Age-structured vectorial capacity in a ZIKV-*Ae. aegypti* model system

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ABSTRACT

The transmission of vector-borne diseases is a dynamic process dependent on a multitude of factors. A better understanding of the temporal factors that modify transmission is needed. Here, we examined the impacts of age at the time of infectious bloodmeal on the transmission potential of Zika virus (ZIKV) by *Aedes aegypti.* To do this, we applied three treatments: treatment Z, where mosquitoes were exposed to ZIKV at 5 days post-emergence; treatment M.Z, where mosquitoes were given a non-infectious bloodmeal at 5 days post-emergence, followed by a ZIKV-infectious bloodmeal at 12 days post-emergence; and treatment S.Z, where mosquitoes were exposed to ZIKV at 12 days post-emergence. We then observed the impact of these treatments on mosquito mortality, vector competence, and biting rate where we introduce a modification of the human landing rate to measure “laboratory bloodmeal landing rate” in the laboratory. Mosquitoes from treatment Z survived significantly longer than treatments M.Z and S.Z, though this difference was modest. There were no statistically significant differences observed in either vector competence or in biting

rates among the three treatment groups when assessed as functions of days post exposure. When results were put in the context of mosquito age, we found a significant age effect in both vector competence and biting rate. Subsequently, we combined the life traits and viral kinetics results and modified the vectorial capacity equation in a way that addresses these age-dependent interactions, providing insight into the transmission potential of arboviruses as a function of mosquito age. Our principal finding is that mosquito age at the time of the infectious blood meal affects the subsequent transmission potential of the arbovirus and offers a means by which to quantify this effect in our modified vectorial capacity equation.

Introduction

ZIka virus (ZIKV) is an arthropod-borne virus belonging to the family *Flaviviridae*. ZIKV was first isolated from a primate in Uganda in 1947, and shortly thereafter an *Aedes* mosquito in 1948 [1]. The primary vector in subsequent outbreaks is believed to be *Ae. aegypti* [2]. The virus emerged in the Americas in 2015 leading to an unprecedented outbreak, resulting in the WHO declaring ZIKV a public health emergency [3]. ZIKV rapidly spread across South America and there was short-lived autochthonous transmission in the Southern United States [4]. Unlike previous outbreaks, however, ZIKV has been associated with congenital malformations and autoimmune problems [1]. Because there are no antiviral treatments or vaccines available, prevention relies on vector control and understanding the temporal dynamics of virus-mosquito interactions.

Specifically, understanding the temporal nature of transmission and the heterogeneities inherent in such is key for precision in estimates of risk [5-9]. The most widely utilized measure of transmission potential is vectorial capacity (VC) given in the equation below.

$$VC = \frac{ma^2bp^N}{-\ln(p)}$$

where ‘m’ is the density of mosquitoes, ‘a’ is the biting rate, ‘b’ is the vector competence of the mosquito for a particular virus, ‘N” is the extrinsic incubation period, and ‘p’ is the daily probability of mosquito survival [10].

Vector competence is the ability of a mosquito to acquire and ultimately transmit a virus. In order for the mosquito to become infectious and transmit a virus, several events must occur. First, the mosquito must acquire an infectious bloodmeal and the virus must establish infection in the midgut. Next, the virus escapes the midgut into the hemocoel and hemolymph of the mosquito circulatory system, eventually making it to the salivary glands. Shortly after the virus establishes infection in the salivary glands, the mosquito becomes competent to transmit the virus to vertebrate hosts [5, 10]. The time it takes for this process to occur is referred to as the extrinsic incubation period (EIP). Not all mosquitoes that are exposed will be able to transmit, and the EIP is not a constant. EIP has often been inferred from static sampling done at days 7 and 14 dpi, which does not capture the dynamism of the process of vector competence [11-14]. The EIP_{50} is the time it takes for 50% of the exposed mosquito population to become infectious and offers a standardized means of reporting transmission potential of arboviruses and other vector-borne diseases [15, 16].

Additionally, mosquito mortality is a critical factor in the spread of arboviruses like ZIKV. Shorter lifespans may result in reductions in viral transmission as the mosquito may not survive the EIP [15, 17-19]. However, in the traditional vectorial capacity equation, the average probability of daily survival is used to determine the probability that a mosquito will live through the EIP, though the EIP should be thought of in the context of the age of the mosquito at the time of infectious bloodmeal [20, 21]. That is, the length of the EIP has differing consequences for a mosquito that receives an infectious bloodmeal at age 10 days old versus one that acquires virus at 20 days old.

Further, the biting rate of the mosquito is the most critical factor in determining transmission potential. Biting rate is difficult to quantify and is not often measured in laboratory studies [22, 23]. More often, biting rates are estimated by proxy. Human landing rates are the gold standard as they measure the number of female mosquitoes that land on a human during a given time [24]. While laboratory bloodmeal landing rates appear to have limited field relevance, it may be able to inform models which can then be compared to field data to potentially form suppositions about the makeup of the effective density of transmitting mosquitoes compared to the overall density of mosquitoes.

