Vector competence for Zika virus unaffected by mosquito age but vectorial capacity of *Ae. aegypti* is multifactorial and age-dependent

E. Handly Mayton[1], A. Ryan Tramonte[1], Rebecca C. Christofferson*[1,2]

[1]Department of Pathobiological Sciences, School of Veterinary Medicine, Department of Pathobiological Sciences, Baton Rouge, USA

[2]Center for Computation and Technology, Louisiana State University, Baton Rouge, USA

* Indicates corresponding author

Email: rcarri1@lsu.edu

ABSTRACT

The transmission of Zika virus (ZIKV) is a dynamic process defined by multiple factors. Specifically, the interaction of factors like vector competence and extrinsic incubation period (EIP) and age-dependent life traits has not been well quantified. We investigated the impact of mosquito age at time of exposure on the vector competence/EIP of *Aedes aegypti* for ZIKV, and found no significant differences between mosquitoes exposed at 5- or 12-days post-emergence. However, when these results were coupled with age-dependent life traits determined experimentally - lifespan and biting rate – we illustrate the necessity of putting vector competence and EIP into an age-structured construct. We demonstrate this by modifying the vectorial capacity (VC) equation, which describes the number of secondary cases of vector infection given the introduction of an infectious individual into a naïve population. By deriving an age-structured measure (VC_{age}), we are able to quantitatively demonstrate the dynamism of the interaction of viral:vector transmission factors. These impacts of age are intuitive; however, our model puts such intuition into a quantitative framework. As technologies to age mosquitoes (or other vectors) in the field are pursued, VC_{age} can inform hypotheses regarding the factors identifying the proportion of vectors that transmit relative to the total population.

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 has been identified as *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, which can be aided by an in-depth understanding of the temporal dynamics of virus-mosquito interactions.

The most important virus-mosquito interaction is that of vector competence. 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 [5,6]. The time it takes for this process to occur is referred to as the extrinsic incubation period (EIP).

The union of vector competence and EIP into a single, dynamic measure of the temporal process of mosquito infectiousness allows for a more comprehensive understanding of this process [5,7-10]. Not all mosquitoes that are

exposed will be able to transmit (vector competence) and the time it takes for those mosquitoes that will transmit is not a constant (EIP). 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].

Many things affect vector competence including vector species, discrete populations within species, and environmental factors [5,8,12,13,17,18]. Several recent studies have focused on environmental factors, such as temperature, and found that temperature not only affects vector competence of ZIKV and related arboviruses in *Aedes aegypti* but also the life traits of the mosquito [19-21]. Thus, we wanted to determine if the vector competence and the EIP of ZIKV was altered due to the age at which a mosquito becomes exposed.

A study using near-infrared spectroscopy was able to predict the age of female *Ae. aegypti* +/- 2 days, indicating that determining the age-structure of a mosquito population is possible, and that such technology could be refined for field studies[22]. As these technologies are pursued and refined, there will be a need for ways to understand and quantify interactions among vector competence and EIP and lifespan and biting rate[23]. Mosquito mortality is a critical factor in the spread of arboviruses, as shorter lifespans may result in reductions in viral transmission as the mosquito may not survive the EIP [15,24-26]. Biting rate is another critical factor in determining transmission potential but is difficult to

quantify and not often measured in laboratory studies [27,28]. More often, biting rates are estimated by proxy, and human landing rates (HLR) is considered the gold standard. HLR measure the number of female mosquitoes that land on a human during a given time [29]. We undertook to develop a modification of this method – the laboratory bloodmeal landing rate – to determine and quantify the extent to which age effects transmission. *Ae. aegypti* take a blood meal more than once and the timing at which an individual will acquire an infectious bloodmeal is not uniform [30]. 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.

Vectorial capacity (VC) was derived as a measure of transmission potential of a vector-borne pathogen by a competent vector [6,7,15,31-33]. VC is the vector-centric equivalent to the basic reproduction number (R_0) and describes the number of secondary cases of vector infection given the introduction of an infectious individual into a naïve population.

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

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 [6]. Studies have addressed some of the issues of age-structure in vectorial capacity, and this work builds on those studies [8,17,24,25].

Through a series of laboratory experiments, we are able to determine the effect of mosquito age at the time of ZIKV exposure on vector competence and

EIP, as well as quantify the interaction with of these results with age-dependent life traits. And while determining the precise age of mosquitoes in the field is currently unavailable, our results provide data-driven age distributions of key parameters, and also provide insights into the age-structured transmission potential of ZIKV in *Ae. aegypti*.

