance for the cold end or a cold trap and strong avoidance of the warmest temperatures. However, mosquitoes reared and held at 22°C in our study still had a high resting density at the coldest temperatures, suggesting rearing and acclimation to 22°C either does not improve cold tolerance or that there is a true preference for the coldest temperatures in our arena.

A potential limitation in thermal gradient studies is the inability to maintain a constant humidity gradient. We ran trials in a chamber with constant 60%RH at 23°C, meaning humidity is <60% at temperatures higher that 23°C and >60% at temperatures lower than 23°C. In other studies, moist paper towels or hydrated salts were used to increase or decrease humidity. Verhulst et al. concluded that humidity had no effect on temperature preferences and mosquitoes still preferred a cold side with low relative humidity over a warm side with high relative humidity, which supported previous research on this topic [49]. All mosquitoes in our study had constant access to 10% sucrose before experimental trials and were left in the arena for 20 minutes. It is unlikely, but possible that avoidance of highest temperatures is due to humidity.

This laboratory study supports and adds to the literature on *Ae. aegypti* thermal preferences and is our first step at understanding the thermal preferences specifically of temperate mosquitoes from California's central valley. These results should not be extrapolated to *Ae. aegypt*i from other regions as there may be differences in thermal preferences. Further, microhabitat availability should be studied to understand the realistic temperatures available to mosquitoes in addition to their preferences. Conclusions and data from studies like this one along with data from future field studies on thermal preferences can be incorporated into pathogen transmission models to understand mosquito-borne pathogen risk more accurately and improve mosquito control decisions.

Acknowledgements

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Figures and Tables

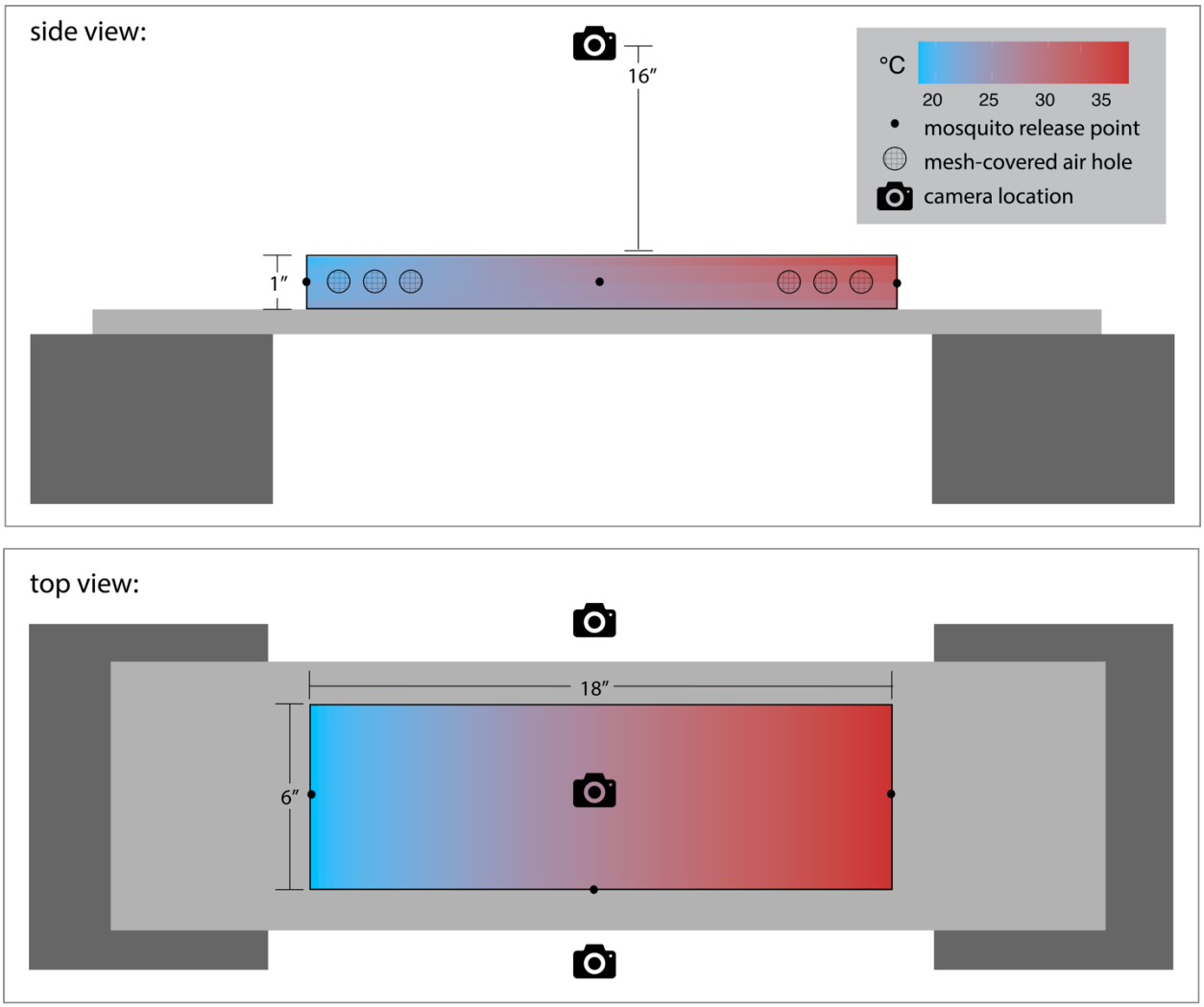


Figure 1: Thermal gradient bar with Plexiglas arena set-up. Dark grey boxes are Versatile Cold/Hot Plates AHP-1200CPV, light grey is aluminum thermal gradient bar. Colors indicate gradient from 17.5°C to 36.5°C within the Plexiglas arena. Gradient direction was randomly switched from left to right, right to left, or a no gradient control every 3-4 trials.

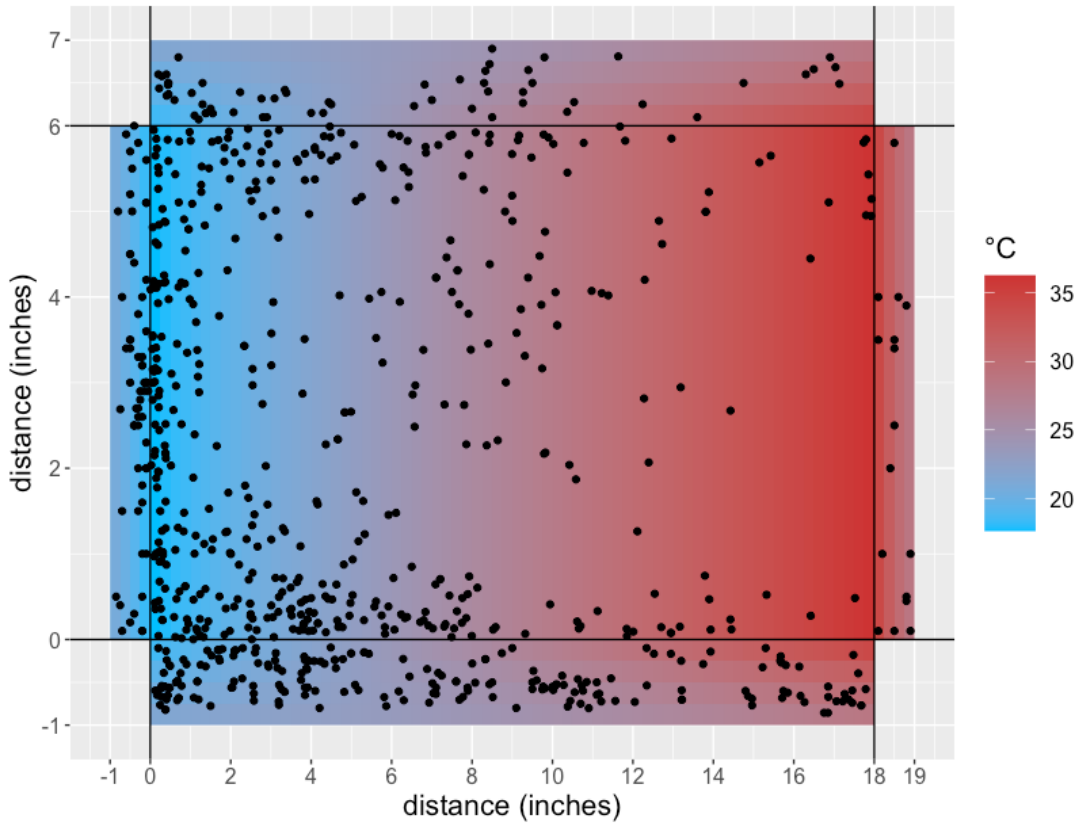


Fig 2: Mosquito spatial locations in the gradient arena from trials combined for nulliparous mosquitoes reared at 26°C.

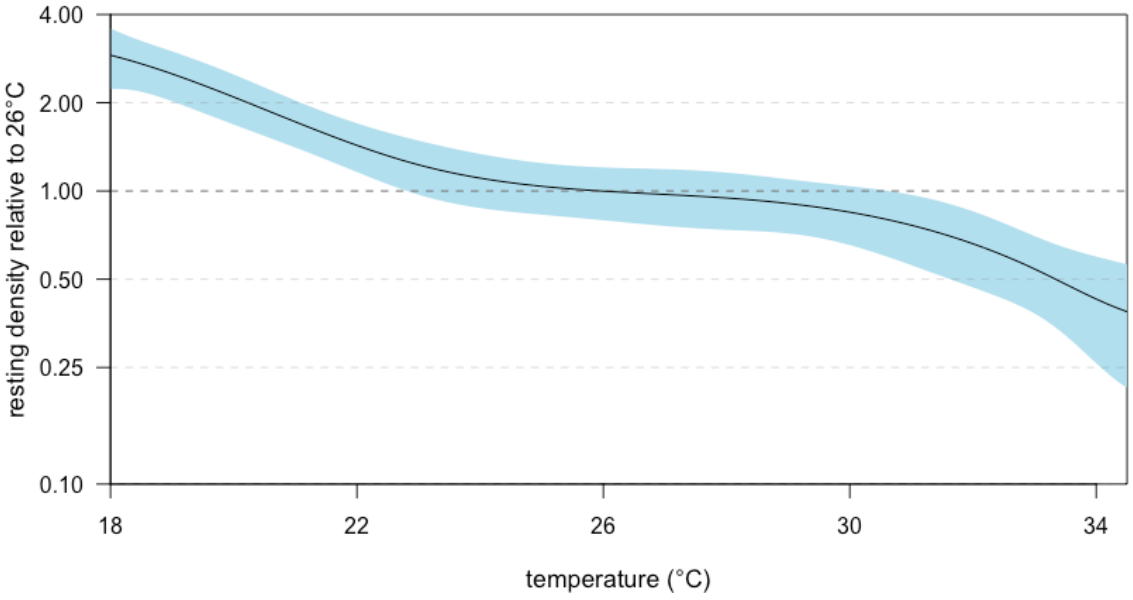


Fig 3: Relative rate plot for all gradient trials for nulliparous female *Ae. aegypti* reared at 26°C combined. Resting density is relative to 26°C. Black line shows maximum likelihood and blue shading represents 95% CI.