Ae. aegypti feed more than once and the timing at which an individual will acquire an infectious bloodmeal is not uniform [25]. Thus, the age at which an infectious bloodmeal occurs will determine the likelihood that the virus will get back out of the mosquito and transmission will continue. Studies have addressed some of the issues of age-structure in vectorial capacity, and this work builds on those studies [7, 17, 18, 21]. We were interested to know whether timing of the mosquito-infecting bloodmeal would

affect the transmission potential of ZIKV. To that end, we investigated age-specific life traits of *Ae. aegypti* mosquitoes – mortality, biting rate, and vector competence for ZIKV – and altered the vectorial capacity equation to account for these interactions relative to infectious bloodmeal timing. As many of the parameters of vectorial capacity vary across viral strain, genotype, and species, as well as mosquito species and conspecific populations, this model will have utility to investigate differential age-dependence of vector-pathogen interactions for determination of transmission potential.

Materials and Methods

Virus and mosquitoes

ZIKV strain PRVABC59 (ZIKV-PRV), originally isolated from a human patient in Puerto Rico in 2015, was provided by Dr. Barbara Johnson at the US Centers for Disease Control and Prevention. Prior to use, it was passaged three times in Vero cells and cultured as in [26]. Supernatant was collected 3 days post-inoculation and titer determined as previously described [26]. Titer of ZIKV was verified by qRT-PCR at approximately 8×10^7 plaque forming units (pfu)/mL, matched across all exposure experiments. Virus used for mosquito exposure was never frozen. Colony *Aedes aegypti* (Rockefeller) were provided by Dr. Daniel Swale of the LSU Entomology Department. Mosquitoes were maintained at constant conditions as in [27] with 16:8 light/dark periods and at 28°C constant temperature. Sucrose solution was removed 24 hours before experiments.

Blood-feeding and oral exposure of Ae. aegypti

At approximately 5 days post-emergence, mosquitoes were either exposed to an infectious bloodmeal at a 1:2 virus supernatant-to-blood ratio as in [27] or to a mock infected bloodmeal containing uninfected cell culture media at the same ratio as in [27]. Mosquitoes were allowed to feed via the Hemotek (Discovery Labs, UK) membrane feeding system for 45 minutes, after which mosquitoes were cold anesthetized and blood-fed females were placed into clean cartons until further treatment. Each treatment was repeated three times. The three treatment groups were: ZIKV-infectious bloodmeal given at 5 days post emergence (group ‘Z’), mock bloodmeal at 5 days post emergence followed by a ZIKV-infectious bloodmeal at approximately 12 days post emergence (group ‘M.Z’), and a last group that got only a single ZIKV-infectious bloodmeal at 12 days post emergence (group ‘S.Z’). To prevent confounding, we starved all groups 24 hours prior to days 5 and 12 post-emergence (regardless if that group received a bloodmeal) and all groups were cold anesthetized and moved to a new carton at days 5 and 12 post-emergence (again, regardless of whether they got a bloodmeal). Thus all cohorts were treated in the exact same way with the exception of treatment conditions. The experimental design is depicted in Figure 1.

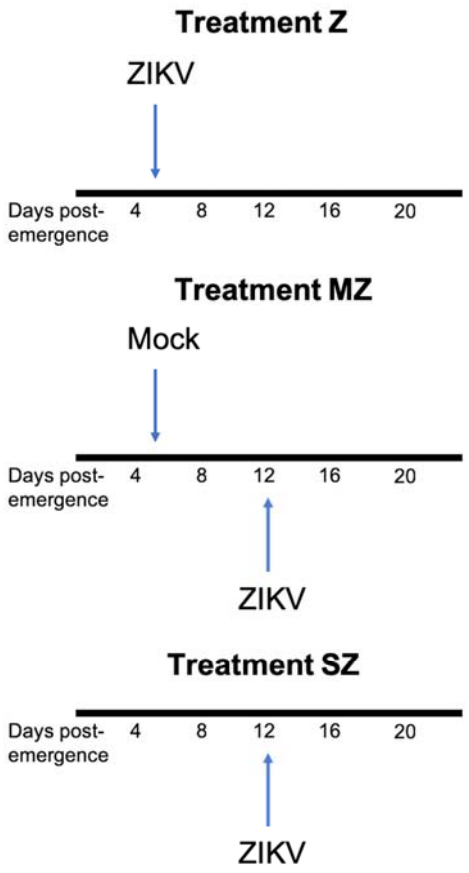

Figure 1. Illustration of treatment design.
Three treatments were applied to assess our hypothesis. Treatment Z (ZIKV) received an infectious bloodmeal at 5 days post-emergence (dpe); Treatment M.Z (mock-ZIKV) received a mock infectious bloodmeal at 5 days dpe, followed by an infectious bloodmeal at 12 dpe; and Treatment S.Z (sugar-ZIKV) received an infectious bloodmeal at 12 dpe.

Infection kinetics of ZIKV

For vector competence studies, mosquitoes were sampled at 5, 8, and 11 days post-exposure to test for infection and dissemination. Mosquito legs and bodies were put into separate tubes containing 900 uL BA-1 diluent media and nickel plated BBs. Samples were then homogenized twice at 25 Hz for 3 minutes using a Qiagen Tissuelyzer. RNA was extracted and tested for the presence of viral RNA (see below). Each treatment was replicated three times, and data are averages of all three replicates per treatment.

Transmission and determination of EIP50

In order to test for transmission, an additional 10 mosquitoes per ZIKV treatment were force-salivated. ZIKV-exposed mosquitoes were immobilized on ice before removing legs and wings. Mosquitoes were then placed on double-sided tape, and the proboscis of each mosquito was placed into a pipette tip containing 35 uL FBS with 3 mmol/L ATP for 30 minutes, as previously described in [28]. Contents of the pipette tip were ejected into 100 uL BA-1 diluent and stored before testing (see below).

The EIP_{50} was calculated by fitting a logistic regression (SSlogis function in R) to the days post infection (DPI) and obtaining the value of the function at 50%.

$$T(t_{DPI}) = \frac{s}{1 + exp^{-\frac{DPI-x}{k}}}$$

Where s is the asymptote, x is the value of x at the inflection point, and k is the scale parameter.

Mortality

Mortality studies were performed for the same three treatments as those used for vector competence (Z, M.Z, and S.Z) as well as appropriate negative controls where a mock bloodmeal was used in place of infectious bloodmeals. The three negative controls were: 1) a mock bloodmeal at 5 days post-emergence (M) to correspond to the Z treatment, 2) a mock bloodmeal at 5 days post-emergence, followed by another mock bloodmeal at 12 days post-emergence (M.M) to correspond to the M.Z treatment, and 3) a mock bloodmeal at 10 days post-emergence (S.M) to correspond to the S.Z treatment. Another treatment was created where the mosquitoes were cold anesthetized and sorted at approximately 5 days and 12 days post-emergence, but

were never blood fed (S). All treatments were cold anesthetized at 5 days, regardless of whether they were offered a bloodmeal so that all mosquitoes experienced the same treatment and mosquitoes were kept at relatively similar density/carton with an average of 47 mosquitoes/carton (range 36-58). Each mortality treatment was repeated a total of three times and data are averages of the three replicates.

Mosquitoes were checked daily and, when applicable, dead mosquitoes counted for 23 days post initial treatment. For the purposes of this illustrative study, we took the mortality study out to 23 days post exposure, or 28 days old (approximately 1 month), as this has been shown to be the upper limit of believed survival of *Ae. aegypti* and is similar to the range used in Tesla, et al. [28, 29]. For treatments where mosquitoes were offered a later bloodmeal (M.M, S.M, M.Z, and S.Z), only mosquitoes that took one or both bloodmeals were included in the mortality analyses. During the mortality study, dead mosquitoes of ZIKV-exposed treatments were removed from the carton, processed, and tested for infection and dissemination. Studies have previously shown that viral RNA detection from dead mosquitoes was successful [30]. At 28 days old, remaining live mosquitoes were taken down, counted, processed, and tested for infection and dissemination.

Daily mortality estimates relative to age ($M(t_{age})$) were then predicted fitting an asymptomatic regression (SSasymp function) using a non-linear least squares method where:

$$M\left(t_{age}\right) = s + (r - s) * exp^{-exp^{c} * (-age)}$$

where s is the asymptote, r is the zero response parameter, and c is the logarithmic rate constant.

Laboratory blood-meal landing rates

Ten to twelve mosquitoes per day post exposure from each ZIKV-infection treatments (Z, M.Z, and S.Z) were placed in individual, clear plastic canisters (Bioquip) twenty-four hours before being provided a bloodmeal via membrane feeder using 1 mL discs with 800 μL of blood (Hemotek, Discovery Labs, UK). Blood was kept at constant temperature of 37°C and placed on top of the canisters. Herein we devised a method of measuring a proxy biting rate similar to human landing rates, "laboratory bloodmeal landing rate" [23]. This was assessed in two ways. First, mosquitoes were observed through the clear canister for their general position in the canister and second, the disc was removed to determine if they were on or near the mesh at the top of the canister. In all cases, these two methods of observation matched. That is, if a mosquito was observed to be at the blood meal prior to disc removal (looking through the canister), she did not move to the bottom of the canister upon disc removal.

A mosquito was assessed as "landed" and recorded as "1" if the female was at the top of the canister or "not landed" and recorded as "0" if she was at the bottom of the canister. This observation was done at 1, 20, and 45 minutes post placement of the disc and the disc was replaced between observation time points.

The sum of "landings" was taken for each mosquito as the individual frequency of biting. The daily biting rate relative to age ($B(t_{age})$) was then predicted using the same asymptotic regression model as described above.

$$B(t_{age}) = s + (r - s) * exp^{-exp^{c} * (-age)}$$

Additionally, we calculated a probability of biting where the frequency of biting from above was characterized binomially as either having bitten at least once of the

three observations (coded as “1”) or not at all (coded as “0”). The probability of biting as a function of age ($Z(t_{age})$) was determined by fitting the proportion of mosquitoes that landed or fed at least once a day using a self-starting non-linear least squares regression as above (SSasymp).

$$Z(t_{age}) = s + (r - s) * exp^{-exp^{c} * (-age)}$$

For the ZIKV treatment, biting behavior was assessed at days post emergence 10, 13, 16 (to represent 5, 8, and 11 days post exposure) as well as 17, 20, 23, and 28 days post emergence to match age with the M.Z and S.Z treatments. (For clarity, days 17, 20, and 23 in M.Z and S.Z treatments correspond to days 5, 8, 11 post-exposure).

The blood that individual mosquitoes were exposed to were collected and serum was separated by centrifugation at 6,000 rpm for 4 minutes at 4 degrees. Serum was tested for the presence of ZIKV via RNA extraction and qRT-PCR.

Viral detection

RNA extractions performed using the MagMax-96 kit on a King Fisher nucleic acid extraction instrument according to the manufacturer’s instructions as in [26]. Viral RNA was then detected via qRT-PCR using the SuperScript III One-Step RT-PCR system with Platinum Taq on a Roche Lightcycler 480. Protocols and primers used were previously described [31]. Samples with suspected positive Cp values above 35 were inoculated onto confluent 6-well plates of Vero cells, rocked for 45 minutes, and supernatant collected at 5 days post inoculation. For abdomen, leg, and forced salivation samples, plating was performed with M199 Medium containing 2% FBS and 2% PSF. For serum samples, plating was performed with M199 Medium containing 2%

FBS and 4% PSF. Wells containing serum samples were also washed after rocking. RNA was extracted and tested for the presence of viral RNA and tested as described. Upon second testing, if the Cp value was again greater than 35, they were considered negative.