Variable tested	Model	18°C (95% CI)	22°C (95%CI)	26°C (95%CI)	30°C (95%CI)	34°C (95%CI)
-	All nulliparous trials reared at 26°C combined	2.91 (2.22, 3.59)	1.43 (1.16, 1.70)	1.00 (0.80, 1.20)	0.85 (0.66, 1.04)	0.43 (0.26, 0.60)
Entry position	Cold release	2.51 (0.55, 4.48)	1.56 (0.80, 2.32)	1.00 (0.43, 1.57)	0.80 (0.29, 1,32)	0.10 (-0.04, 0.23)
	Middle release **	1.24 (0.19, 2.30)	1.03 (0.50, 1.58)	1.00 (0.46, 1.54)	0.34 (0.10, 0.59)	0.08 (-0.08, 0.23)
	Hot release	2.62 (0.11, 5.14)	1.22 (0.55, 1.88)	1.00 (0.45, 1.55)	0.71 (0.28, 1.15)	0.62 (-0.01, 1.26)
Age	4 day old nulliparous ***	6.30 (1.51, 11.09)	2.07 (0.79, 3.34)	1.00 (0.30, 1.70)	0.71 (0.12, 1.31)	0.05 (-0.07, 0.16)
	5 day old nulliparous	5.12 (0.92, 9.32)	0.88 (0.42, 1.35)	1.00 (0.33, 1.67)	0.08 (-0.07, 0.23)	0.00 (0.00, 0.00)
	7 day old nulliparous	15.18 (4.51, 25.84)	2.06 (0.86, 3.24)	1.00 (0.31, 1.69)	0.57 (-0.12, 1.25)	0.00 (0.00, 0.00)
	9 day old nulliparous	28.73 (5.56, 51.90)	2.70 (0.94, 4.46)	1.00 (0.15, 1.85)	0.42 (-0.07, 0.91)	0.29 (-0.19, 0.78)
	14 day old nulliparous	16.90 (3.68, 30.11)	2.49 (1.01, 3.99)	1.00 (0.29, 1.71)	0.47 (0.05, 0.90)	0.00 (0.00, 0.00)
Gonotrophic status	4 days old – 0dpf	2.30 (0.71, 3.89)	0.33 (0.14, 0.54)	1.00 (0.56, 1.44)	0.71 (0.34, 1.07)	0.14 (-0.03, 0.31)
	5 days old – 1dpf	7.39 (1.15, 13.63)	3.60 (1.08, 6.11)	1.00 (0.17, 1.83)	0.72 (0.03, 1.40)	0.17 (-0.11, 0.44)
	7 days old – 3dpf	11.41 (3.22, 19.60)	1.76 (0.83, 2.69)	1.00 (0.37, 1.63)	0.12 (-0.12, 0.36)	0.00 (0.00, 0.00)
	9 days old – 5dpf	130.13 (39.29, 220.97)	44.72 (23.89, 65.55)	1.00 (-0.44, 2.44)	0.48 (-0.30, 1.25)	0.00 (0.00, 0.00)
Rearing temperature	Reared at 22°C	6.75 (-0.88, 14.38)	0.86 (0.28, 1.44)	1.00 (0.32,1.68)	1.62 (0.59, 2.66)	4.31 (1.17, 7.45)
	Reared at 26°C *	3.05 (-0.04, 6.15)	0.97 (0.39, 1.6)	1.00 (0.41, 1.59)	0.45 (0.13, 0.76)	0.10 (-0.09, 0.29)
	Reared at 30°C	1.69 (-0.08, 3.47)	1.32 (0.47, 2.16)	1.00 (0.35, 1.66)	0.75 (0.24, 1.26)	0.31 (-0.01, 0.64)
Generation from wild	F1*	3.05 (-0.04, 6.15)	0.97 (0.39, 1.6)	1.00 (0.41, 1.59)	0.45 (0.13, 0.76)	0.10 (-0.09, 0.29)
	F2**	1.24 (0.19, 2.30)	1.03 (0.50, 1.58)	1.00 (0.46, 1.54)	0.34 (0.10, 0.59)	0.08 (-0.08, 0.23)
	F3***	6.30 (1.51, 11.09)	2.07 (0.79, 3.34)	1.00 (0.30, 1.70)	0.71 (0.12, 1.31)	0.05 (-0.07, 0.16)

Table 1: Relative rates for all gradient models. Rates for temperatures are relative to 26°C. Asterisks indicate mosquitoes used to test both variables and thus variables with the same number of asterisks represent the same models.

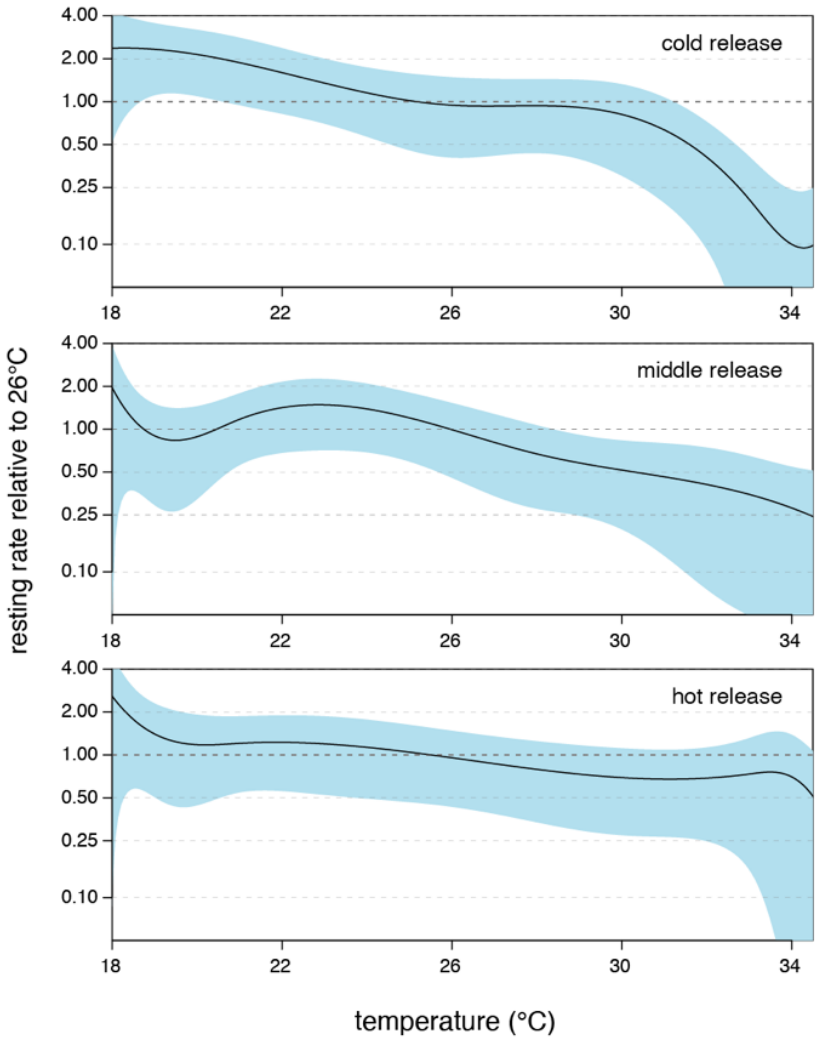


Fig 4: Relative rate plots for entry position. Resting density is relative to 26℃. B**l**ack line shows maximum likelihood and blue polygon represents 95% CI.

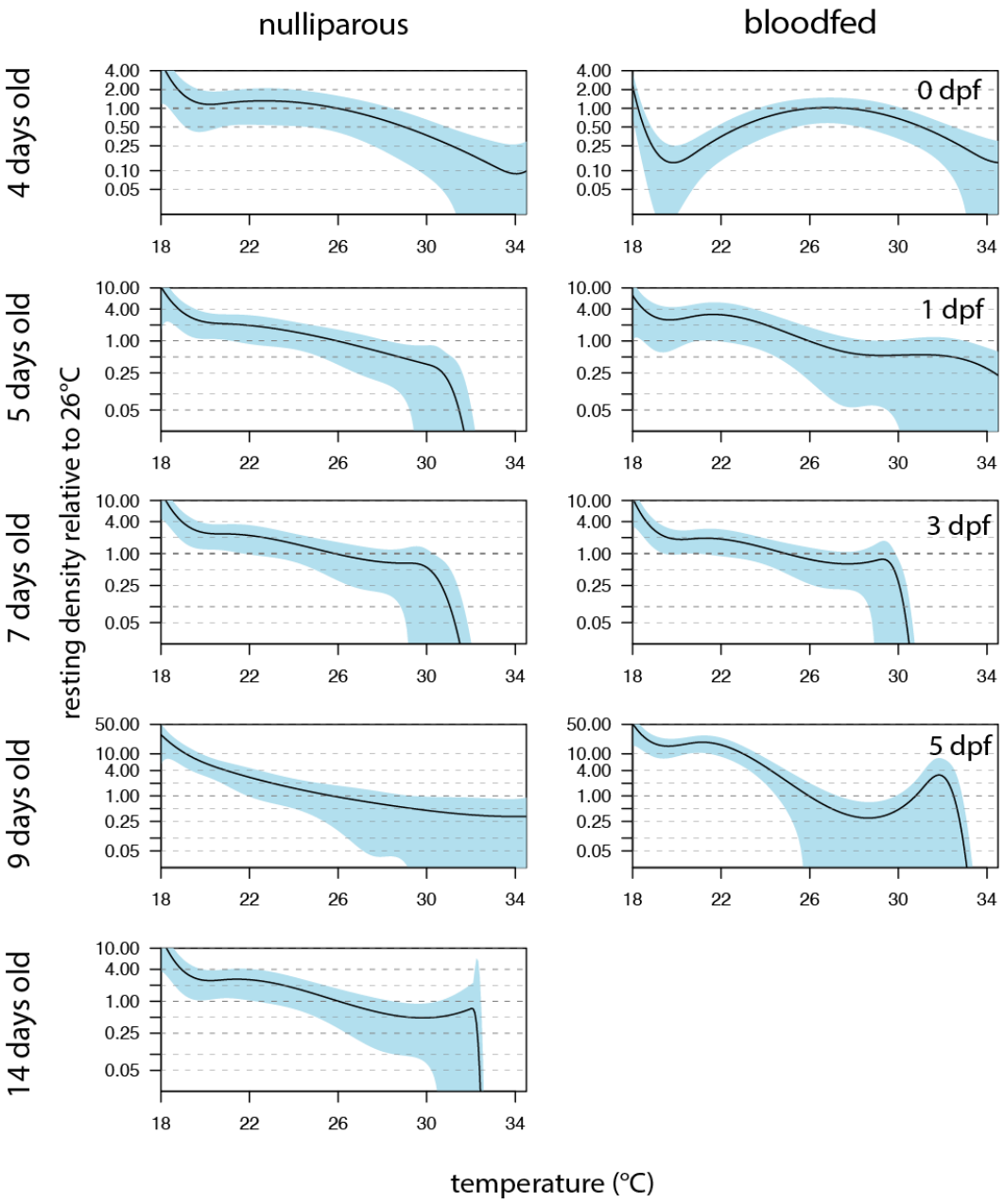


Fig 5: Relative rate plots for age and gonotrophic status. Resting density is relative to 26°C. Black line shows maximum likelihood and blue polygon represents 95% CI. Note Y axis varies between days.

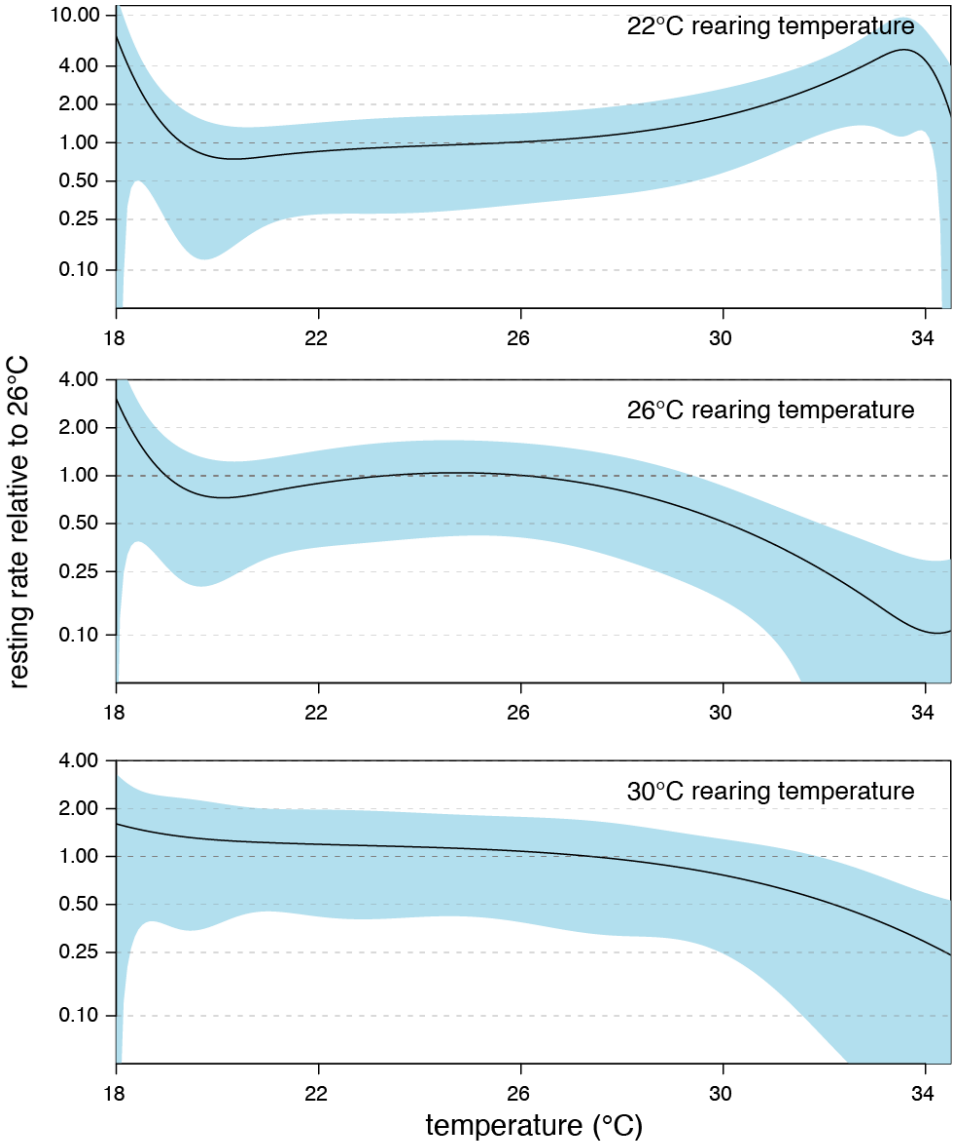


Fig 6: Relative rate plots for rearing temperatures.

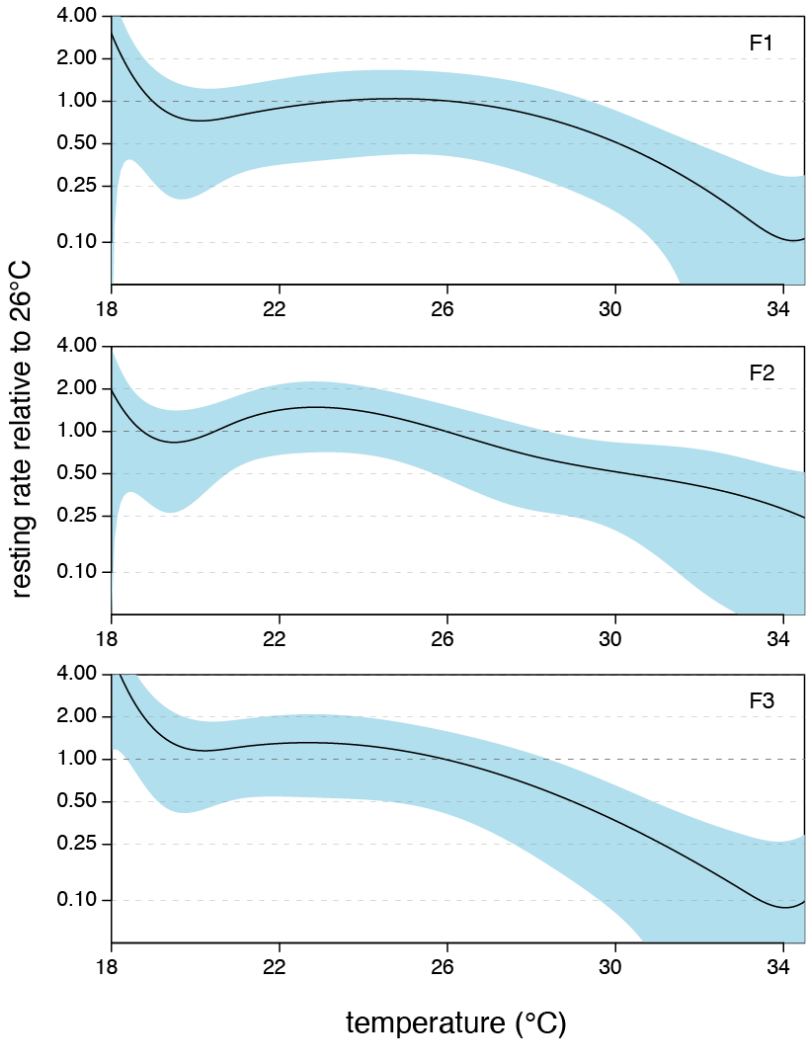
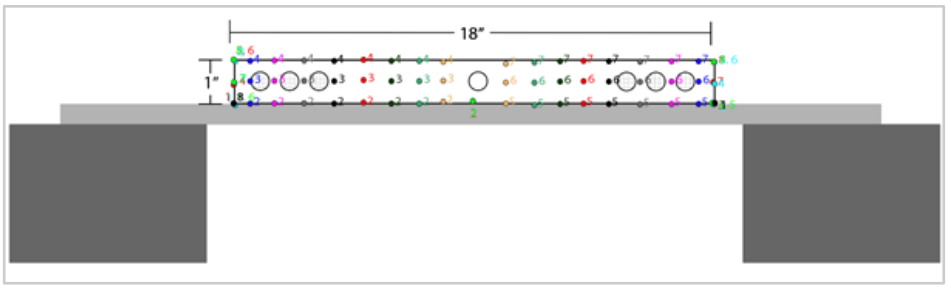
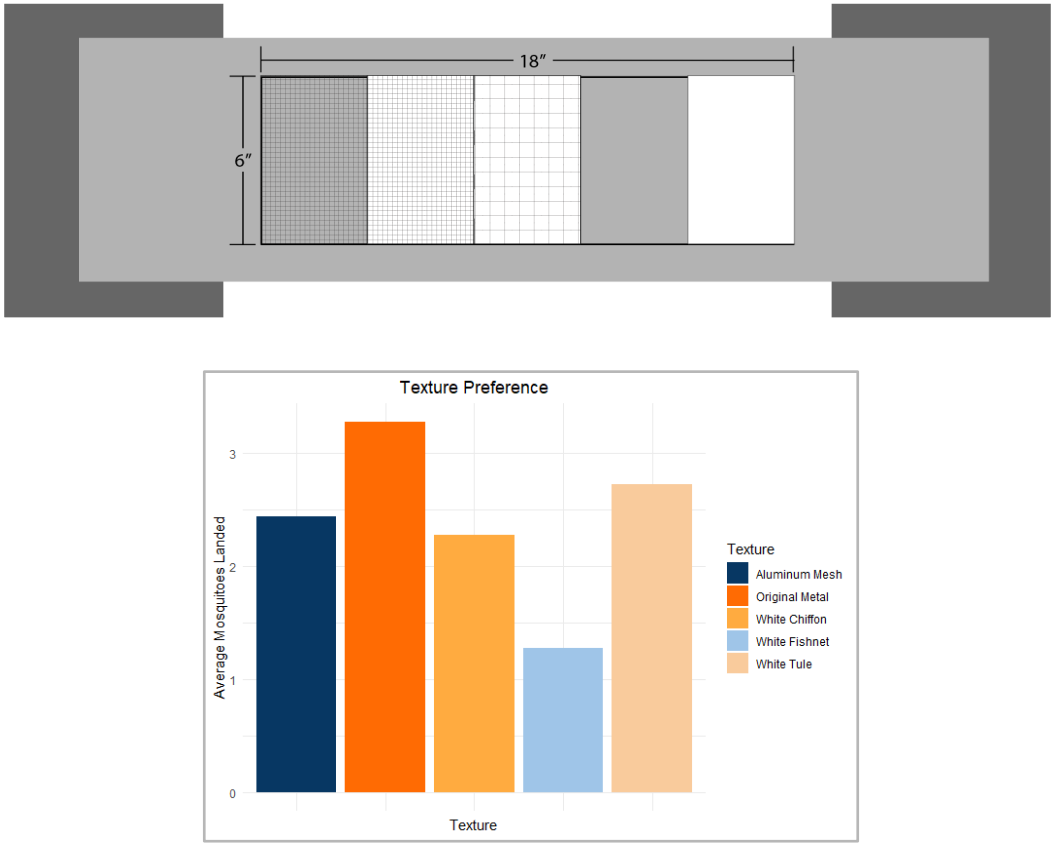