Statistics

All statistics and subsequent graphics were performed and generated using R version 3.4.3. To test for differences in mortality rates among treatments, Kaplan-Meier survival analyses were conducted and the average time to death (TTD) estimated. Differences among the treatment groups for infection, dissemination, and transmission rates were tested by a chi-square test for multiple proportions on a day-by-day basis. Biting rates were tested using a non-parametric analysis of variance (Kruskal-Wallis) for treatment over each day post exposure.

Vectorial capacity as a function of timing of infectious bloodmeal

Using the data generated for blood feeding as a proxy for biting rate (see Results), the mortality data to estimate daily probabilities of survival, and our transmission data from forced salivation to predict the EIP_{50} of ZIKV in our mosquito colony, we altered the vectorial capacity equation as follows to estimate vectorial capacity as a function of when the mosquito acquires an infectious bloodmeal. In addition, we add the parameter of probability of biting as a function of age (z).

$$VC_{age} = \frac{m(z_{age}a_{age} * z_i a_i)b\prod_1^i p_i}{-\ln\,(p_{age})}$$

Here we derive an age-structured vectorial capcity (VC_{age}) with the average time to transmission of EIP_{50}. We then allow VC_{age} to be customized based on the timing of the infectious bloodmeal taken by the mosquito.

To account for age-dependent mortality, we assign two different mortalities. The traditional calculation of p^N calculated the probability of a mosquito living through to an age of N, the EIP. In the context of an age-dependent vectorial capacity framework, we can calculate a more precise probability of surviving based on the day the mosquito bit (and obtained an infection) as p_i where *i* is the age at the time of a mosquito-infecting bloodmeal + EIP_{50}. Allowing for the daily survival correction (denominator), p_{age} is the probability of daily survival at the age of the mosquito acquiring a ZIKV infection. Where p_{age} is 100%, we substituted 99% in order to satisfy non-zero denominator. Similarly, we derive an age-dependent biting rate. Whereas a^2 – as the average biting rate – is squared to account for the bite at which point a mosquito acquires an infection and the second bite when the mosquito transmits this infection. Putting this in the context of age-dependence, a^2 becomes the product of a_{age} (the biting rate at the age of infection acquisition) and a_i (the biting rate at *i*). In addition, we introduced an additional parameter to determine if accounting for the probability of biting per day is impactful. This was parameterized as z_{age} and z_i to account for the probability of biting at times *age* and *i*, as defined above.

Results

<u>Mortality of *Aedes aegypti* is affected by exposure status and timing of bloodmeal</u>

Pairwise comparisons of the ZIKV-infection treatments determined that the Z group had a significantly longer TTD when compared to M.Z and S.Z (Figure 2). The average time to death for the Z group was 25.9 days, 25.3 days for the M.Z group, and 24.5 days for the S.Z group. When the appropriate unexposed controls were compared to each infection treatment group, there was a significant difference between the S.Z and S.M comparison only, with an estimated difference in TTD of two days (Supplemental Table 1). Of interest, the non-blood fed sugar-only controls died significantly faster than any of the other treatments with an average TTD of 19.6 days (Supplemental Table 1).