Fig 7: Relative rate plots for generation from wild. Trials from the previously listed variables that were age matched, nulliparous, reared at 26°C, and released in the middle of the arena were used to assess the effect of three generations of generation from wild (F1, F2, F3; denoted by asterisks in table 1). Resting density is relative to 26°C. Black line shows maximum likelihood and blue polygon represents 95% CI.



S1 Fig: **Gradient determination. A. Thermocouple sensor map on Plexiglas walls.** Thermocouple sensors were moved in 1" increments down the length of the 18" side of the Plexiglas cover at three different heights (on metal, 0.5", 1"), with three constant sensors spanning the metal for all trials to ensure same temperature on metal. Temperature was not measured in the holes of the Plexiglas. The different colors represent the location of the 8 sensors in each of the different trials. The temperature along the metal in gradient trials changed linearly, increasing or decreasing 1.05°C per inch. The temperature on the long Plexiglas sides changed quadratically.



S2 Fig. Texture and device orientation trials. Trials presenting five different textures for mosquitoes to land on showed that mosquitoes did not prefer any texture over the original aluminum metal. 16.6% of mosquitoes landed on the original aluminum metal in our texture trials which was higher than any other texture. Only the white fishnet material severely decreased the number of mosquito landings, to 6.3%. All texture trials were conducted with no temperature gradient and a constant 25°C on all surfaces of the arena. The distribution of mosquitoes in these control conditions showed the highest density of mosquitoes at the edges of the Conducted at constant control temperatures of 25°C, the majority of mosquitoes preferred to land on the original aluminum metal of the device. No other materials would increase resting directly on the heat source. **Device Orientation.** In both device orientations,

the temperature on the long, 18" sides of the Plexiglas arena changed in two dimensions - across the length of the metal plate as well as in the z (away from plate) direction, moving off of the surface of the metal plate and up the Plexiglas. In the vertical orientation, temperature changes up to 10 degrees on the Plexiglas sides at the furthest point from the metal gradient and up to 7 degrees halfway up the side. In general, surface temperatures move towards the ambient temperature (23°C) the further away from the metal gradient the surface is. So, the gradients on surfaces at the top of the setup gave a smaller temperature range than at the bottom. Consequently, in the vertical orientation, the two long Plexiglas sides have different temperatures from each other. However, in the horizontal orientation, the long sides display identical temperature gradients.

Model	***knot points***
All nulliparous gradient trials reared at 26°C	23, 33
Middle release	21,28
Cold release	26, 33
Hot release	21, 33
4 days old nulliparous	21, 33
5 days old nulliparous	21, 30
7 days old nulliparous	21, 30
9 days old nulliparous	26, 32
14 days old nulliparous	21, 32
0 dpf (4 days old)	21, 33
1 dpf (5 days old)	21,28
3 dpf (7 days old)	21, 29
5 dpf (9 days old)	21, 32
30°C rearing temp	21, 28
26°C rearing temp	21, 33
22°C rearing temp	21, 33

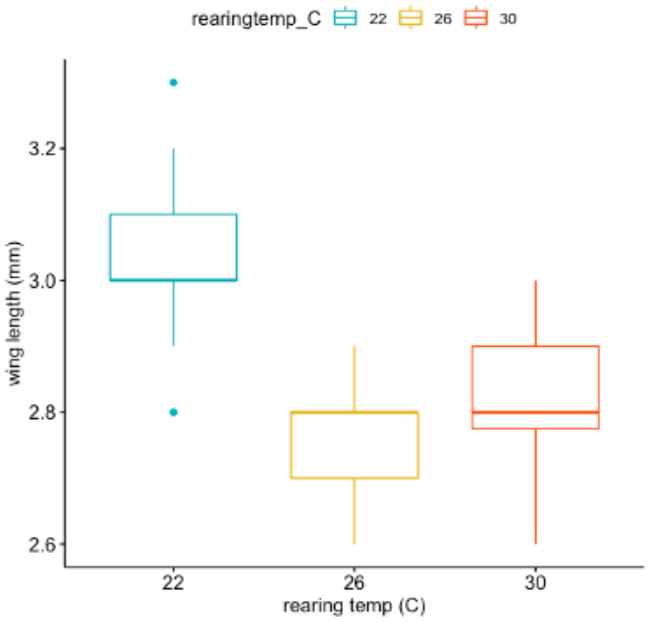
S1 Table: knot points for point-pattern Poisson GAMs based on lowest AIC.

	Control trials		Gradient trials	
	# mosquitoes	% of total	# mosquitoes	% of total
Mesh holes	95	18.3	189	12.6
Plexiglas walls (not including holes)	167	32.1	535	35.7
Metal total	258	49.6	776	51.7
Metal within 0.5" of walls	168	32.2	436	29.1
Middle (Metal total - metal within 0.5" of walls)	90	17.3	340	22.7
Total	520	-	1500	-

S2 Table: Mosquito counts on each surface for control and gradient trials.

Model	terms	Edge estimate (95% CI)	Rate ratio (95% CI)	P-value	Walls estimate (95% CI)	Rate ratio (95% CI)	P-value	AIC
2" + walls	1	1.13 (0.69, 1.57)	3.06 (1.99, 4.81)	<.001	-	-	-	2333
1" + walls	1	1.39 (1.11, 1.67)	4.01 (3.03, 5.31)	<.001	-	-	-	2237
0.5" + walls	1	1.52 (1.27, 1.76)	4.57 (3.56, 5.81)	<.001	-	-	-	2177
0.25" + walls	1	1.37 (1.15, 1.59)	3.94 (3.12, 4.90)	<.001	-	-	-	2193
walls	1	-	-	-	0.40 (0.20, 0.60)	1.49 (1.22, 1.82)	<.001	2354
2" + walls	2	1.04 (0.59, 1.48)	2.83 (1.80, 4.39)	<.001	1.31 (0.85, 1.77)	3.71 (2.34, 5.87)	<.001	2328
1" + walls	2	1.44 (1.15, 1.73)	4.22 (3.12, 5.64)	<.001	1.32 (1.02, 1.63)	3.74 (2.77, 5.10)	<.001	2238
0.5" + walls	2	1.72 (1.46, 1.98)	5.58 (4.31, 7.24)	<.001	1.29 (1.01, 1.56)	3.63 (2.75, 4.76)	<.001	2163
0.25" + walls	2	1.67 (1.43, 1.91)	5.32 (4.18, 6.75)	<.001	1.09 (0.84, 1.34)	2.97 (2.32, 3.82)	<.001	2168

S3 Table: Coefficients and AICs for point-pattern Poisson models of control data (no gradient, constant metal temperature of 25C). Models were tested for the metal gradient within 2", 1", 0.5", and 0.25" of the edge and for the walls to create an edge effect term for control trials to include in the gradient trials. Middle is referent.



S4 Fig: Wing lengths of mosquitoes reared at 22°C, 26°C, and 30°C (n=20/temperature). Wing length of mosquitoes reared at 22°C (mean=3.0 mm; range: 2.8-3.3 mm) was significantly longer than those reared at 26°C (mean=2.8; range: 2.6-2.9 mm) or 30°C (mean=2.8; range: 2.6-3.0 mm) (ANOVA and Tukey's HSD, $P < 0.001$) (Fig 3). Within-group variation didn't deviate significantly from normal distribution (Shapiro-Wilks test, $P = 0.346$).

Break (°C)	**Density**
18	0.038
18.5	0.059
19	0.042
19.5	0.063
20	0.066
20.5	0.034
21	0.074
21.5	0.061
22	0.064
22.5	0.067
23	0.068
23.5	0.082
24	0.058
24.5	0.058
25	0.058
25.5	0.038
26	0.057
26.5	0.058
27	0.058
27.5	0.058
28	0.052
28.5	0.054
29	0.069
29.5	0.050

30	0.050
30.5	0.068
31	0.027
31.5	0.044
32	0.064
32.5	0.042
33	0.043
33.5	0.042
34	0.042
34.5	0.059
35	0.038
35.5	0.038
36	0.019
36.5	0.038

S4 Table: Densities of temperature availability in gradient arena.