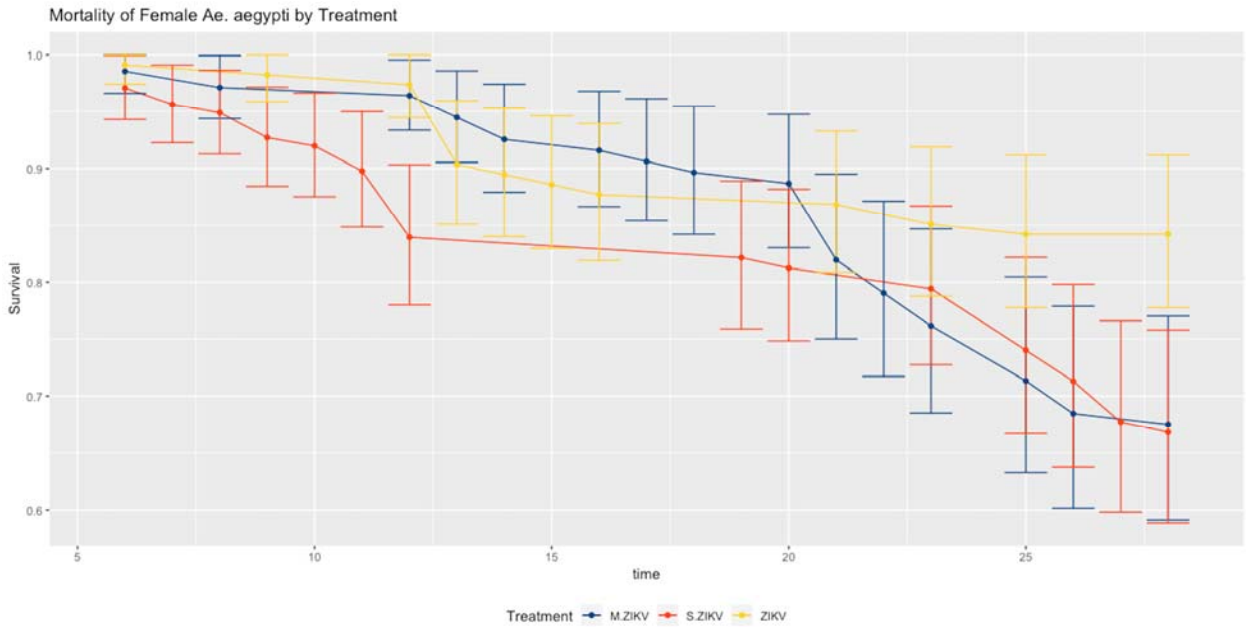

Figure 2. Survival curves of female *Ae. aegypti* by treatment.
Each line represents the combined data from three replicates per treatment: Zika (Z, blue line), Mock-ZIKV (M.Z, red line), and sugar-ZIKA (S.Z, yellow line). Average time to death of treatment Z was significantly longer than treatments M.Z and S.Z.

To determine if there was a generalized effect of exposure on mortality we compared all groups with a ZIKV exposure to those without, excepting the group that received no bloodmeal at all which was removed from the analysis. There was no significant difference between ZIKV-exposed mosquitoes (groups Z, M.Z, and S.Z) and the non-exposed groups ($p>.05$). When we predicted the daily survival rates, we pooled all three ZIKV treatments together (Z, M.Z, S.Z) and obtained the parameter estimates for the non-linear fit (Supplemental Figure 1). Though there was a significant difference between Z and the M.Z and S.Z groups, the difference was relatively modest (0.6 and 1.4 days, respectively). The infection and dissemination rates of the dead mosquitoes are given in Supplemental Table 2.

ZIKV Infection Kinetics and Transmission Potential

When we tested for differences in the infection and dissemination rates of *Ae. aegypti* after ZIKV exposure, we found that the kinetics were virtually the same across all three treatments when assessed as a function of days post exposure (dpe) (Figure 3A and3C). When we tested the proportion of infected and disseminated mosquitoes per day among treatments, there were no significant differences noted (Supplemental Table 3). Figure 3B and 3D show the same data as a function of age, rather than dpe.

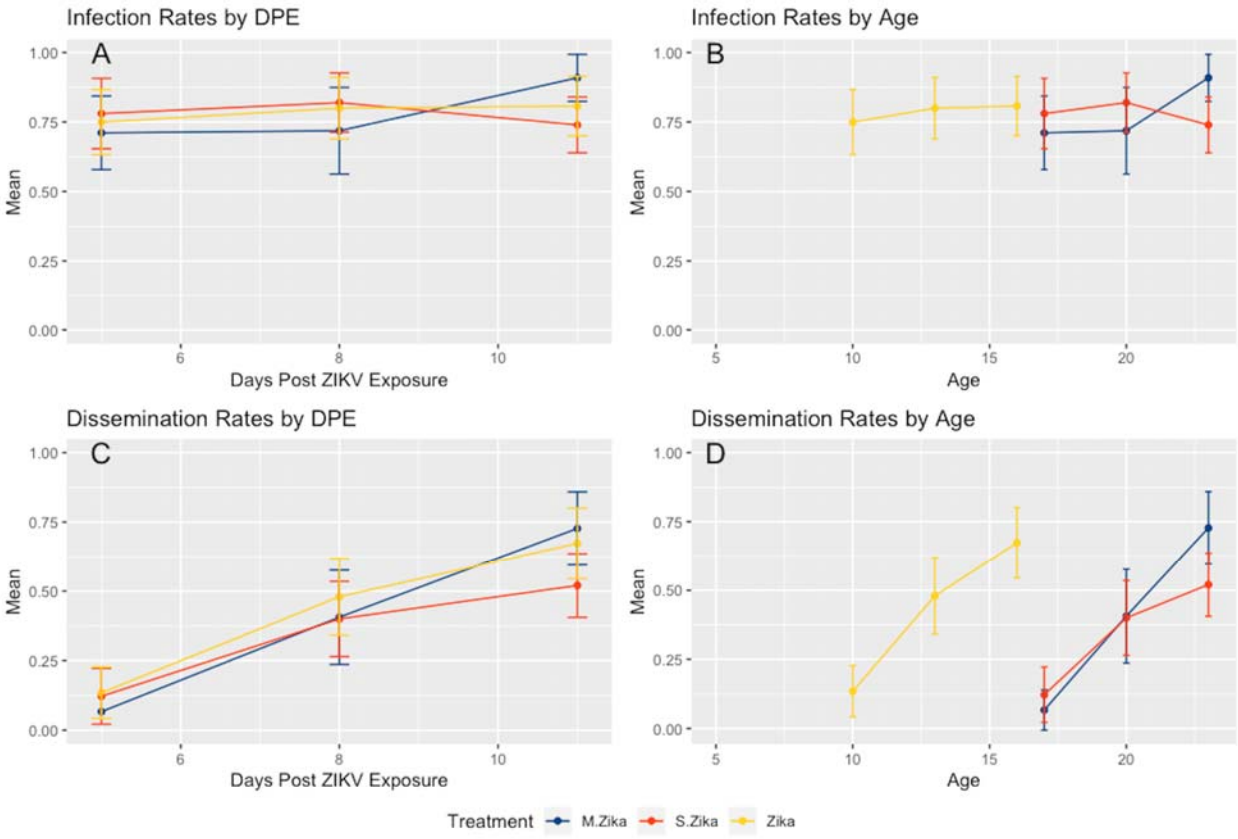

Figure 3. Infection and dissemination rates per treatments.
Each line represents the combined data from three replicates per treatment. Abdomens/legs sampled on days 5, 8, and 11 post-exposure. Rates of infection/dissemination examined in the context of days post-exposure (A)(C) and mosquito age (B)(D). No significant differences were noted between treatments at any days post-exposure.

<u>EIP_{50} and interaction with mosquito age</u>

We first compared the proportion of transmitting mosquitoes at shared days post exposure (5, 8, and 11 dpe). There was no transmission from any of the groups at days 5 and 8 post exposure. At day 11 post exposure, there was no significant difference in transmission between the three treatments ($p>0.05$). Based on our findings with infection and dissemination, we wanted to examine transmission in the context of age. We compared the proportion of transmitting mosquitoes at shared ages (17, 20, 23, and 28 days post-emergence). At ages 17 and 20, only 10% of mosquitoes from Z showed

transmission, with no signs of transmission for the other two treatments. At age 23, we found a significant difference among the three treatments ($p<0.05$). Z had the highest proportion of transmission (60%), while S.Z had low transmission (10%) and M.Z had no signs of transmission. Significant differences were also found among the three treatments at age 28 ($p<0.05$), with no signs of transmission for M.Z or S.Z, and 60% transmission for Z. This is summarized in Table 1.

We determined the EIP_{50}, or the time needed for 50% of the mosquitoes to become infectious, for the Z-group only, as this was the only treatment to reach 50% transmission rates. The EIP_{50} was determined to be 18.8 days post-exposure. This translates to 23.8 days old in our system.

Table 1: Transmission rates for each day post exposure (DPE) and corresponding mosquito age for each of the three treatment groups.

Treatment	DPE (Age)	% Transmission (n)
Z	5 (10)	0 (10)
	8 (13)	0 (10)
	11 (16)	10 (10)
	12 (17)	10 (10)
	15 (20)	10 (10)
	18 (23)	60 (10)
	23 (28)	60 (10)
M.Z	5 (17)	0 (10)
	8 (20)	0 (10)
	11 (23)	0 (10)
	16 (28)	0 (10)

S.Z	5 (17)	0 (10)
	8 (20)	0 (10)
	11 (23)	10 (10)
	16 (28)	0 (10)

Biting Rates

Using a modification of human landing rates which we term “bloodmeal landing rate”, we were able to estimate the biting rates as a function of age. There was no difference among the three treatments with regard to biting rates across days post exposure ($p>.05$) (Figure 4A). When we re-center the data and test for differences among the groups as a function of age, there were no significant differences ($p>.05$) (Figure 4B). We then combined all three treatments into a single exposed group and determined whether infection or dissemination status affected the biting rate. There were no significant differences in biting rates between infected mosquitoes and uninfected mosquitoes, as well as those with a disseminated infection and those without at each day post exposure ($p>.05$), though an effect of infection has been shown for DENV-2 and DENV-3 [32-34]. Again combining all the treatments into a single group due to no statistical differences, we determined that there was a day-to-day difference in biting rate by age ($p<.0001$), indicating that age does affect the biting rate of *Ae. aegypti*. The non-linear least squares fit is shown in Figure 4C, with a lower asymptote of 0.