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CHAPTER 3:

Thermal preferences of *Aedes aegypti* mosquitoes in California's Central Valley alters current R_0 estimates

Olivia C. Winokur, Trinidad Reyes, Sarah T. Abusaa, Christopher M. Barker

Abstract:

Estimating mosquito-borne pathogen transmission risk is important to inform mosquito control decisions to mitigate disease burden. Current pathogen transmission models predominantly use widely available air or land surface temperatures as a proxy for mosquito exposure temperatures and do not incorporate microhabitat availability or thermal preferences of mosquitoes. Here, we report results from the first study of *Ae. aegypti* thermal preferences in the field and how thermal preferences can alter viral transmission risk. Mosquito resting boxes with temperature sensors were aspirated twice daily during a 6 week period spanning the late-summer peak of adult *Ae. aegypti* abundance in Madera, California. The boxes represented a wide range of available microhabitats. Female *Ae. aegypti* were found resting at temperatures that were increasingly cooler than ambient as ambient temperature increased. A quadratic model was fitted to thermal preferences and was used to estimate expected resting temperatures of female *Ae. aegypti* based on ambient temperature on representative "hot" or "mild" days during our study. Overall, resting temperatures increased with ambient temperatures, although the rate of increase slowed at the highest temperatures. Accounting for *Ae. aegypti* thermal preferences yielded lower estimates for Zika virus transmission risk compared to models based on air temperatures alone.

Introduction:

Temperature modulates transmission of mosquito-borne pathogens by altering traits of the ectothermic vector and interactions between the vector and pathogen [1–3]. Pathogen

transmission models predominantly use remotely sensed temperature data or air temperature from nearby weather stations as a proxy for the temperatures mosquitoes experience, however, these sources do not represent the varied microhabitats available to adult mosquitoes [4,5] and certainly do not account for any microhabitat preferences of the mosquito.

Mosquito thermal preferences have been studied only in a few laboratory studies. *Culex tarsalis and Cx. quinquefasciatus,* which are vectors of West Nile and St. Louis encephalitis viruses, and related species *Cx. territans* demonstrated thermal preferences in the lab; *Cx. quinquefasciatus* females sought the coolest locations available on both low- and high-temperature gradients (15-35°C and 25-50°C), whereas *Cx. tarsalis* and *Cx. territans* females rested at temperatures toward the middle in both gradients [6]. In another study, *Cx. quinquefasciatus (*recognized as *Cx. fatigans* at the time of the cited study), showed no marked preference on gradients spanning 5°C between 5°C and 25°C, but strongly avoided the warm end on a gradient spanning 25°C to 30°C [7]. *Anopheles stephensi,* a vector of malaria parasites, avoided the cold and warm temperatures at the edges of a gradient spanning 14°C to 38°C, regardless of *Plasmodium* infection [8]. *Aedes aegypti,* the primary vector of Zika, dengue, chikungunya, and yellow fever viruses, nulliparous females and females one day after bloodfeeding tolerated cooler temperatures down to 15°C and avoided temperatures above 31°C [9]. Similar results were seen for *Ae. japonicus* that transmit Japanese encephalitis virus, although they tolerated temperatures up to 33°C [9]. In chapter 2 of this dissertation, we reproduced the results Verhulst et al. found in *Ae. aegypti*, concluding that nulliparous females and females one day post blood feeding reared at 26°C tolerated the coldest temperatures on our gradient (17.5°C) and avoided temperatures above 31°C. Additionally, we found that age, laboratory colonization (F1 vs. F3) and gonotrophic status beyond one day post feeding showed

increasing tolerance for cold temperatures, but did not have marked effects on the warm end of thermal preference curves, however mosquitoes reared at cooler temperatures (22°C vs 26°C) were larger and showed a significant increase in resting density between 31.5°C and 34°C.

In this study, we conducted the first assessment of adult *Ae. aegypti* thermal preferences in the field and examined how accounting for thermal preferences affect relative Zika virus (ZIKV) transmission potential in California's Central Valley.

Materials and Methods:

Resting Boxes

Mosquito resting boxes were adapted from Edman et al. [10] and constructed of a sturdy 42cm x 32cm x 32cm cardboard moving box with a 20cm x 32cm opening on the front side and the interior and front cardboard covered with black muslin fabric (S1 Fig A). This resting box design was selected based on a preliminary trial comparing the box design to collapsible resting shelters adapted from [11] (S1 Fig B). Thirty resting boxes were placed in the front or back yard of 10 homes (three to four boxes per yard, nine homes in back yard, one home in front yard) for six weeks from August 24th, 2021 until September 30th, 2021. Boxes were placed to represent a variety of microhabitats (i.e. full shade, afternoon sun, under covered patio, near or under bushes or wall, fully exposed, etc.). Temperature sensors (iButton, Maxim Integrated, San Jose, California) were placed on the bottom back left of the box to avoid direct heat from any walls exposed to the sun and to avoid moisture from any potential irrigation sprinklers. Sensors recorded temperature in 15-minute intervals for the duration of the study. Six of the sensors also recorded relative humidity. There was no humidity added to the boxes for the first week; however, for the remaining five weeks, a dog bowl (19cm diameter) with water was placed in the bottom of the resting box to increase humidity. The water in the dog bowl was replaced every 4-

5 days to prevent mosquito breeding. Resting boxes were aspirated two times a day on weekdays only from 7-8am before the morning peak of *Ae. aegypti* activity and from 2-3pm before the afternoon peak of *Ae. aegypti* activity for the first four weeks, and then only from 2-3pm for the remaining two weeks. Each box was aspirated for 30 seconds using a modified leaf blower (Ryobi Power Tools, Anderson, South Carolina, USA) with the wires reversed for suction (henceforth referred to as aspirator). Mesh bags were attached to the end of the aspirator tube to contain mosquitoes from each box. The bags were cinched before the aspirator was turned off to avoid mosquitoes flying out. Each mesh bag was labeled with the box identifier, date, and time and placed in an insulated cooler for transport back to a laboratory, then transferred to a -20°C freezer for future identification. If all 3-4 boxes in a yard did not catch *Ae. aegypti* mosquitoes on two or more consecutive days, the boxes were moved to a new yard, assigned a new number, and placed in varied environments in a similar fashion as the original home (i.e. full sun, full shade, partial sun). This was done to ensure we were surveying mosquitoes in yards with *Ae. aegypti* present. Mosquitoes were identified morphologically to species (for *Aedes* and *Culex*) or genus (for *Anopheles*) under a microscope and their gonotrophic status was recorded.

Weather station data

Hourly temperature data were obtained from NOAA National Centers for Environmental Information Local Climatological Data at Madera Municipal Airport, CA (Station ID: GHCND:USW00093242) for the time period studied [12]. Weather station temperature data are referred to herein as ambient temperature.

Statistical analyses

Analyses was performed using R software version 4.1.3 [13]. A negative binomial model for female *Ae. aegypti* per box was fitted and visualized using the MASS [14] and lattice packages

[15], respectively. A quadratic regression model was fitted to the temperature differences of the boxes where *Ae. aegypti* were collected and the model was used to estimate expected resting temperatures for female *Ae. aegypti* at hourly ambient temperatures on representative days.

Transmission Risk modeling

Daily basic reproductive rates, R_0, were calculated for representative days using ambient temperature and the mosquito preference model according to the classical formula:

$$R_0 = \frac{ma^2bcp^n}{-\ln(p)r}$$

where *m* is the ratio of female *Ae. aegypti* to humans held constant at a "high" value of 5 mosquitoes/person, *a* is the temperature-dependent human biting rate fitted from Sharpe-DeMichele functions [16], *b* is the human host competence held constant at 0.8, *c* is ZIKV vector competence held constant at 0.8 [17], *p* is the daily probability of survival based on temperature and calculated from SkeeterBuster [18], *n* is ZIKV EIP calculated from daily temperature means [19], and *r* is human recovery rate (1/infectious period) held constant at 1/5.

Here, R_0 is used as a metric for the relative transmission risk when thermal preferences are considered vs. when ambient temperature is used as a proxy for mosquito temperature, rather than as a definitive metric for transmission risk.

Results

Resting box collections

Resting boxes were aspirated a total of 1,008 times and 1,479 mosquitoes were captured, of which 205 (13.9%) were female *Ae. aegypti* (Table 1). Of the female *Ae. aegypti* collected, 158 (77.1%) did not have blood or eggs visible in their abdomen, 22 (10.7%) were bloodfed or partially gravid with blood still visible in the abdomen, and 25 (12.2%) were gravid with no blood visible in the abdomen (Table 2). Male *Ae. aegypti* represented 21.9% of the total

collection, and 61.2% of the *Ae. aegypti* collected. A total of 928 *Culex* mosquitoes were collected, representing 62.7% of the total mosquitoes collected. Of the *Culex* collected, 378 (40.7%) were female *Cx. quinquefasciatus,* 58 (6.3%) were female *Cx. tarsalis*, and 492 (53.0%) were male, which were not identified to species. We collected 22 total *Anopheles* mosquitoes (1.5% of total collection).

Thermal preferences of Aedes aegypti

The number of female *Ae. aegypti* per box was dependent on both ambient temperature and each box temperature's deviation from ambient temperature (Fig 1, Table 3). *Ae. aegypti* females were much more abundant in boxes that were much cooler than ambient temperature as ambient temperature increased. The coefficients from the negative binomial model (Table 3) describe the predicted number of female *Ae. aegypti* per box for any given ambient temperature and temperature difference, according to the formula:

$$\ln\left(\frac{female\ Ae.aegypti}{box}\right) = -10.92 + 0.67A + 0.48D - 0.01D^2 - 0.02A \times D$$

where A is ambient temperature in Celsius and D is temperature difference (box temperature - ambient temperature).