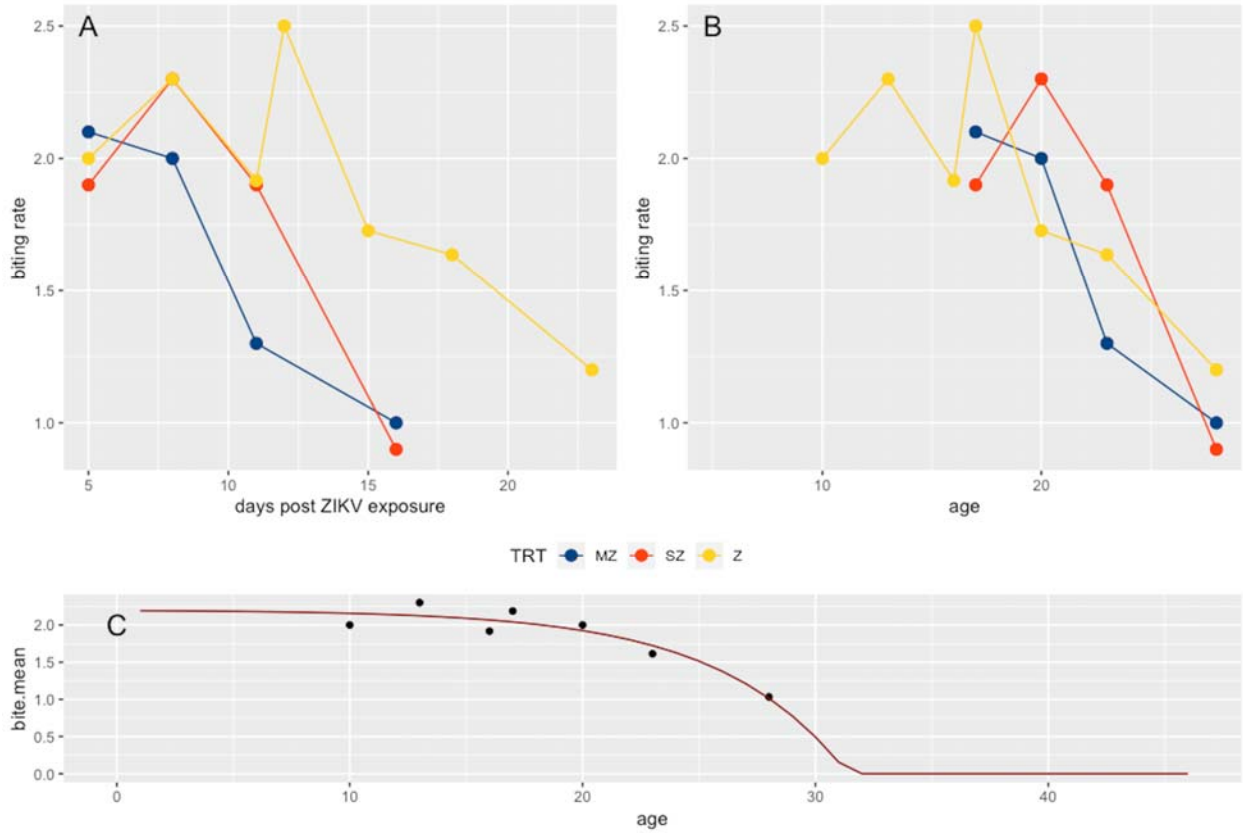

Figure 4. Biting rates per treatment. (A) Estimated biting rate per each treatment as a function of day post ZIKV exposure. (B) Estimated biting rates per treatment as a function of mosquito age. (C) Collapsed biting rates as a function of age across all three treatments (dots) and daily biting rate predictions from fitted model (red line).

In addition, the proportion of mosquitoes that fed per day post emergence was not significantly different among the treatment groups (p>.05). The individual per-day biting proportions and the results of the non-linear least squares fit of probability of biting as a function of age are given in Supplemental Figure 2.

<u>Vectorial Capacity as a function of age at the time of mosquito-infectious bloodmeal</u>

When we calculated VC_{age} with all average values (and assuming a constant density of 1), we determined that the VC_{age} of ZIKV in our colony mosquitoes was 0.3119338 indicating a low likelihood of outbreak. However, when we calculated VC_{50}

as a function of the timing of the infectious bloodmeal, only days 1-10 post emergence resulted in VC_{age} values greater than 1, indicative of outbreak potential [20]. The range of VC_{age} over these 10 days was 66 at its height (day 1 post emergence) and 1.06 at day 10 post emergence. This is shown in Figure 5, where we demarcate the window of opportunity for biting based on age.

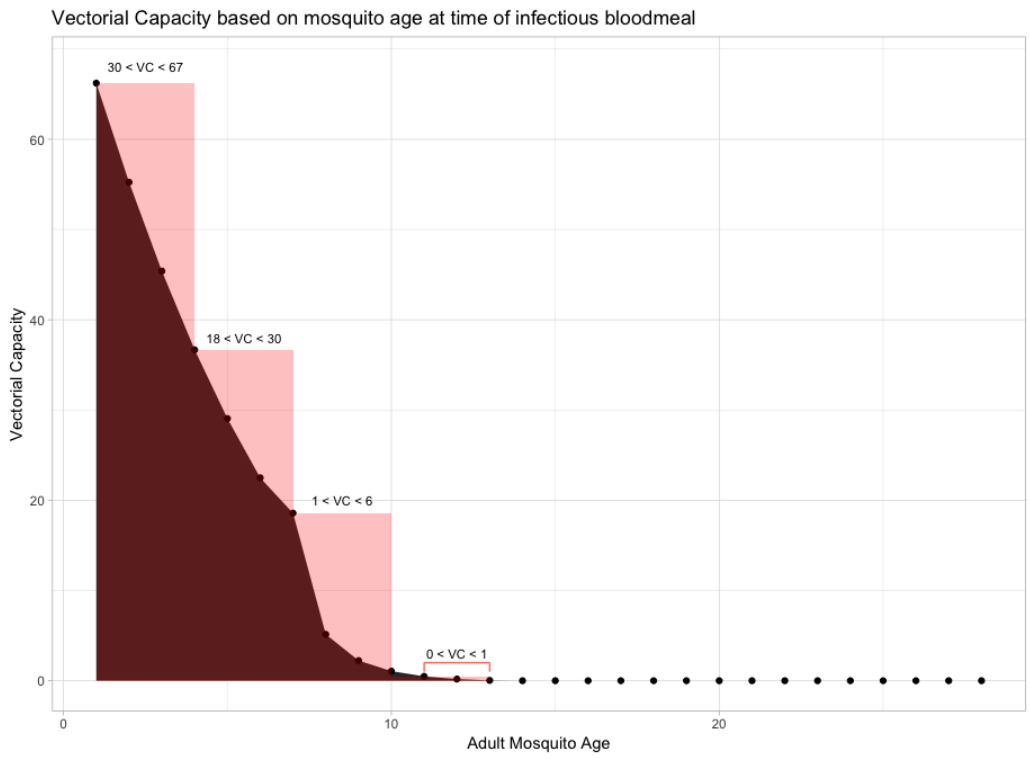

Figure 5. Age-structured vectorial capacity (VC_{age}) based on mosquito age at time of infectious bloodmeal. The extrinsic incubation period 50 (EIP_{50}), or the time it takes for 50% of the population to be transmitting, was used to calculate the VC_{age}. Red shading shows the time range post-emergence in which mosquitoes must bite in order to survive the EIP_{50} (days 1-10).