Across ambient temperatures, resting box temperatures generally had wide variability, with the deviation of box temperature from ambient generally decreasing as ambient temperature increased (Fig 2a, Table 4). The number of boxes in each 1°C ambient temperature bin ranged from 0 to 89, with a median of 30 boxes per temperature bin (Table 4). Temperature differences of female *Ae. aegypti* were plotted (Fig 2B) and a quadratic regression model was fitted to estimate typical mosquito exposure temperature across the range of observed ambient temperatures (R^2=0.60, P<0.001, Fig 2B, Table 5). Mosquitoes rested at warmer temperatures as

ambient temperature increased, however the rate of increase in temperature is much slower than ambient temperatures themselves (Fig 3).

The quadratic model for female *Ae. aegypti* temperature difference was used to estimate typical mosquito temperatures throughout the day for representative hot and mild days during our study period (Fig 3, Table 5). After accounting for female *Ae. aegypti* thermal preferences, the mean mosquito temperature on the hot day was 3.4°C cooler than the ambient mean temperature and 2.1°C cooler than ambient on the mild day.

Relative ZIKV transmission risk

On the hot day, ZIKV R_0 was 2.2 times lower when mosquito thermal preference was accounted for (3.26 using ambient temperature vs. 1.03 using mosquito preference model) and on the mild day, R_0 was near zero after accounting for mosquito thermal preference and 0.82 when using ambient temperature (Table 5).

Discussion

Understanding and incorporating microhabitat availability and thermal preferences will improve mosquito-borne pathogen transmission models that are used to inform mosquito control. We determined that during peak season in California's Central Valley, female *Ae. aegypti* were more likely to be found resting at temperatures that were below ambient air temperature as ambient temperature increased. Incorporating these thermal preferences lowered estimated ZIKV risk during the study period relative to a model that used nearby weather station air temperature as a proxy for mosquito temperature.

The risk estimates provided in this paper depend on the assumptions inherent in our parameterization and therefore cannot be regarded as absolute predictions of the potential for

sustained ZIKV transmission. Rather, we conclude that incorporating thermal preferences of *Ae. aegypti* resulted in a relative reduction in estimated transmission risk in the study area during peak *Ae. aegypti* season. The parameters we used to estimate ZIKV R_0 are best estimates from the literature, though many of these parameters have not been resolved in subtropical and temperature regions. For example, biting rate assumes any preferential feeding on humans is ignorable, however this can vary by host availability [20], and it is likely that the feeding rate on humans is lower in Madera, California than in tropical regions where *Ae. aegypti* feed almost exclusively on humans [21]. In this study, vector competence was treated as an inherent trait of transmission potential and was averaged based on a laboratory study of *Ae. aegypti* from Los Angeles, California and transmission of two ZIKV outbreak strains 21 days post feeding on an infectious bloodmeal [17], though we note that there are variations in vector competence depending on viral strain and mosquito population. Extrinsic incubation period is based on one study using a ZIKV from the 2016 outbreak in mosquitoes from California's Central Valley [19], which varies slightly from the only other study examining temperature and ZIKV EIP[22]. Additionally, thermal preferences may affect daily probability of survival.

A significant limitation to our study is the lack of resolution of *Ae. aegypti* thermal preferences at colder temperatures outside the late-summer period of peak adult abundance. In this study, fewer mosquitoes were collected in the morning when temperature was cooler outside compared to in the afternoon, when adjusted for number of boxes aspirated (42 female *Ae. aegypti*/ 333 boxes in the morning (12.6%), 163 female *Ae. aegypti*/ 675 boxes in the afternoon (24.1%), and no female *Ae. aegypti* were collected when the ambient temperature was below 17°C (Fig 2). It is possible that mosquitoes sought refuge in microhabitats in the morning that our boxes did not represent. Further, we only aspirated boxes two times per day that represent

coolest and warmest times of the day. Increasing monitoring throughout the day would improve further improve our understanding of mosquito thermal preferences. If mosquitoes prefer warmer microhabitats at the coldest temperatures, our ZIKV transmission risk estimates would increase, however, we expect estimates to remain lower relative to a model using ambient temperature as a proxy for mosquito temperature due to the strong preference for cooler temperatures at high ambient temperatures and the range of microhabitat temperatures at the coolest ambient temperatures likely not being dramatically warmer than ambient.

When our model accounted for thermal preferences of female *Ae. aegypti*, the estimate for R_0 is relatively low, even on the hottest days in Madera. There have been several temperate and subtropical *Ae. aegypti*-borne virus outbreaks, suggesting that at least in some temperate and subtropical regions, ZIKV should be able to sustain transmission. It is possible that these regions have inherently different risk that is not attributable to temperature (e.g. vector density), the R_0 parameter estimates we are using need to be improved and therefore we are underestimating risk, these regions have different weather patterns or microhabitat characteristics and availability, outbreak years had atypical weather patterns that allowed for an outbreak, and/ or mosquitoes have different spatial or thermal preferences in these regions. One study found that the 2015-2016 ZIKV outbreak in the Americas was fueled by an El Niño climate year that resulted in an R_0 anomaly not due to seasonal climate [23]. The same study showed a ZIKV outbreak was possible in California during the anomaly year when calculating risk using ambient temperature (R_0 peak > 5), however, they estimate the highest monthly R_0 for ZIKV in central California between 1980-2015 was between 3 and 5, which, though we used slightly different parameter estimates, encompasses our R_0 estimate on the representative hot day in Madera when using ambient temperature as a proxy for mosquito temperature (R_0=3.26) [23]. This suggests that our

estimates may represent R_0 decently well and that accounting for thermal preferences may decrease transmission to levels where sustained transmission is unlikely in California's Central Valley when peak season temperatures reflect the temperatures in this study period.

Daily ambient temperature maxima in Madera during peak *Ae. aegypti* season are often higher than in many tropical places, reaching 40°C and above, compared to a high around 30-32°C in most tropical regions during peak *Ae. aegypti* season, yet we know that ZIKV is capable of sustained transmission and outbreaks in many parts of the tropics. When the model accounted for mosquito thermal preferences in Madera, the mosquito daily temperature maxima were much lower than ambient temperature maxima and closer to the daily ambient temperatures maxima in tropical regions, leading to a relatively lower R_0 (Fig 3, Table 5). Additionally, expected mosquito temperature minima in Madera (18.5°C and 15.4°C) were much lower than the average ambient temperature minima in the tropics during peak *Ae. aegypti* (~25-26°C). As an exercise to understand how low ambient temperature minima affect R_0, we compared how a realistic ambient daily temperature range (DTR) in Iquitos, Peru vs. an ambient DTR in Madera, California around the same mean temperature affects R_0 using the same parameters listed in the methods section (S1 Table). Even though Madera reached 10°C warmer than Iquitos, R_0 was 0.5 times lower (3.26 in Madera vs 4.94 in Iquitos) due to the low temperature minimum. This suggests that the lower temp minimum and accounting for thermal preferences leads to the low relative R_0 in Madera, even on the hottest days.

Location	**Mean temperature (C)**	**Min temperature**	**Max temperature**	**ZIKV R_0**
Iquitos, Peru	28.5	26	31	4.94
Madera, California	28.5	18.3	40.6	3.26

Table S1: Assessment of how daily temperature ranges around the same mean daily temperature affect ZIKV R_0.

The effects of daily temperature ranges (DTR) on the parameters of R_0 have been examined in a few studies, however, our research shows that *Ae. aegypti* in central California do not experience the large DTRs prescribed to them in laboratory studies. In Madera, the ambient DTR was 21.7°C on both the representative hot and mild days, however, the DTR when mosquito thermal preferences were accounted for was 13.0°C-14.2°C. A large DTR (~19-20°C, mean=26 °C) led to a longer extrinsic incubation period for DENV in *Ae. aegypti* compared to small DTR (~8-10°C, mean=26°C) and constant temperature in one study, and did not affect EIP in another [24,25]. DTRs based on adult exposure temperatures and ranging from 10.8 to 14.2°C did not alter West Nile virus (WNV) EIP in *Culex tarsalis*, however overall WNV transmission risk is modified by DTR, highlighting the importance of understanding mosquito exposure temperatures [26]. Further, under a large DTR (~18°C, mean=26 °C), *Ae. aegypti* development and survival was affected, but a small DTR (~8°C, mean=26°C) led to minor or slightly beneficial changes in life history traits [27]. If affects of DTR are included in risk estimates without accounting for thermal preferences, we may overestimate the impact DTR has on viral transmission risk in subtropical and temperate regions where large DTRs can occur.

It is important to understand whether life history traits, including gonotrophic status and age, affect thermal preferences, which could affect transmission risk estimates. The majority of female *Ae. aegypti* collected in this study (77.1%) had neither blood nor eggs in their abdomen. In chapter 2 of this dissertation, we noted that bloodfed and gravid females 1 day post-feeding and beyond did not have large differences in thermal preferences on a gradient in the laboratory. None of the bloodfed females collected in this study were replete, whereas in chapter 2, all blood fed mosquitoes fed to repletion. Based on the conclusions from chapter 2, we would not expect

the bloodfed or gravid mosquitoes we collected in this study to have marked differences in thermal preferences, however we did not collect enough blood fed females to assess any potential differences gonotrophic status has on thermal preferences in the field. Additionally, in chapter 2 we noted that age did not have a strong effect on thermal preferences.

Thermal tolerance can vary within conspecific mosquito populations [28–31], although this is not well studied in *Ae. aegypti*, and, to our knowledge, thermal preferences have not been studied between conspecific mosquito populations. The risk model based on thermal preferences in California's Central Valley may not apply to other regions, as there may be differences in thermal preferences between populations. Further, the thermal landscape may vary and microhabitat availability and spatial distribution can affect behavioral thermoregulation [32]. Instead, future field studies should be conducted to understand the microhabitat availability in different regions, and to determine if resting preferences vary by region and/or population.

We would expect that female *Ae. aegypti* similarly tend to maximize fitness by avoiding extreme heat in the wet tropics, when possible, although we would expect the microhabitat temperatures to span a narrower range [33,34] than they do in dryer subtropical and temperate regions such as California that generally have larger seasonal variation and extreme daily temperature ranges due to low humidity. In temperate and subtropical regions, relative humidity may play a significant role in microhabitat preference and this should be explored further in relation to microhabitat selection and understanding transmission risk [35]. Another key difference between California and the tropics is that female *Ae. aegypti* are often found resting indoors in the tropics, whereas in regions of higher socio-economic status, the vast majority of *Ae. aegypti* rest outdoors [20,36]. Resting indoors vs. outdoors likely affects the microhabitat

temperatures available to mosquitoes, as would the built environment (air conditioner use, sprinklers, etc.).

To our knowledge, this is the first study to measure *Ae. aegypti* thermal preferences in the field and to examine how thermal preferences affect viral transmission risk relative to models that use ambient temperature as a proxy for mosquito temperatures. Incorporating microhabitat availability and thermal preferences, along with better estimates for the parameters of R_0 in subtropical and temperate regions, will improve mosquito-borne pathogen transmission risk estimates that inform mosquito surveillance and control decisions. Further, understanding thermal preferences may help improve vector control devices and/or techniques that leverage refuge-seeking behavior to attract *Ae. aegypti*.

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Figures and Tables

	Morning	Afternoon	Total
Number of box visits	333	675	1,008
Number of mosquitoes	527	952	1,479
Female *Ae. aegypti*	42	163	205
Male *Ae. aegypti*	54	270	324
Female *Cx. quinquefasciatus*	170	208	378
Female *Cx. tarsalis*	34	24	58
Male *Culex*	224	268	492

Table 1: **Summary of adult mosquitoes collected from resting boxes.**

Status	Number collected (% of total)
No blood or eggs	158 (77.1%)
Bloodfed/ Partially gravid with blood present in abdomen	22 (10.7%)
Gravid (no blood visible in abdomen)	25 (12.2%)
Total	205

Table 2: **Female *Ae. aegypti* collections by gonotrophic status.**

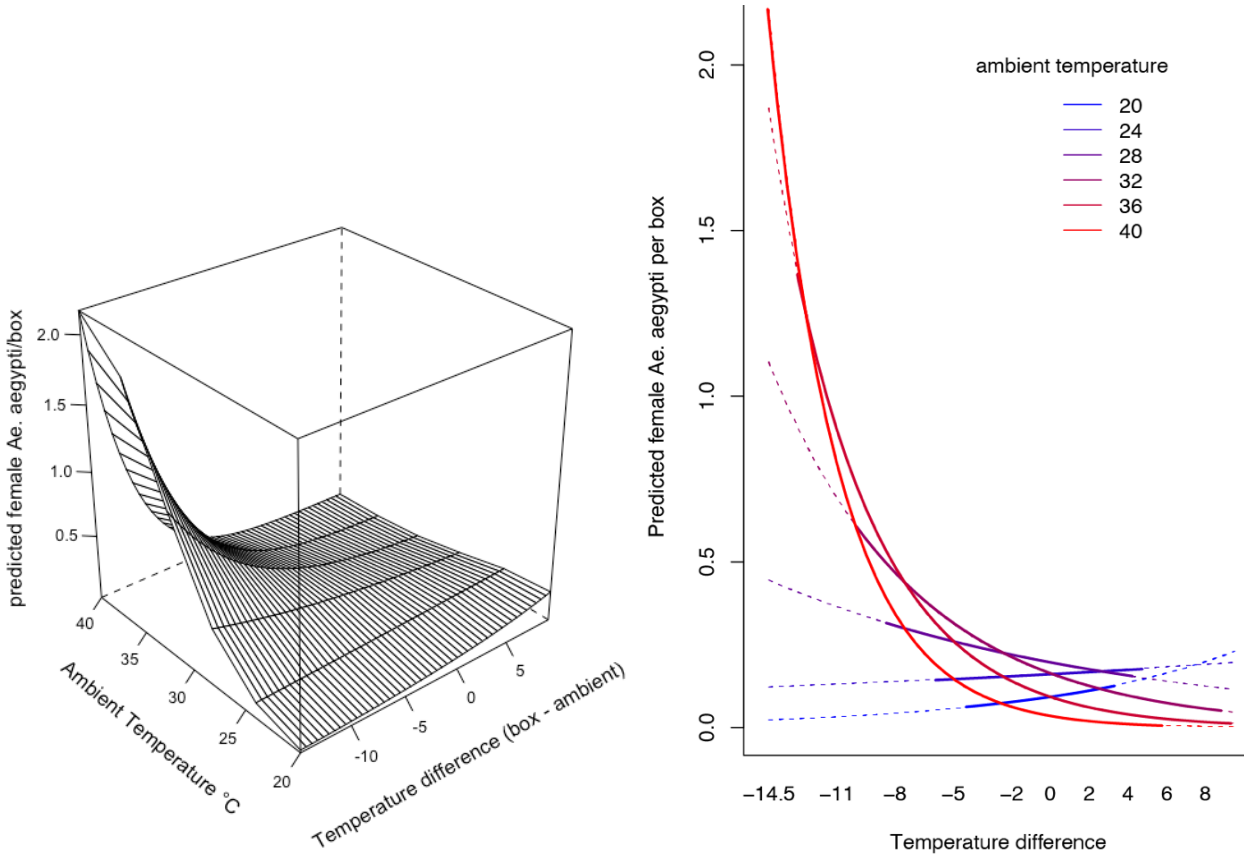

Figure 1: Predicted number of female *Ae. aegypti* per box as a function of ambient temperature and temperature difference (box temperature – ambient temperature). A. Wireframe plot. B. Predicted curves for each temperature. Dotted lines represent the predicted number of female *Ae. aegypti* per box over the maximum temperature difference recorded across ambient temperatures, whereas solid lines represent the range of box temperatures recorded for the listed ambient temperature +/- 2°C during our study period.

Variable	Coefficient (95% CI)	*P-value*
Intercept	-10.92 (-15.94, -6.26)	<0.001
Ambient temperature (°C)	0.67 (0.32, 1.03)	<0.001
Temperature difference (box temp- ambient temp)	0.48 (0.07, 0.89)	0.019
Temperature difference ^ 2	-0.01 (-0.02, -0.01)	<0.001
Ambient temp x temperature difference	-0.02 (-0.03, -0.01)	0.002

Table 3: Coefficients for negative binomial model.

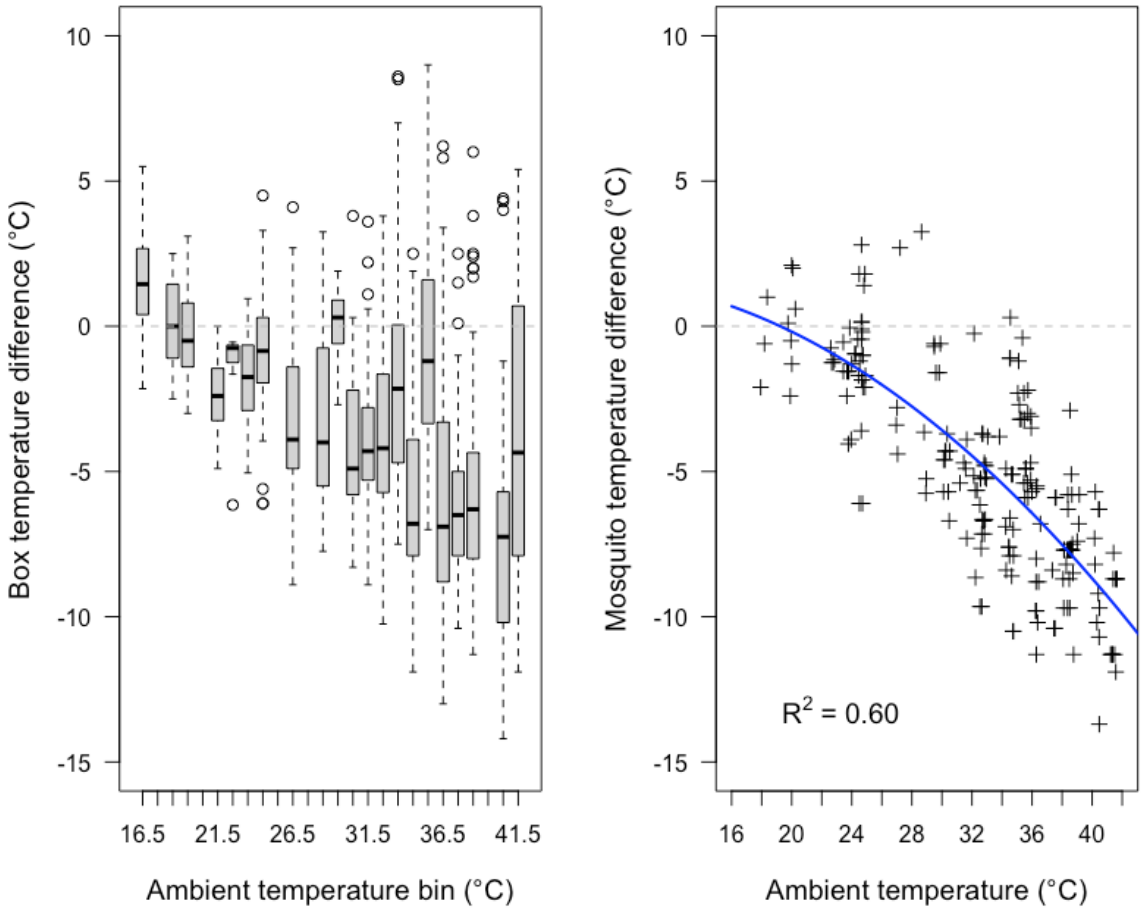

Figure 2: A. Resting box temperature differences for all 1°C ambient temperature bins. Solid black lines represent the median box temperature difference for each temperature bin. The horizontal dashed line represents no difference between box and ambient temperature. Number of boxes in each temperature bin are noted in Table 4. **B. Temperature difference of female *Ae.* aegypti collected.** Crosses represent resting box temperatures for each mosquito collected, and the blue like represents the quadratic model of expected mosquito resting temperatures relative to ambient temperature (R^2=0.60, $P < 0.001$).