Discussion

Heterogeneity in vector-borne disease transmission systems has been shown to alter predictions of disease spread [5, 10, 27]. Temperature, for example, has been shown to be a critical modifier of vector competence and mosquito life traits [28, 35, 36]. Variable infectiousness of the human and the subsequent acquisition of virus by a mosquito has also been shown to have different estimates of transmission compared to average measures of virus acquisition by the vector [37] We demonstrate here that the interactions of several life traits and the viral kinetics of ZIKV can be parameterized in an age-structured framework.

Further, we demonstrate that this framework yields drastically different results relative to a measure of VC_{age} that uses averages for parameterization. At first glance, an EIP_{50} of 18.8 days would suggest that ZIKV transmission is an inefficient process, considering that laboratory mosquitoes likely live longer than populations in the field. However, by taking into account predicted daily survival and biting rates based on our data, we show that there is transmission potential ($VC_{age} \geq 1$) when the infectious bloodmeal is obtained within the first 10 days post emergence. We introduce the inclusion of the probability of biting per day to compliment biting rates. When we excluded the probability of feeding per day, the novel parameter introduced here, the range of VC_{50} increased to 71.3-1.6 over 10 days post emergence. This is likely due to the high values of predicted biting probabilities in the first 10 days post emergence. However, more data is needed to fully define the patterns of biting behaviors in *Ae. aegypti*, especially at early time points.

Of course, our system represents an artificial one where colony mosquitoes have a higher inclination to take an artificial bloodmeal, which is based entirely on heat seeking cues and does not account for other olfactory and chemosensory attractants, and may have altered vector competence than field populations [38-40]. It has been shown, however, that heat alone is sufficient to attract mosquitoes and at least initiate host seeking behaviors [41]. However, the main purpose of our study was to demonstrate, critically, that the timing of an infectious bloodmeal, coupled with heterogeneity in mosquito life-traits and the interactions with viral kinetics alter estimates of VC_{age}.

We show that mosquito age at the time of the infectious bite affects the subsequent transmission potential of the arbovirus, and offers a means by which to quantify this effect in our modified vectorial capacity equation. Indeed, if we alter the EIP_{50} to 8 days, as some data suggests CHIKV to have [42], the VC_{age} reaches 203 if the infectious bite occurs on the first day post emergence and there VC_{age} <1 at 18 days post emergence. However, field infection rates remain quite low in surveillance settings for several *Aedes*-borne viruses, which suggests that the number of mosquitoes that fall within this window are quite small [43-45]. This could be due to several reasons. First, the effective density of mosquitoes, which we did not address in this effort, may not be a straightforward measure. Entomological indices such as the house index (HI), the container index (CI), or the Breteau index (BI) are all indirect measures of density (how many juveniles/houses (HI), how many containers are infected with juvenile stages (CI), or breeding containers/100 houses (BI)) and do not directly assess actual contact and have not been good corollaries of disease [46, 47]. In addition, these measures often

occur outside or adjacent to houses, while transmission of *Aedes*-borne viruses has been shown to occur at the household level [48]. This suggests that a small subset of mosquitoes may be responsible for most of the transmission, and could necessitate further adaptation of the vectorial capacity equation to account for epidemiological relevant measures of mosquito density.

Our study uses a laboratory system to investigate the consequences of alternative parameterization of the vectorial capacity equation. We show that several parameters are age-dependent and offer inclusion of a new parameter of biting probability in addition to the daily biting rate. The influence of individual age-dependent parameters should be carefully studied in the context of field-caught material and outbreak relevant strains in order to fully appreciate the impact and necessity of these modifications.

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S1 Figure. Observed and predicted daily probabilities of survival. Combined observed daily survival rates of all three treatments (dots) and the predicted daily survival rates (red curve).

S2 Figure. Observed and predicted probabilities of daily biting. The observed daily biting rates (dots) from the "blood-meal" landing experiments and the fitted predictions (red curve) with a force lower asymptote of 0.

S1 Table. Average time to death and sample size for ZIKV-infection treatments and unexposed controls used in the mortality study. Exposed groups were the three treatments exposed to ZIKV: Z -- ZIKV at 5 days post exposure (dpe); M.Z – mock bloodmeal at 5 dpe and ZIKV bloodmeal at 12 dpe; S.Z – only a ZIKV bloodmeal at 12 dpe. Unexposed groups were not exposed to ZIKV but matched for bloodmeal uptake: M – mock bloodmeal at 5 dpe; M.M – mock bloodmeals at 5 and 12 dpe; S.M – mock bloodmeal at 12 dpe; and S – no bloodmeal, sugar only. Average times to death (TTD) and total sample sizes per treatment are given below.

S2 Table. Daily infection and dissemination proportions of dead or censored mosquitoes from the three ZIKV-infection treatments of the mortality study. Each day when a mosquito was observed to have died, that mosquito was tested for presence of ZIKV RNA in the abdomen (infection) and legs (disseminated infection). The proportion of daily dead mosquitoes that were infected and/or disseminated are given below, as well as the total number tested (n).

S3 Table. Infection and dissemination rates for each day post-exposure (DPE) and corresponding mosquito age for each of the three treatments. Percent infection was determined by the proportion of infected abdomens over total exposed; and percent dissemination was determined as the proportion of infected legs over total exposed. Three replicates were performed for each treatment, but proportions and sample sizes (n) are combined from all three replicates.

S4 Table. Modeled fits of parameters, the type of model, and the parameter values. For each parameter where predictions were made from experimental data, the type of model (and R self-starting function) and parameter estimates for each fit are given below.