Ambient temperature bin (C)	Number of boxes	Mean temp difference (°C) (box temp – ambient temp)	Number of female aegypti collected
(16-17]	40	1.51	0
(17-18]	0	N/A	0
(18-19]	56	0.08	3
(19-20]	55	-0.25	7
(20-21]	0	N/A	0
(21-22]	27	-2.41	0
(22-23]	29	-1.12	4
(23-24]	58	-1.92	11
(24-25]	89	-0.77	24
(25-26]	0	N/A	0
(26-27]	30	-3.25	4
(27-28]	0	N/A	0
(28-29]	24	-3.05	4
(29-30]	30	0.33	5
(30-31]	22	-3.99	9
(31-32]	29	-3.80	5
(32-33]	79	-3.84	24
(33-34]	28	-1.45	2
(34-35]	49	-5.79	16
(35-36]	87	-0.88	22
(36-37]	50	-4.99	10
(37-38]	30	-6.03	5
(38-39]	83	-5.66	28
(39-40]	0	N/A	0
(40-41]	30	-7.00	10
(41-42]	30	-4.05	11

Table 4: Distribution of boxes, mean box temperatures, female aegypti collected, and mean box temperatures of mosquitoes collected for each 1 degree ambient temperature bin.

Variable	Coefficient (95% CI)	*P-value*
Intercept	1.44	0.72
Ambient temperature (°C)	0.90	0.74
(Ambient temperature)^2	-0.01	<0.05

Table 5: Coefficients for quadratic model of expected mosquito resting temperatures relative to ambient temperature. R^2=0.60, $P < 0.001$.

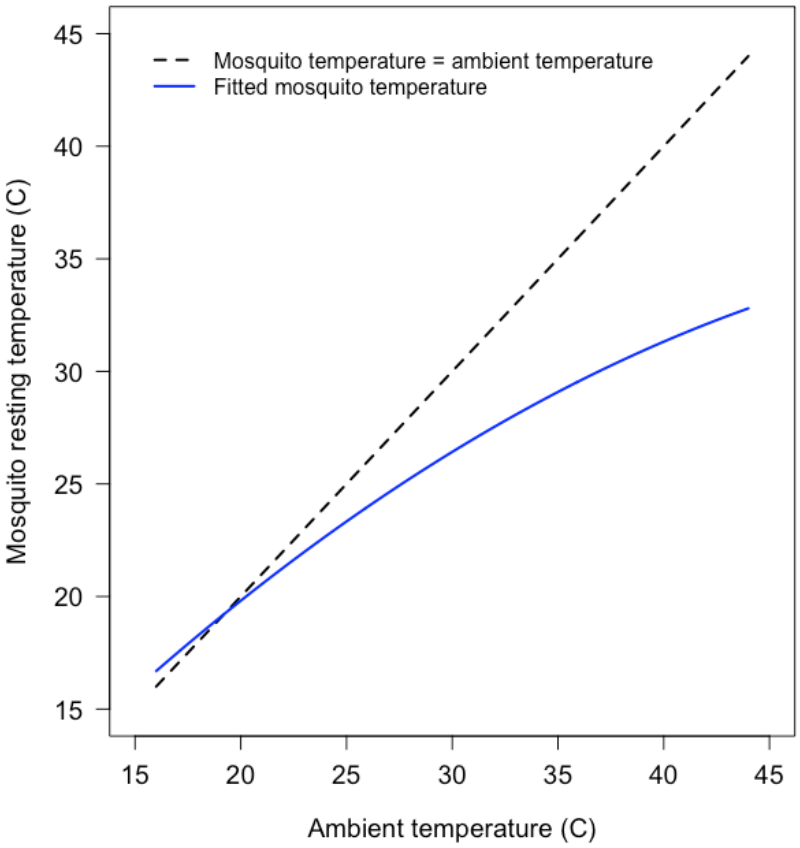

Fig 3: Mosquito resting temperatures with and without accounting for thermal preferences. Fitted mosquito temperature is determined by the quadratic model in table 5.

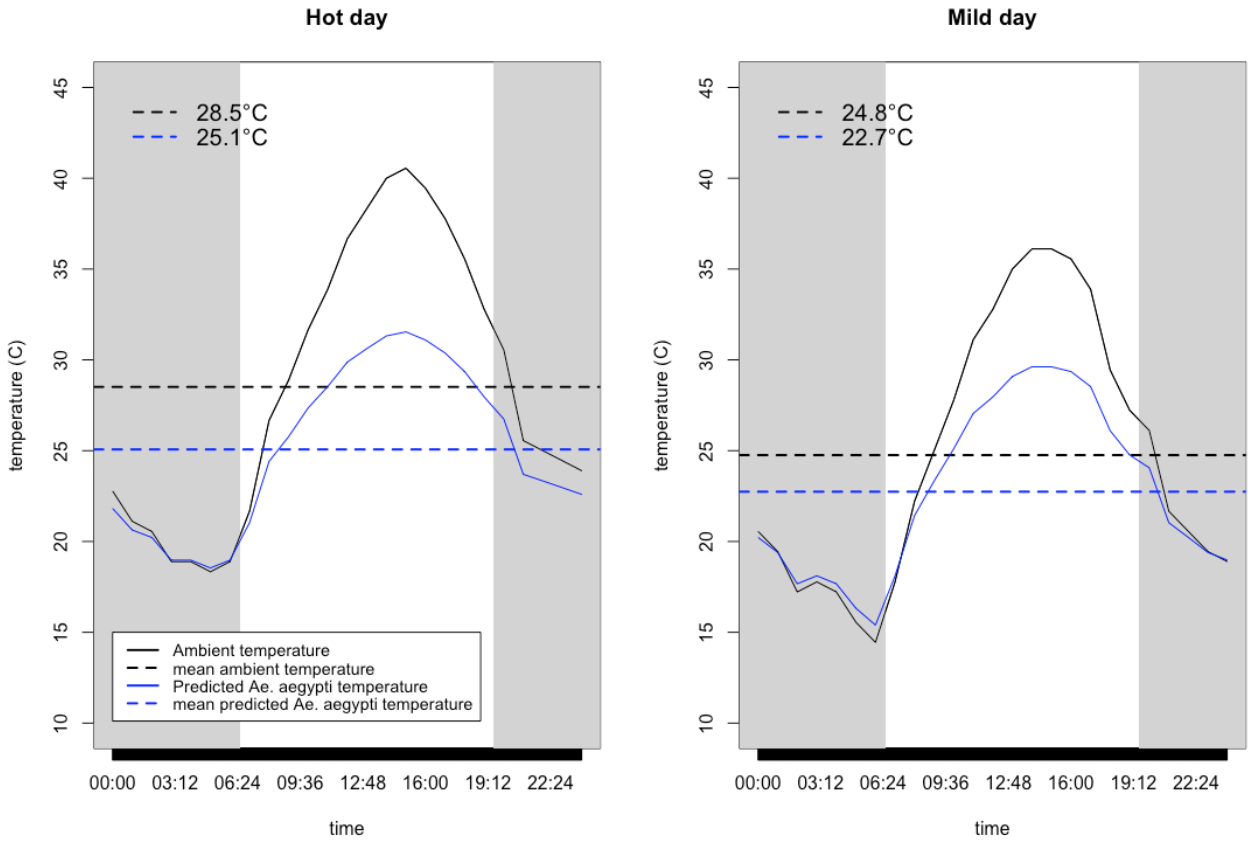

Fig 4: Expected mosquito exposure temperature for a female *Ae. aegypti* compared to ambient air temperature for a representative hot versus mild day during our study period.

Representative day	Temperature source	Mean temperature (C) (min, max)	Mosquito density relative to host	Biting rate	Zika vector competence	Probability of daily survival	EIP (days)	R_0
Hot (Sept 7)	Ambient	28.5 (18.3, 40.6)	5	0.46	0.8	0.79	6.1	3.26
	Mosquito preference model	25.1 (18.5, 31.5)	5	0.32	0.8	0.82	10.4	1.03
Mild (Sept 15)	Ambient	24.8 (14.4, 36.1)	5	0.30	0.8	0.82	11.0	0.82
	Mosquito preference model	22.7 (15.4, 29.6)	5	0.22	0.8	0.82	20.1	0.07

Table 5: Temperature, ZIKV vectorial capacity parameter estimates, and ZIKV R_0 for representative days with and without *Ae. aegypti* thermal preferences accounted for.

Location	Mean temperature (C)	Min temperature	Max temperature	ZIKV R_0
Iquitos, Peru	28.5	26	31	4.94
Madera, California	28.5	18.3	40.6	3.26

Table S1: Assessment of how daily temperature ranges around the same mean daily temperature affect ZIKV R_0.

S1fig: Resting box design.

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CONCLUSION

As the range of *Aedes aegypti* expands due to global connectivity and climate change [1–4], we will continue to see emerging and re-emerging pathogens transmitted by these mosquitoes. Mitigating the burden of disease from *Ae. aegypti*-borne viruses strongly relies on mosquito control. To make evidence-based mosquito control decisions to allocate limited resources and limit insecticide resistance, it is imperative to be able to accurately predict transmission dynamics of mosquito-borne pathogens.

When Zika virus emerged as a global human health threat in 2015-2016, our understanding of transmission risk relied on estimates from dengue virus, however species-specific estimates were needed. In chapter 1, we determined that ZIKV extrinsic incubation period is temperature-dependent and follows a similar trend to the EIPs of other mosquito-borne flaviviruses with warmer temperature resulting in shorter EIP. The temperature-EIP association for ZIKV was most similar to that of dengue virus, which is also transmitted by *Ae. aegypti*, compared to West Nile virus isolated from a temperate region. Overall, ZIKV EIP was shorter than DENV EIP across temperatures.

In chapters 2 and 3, we determined that female *Ae. aegypti* generally avoid high temperatures on a gradient in the laboratory, and field populations commonly selected microhabitat temperatures that differed from ambient air temperatures from nearby weather stations. Thus, "airport air temperatures" are often a poor proxy for the temperatures mosquitoes experience. Incorporating microhabitat availability and *Ae. aegypti* thermal preferences in California's Central Valley typically yielded reduced estimates of Zika virus transmission risk.

Taken together, these studies can improve transmission risk models to better inform mosquito control decisions, especially as *Ae. aegypti* continues to expand into subtropical and temperate environments.

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