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 [34]. Supernatant was collected 3 days post-inoculation and titer determined as previously described [34]. Titer of ZIKV was verified by qRT-PCR at approximately 8 x 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. To isolate the effect(s) of age, mosquitoes were maintained at constant conditions as in [35] 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*

To test for differences in vector competence/EIP due to age, we designed the following experiment. Group ZIKV mosquitoes were exposed to a ZIKV-infectious bloodmeal at 5 days post-emergence. Group S.ZIKV mosquitoes were exposed to a ZIKV-infectious bloodmeal at approximately 12 days post-emergence. An additional group (Group M.ZIKV) was included to determine if an older group that had a previous bloodmeal altered the virus:vector interactions of interest. This group was given a mock-infected bloodmeal at 5 days post-emergence and an infectious bloodmeal at 12 days post-emergence.

Bloodmeals were prepared with either ZIKV-infected cell culture supernatant or non-infected cell culture supernatant. Bovine blood in Alsever's solution from Hemostat Labs (Dixon, CA) was used in a 2:1 blood to supernatant ratio [35]. Mosquitoes were fed 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. 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 Supplementary Fig. S1.

Vector competence and EIP of ZIKV

Because studies have linked mortality and infection and/or dissemination status of mosquitoes with arboviruses, we wanted to better understand the infection and dissemination process of our system. Thus, mosquitoes were sampled at 5, 8, and 11 days post-infection (dpi) to test for infection and dissemination. Mosquito legs and bodies were put into separate tubes containing 900 μL 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 details below). Each treatment was repeated a total of three times and data are averages of the three replicates.

To determine the effects of age on transmission and to derive the EIP_{50}, an additional 10 mosquitoes per treatment per dpi were force-salivated at the 5, 8, and 11 dpi. For context, 10 mosquitoes from the ZIKV group were also force salivated at days 17, 20, and 23 dpi, which corresponds to an age-match of 5, 8, and 11 dpi of the M.ZIKV and S.ZIKV groups. Following the mortality study, each group had an additional 10 mosquitoes/day force salivated at the *a priori* end of the mortality study, corresponding to 23 dpi (see details of mortality study below).

Briefly, 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 μL FBS with 3 mmol/L ATP for 30 minutes, as previously described in [19]. Contents of the pipette tip were ejected into 100 μL 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 (ZIKV, M.ZIKV, and S.ZIKV). We added additional mock bloodmeal controls (that is, accounting for any infectious bloodmeal-associated alteration of mortality) where a mock bloodmeal was used in place of infectious bloodmeals. The three controls were: 1) a mock bloodmeal at 5 days post-emergence (M) to correspond to the ZIKV 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.ZIKV treatment, and 3) a mock bloodmeal at 12 days post-emergence (S.M) to correspond to the S.ZIKV treatment. An additional negative control treatment was performed where the mosquitoes were never blood fed (S). All treatments were cold anesthetized at 5 and 12 days post-emergence, regardless of whether they were offered a bloodmeal so that all mosquitoes experienced the same treatment, and mosquito density per carton was kept relatively constant 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.

Cartons were checked daily and, when present, dead mosquitoes were counted and removed up to 28 days post emergence (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. [18,19]. Only mosquitoes that took all offered bloodmeals were included in the mortality analyses. For ZIKV-exposed treatments, dead mosquitoes were removed from the cartons when observed, processed, and tested for infection and dissemination as above [36]. At 28 days old, remaining live mosquitoes were freeze-killed and counted (coded as censored). For those in ZIKV-exposed treatments, these mosquitoes were processed and tested for infection and dissemination as above.

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:

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

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

Laboratory blood-meal landing rates

We devised a method of measuring a proxy biting rate similar to human landing rates, “laboratory bloodmeal landing rate (LBLR)” [28]. Ten to twelve mosquitoes per each ZIKV-infected treatment (ZIKV, M.ZIKV, and S.ZIKV) 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). This was done at the same DPI schedule as the vector competence studies above. LBLR was assessed using a two-tiered approach. 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.

Thus, 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” per mosquito per day is used as the individual frequency of biting. The daily biting rate relative to age ($B(t_{age})$) was then predicted over all the summed biting frequencies using the same asymptotic regression model as described above.

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

Additionally, because not all mosquitoes showed interest in ‘biting’ each day, we calculated a daily probability of biting where mosquitoes were characterized binomially as either having “landed” 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.

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

For treatment ZIKV, biting behavior was assessed at days post emergence 10, 13, 16 (to correspond to 5, 8, and 11 dpi) as well as 17, 20, 23, and 28 days post emergence to match age with the M.ZIKV and S.ZIKV treatments. (For clarity, days 17, 20, and 23 post-emergence corresponds to 5, 8, and 11 dpi for treatments M.ZIKV and S.ZIKV). Day 28 day post-emergence was, again, the final day of the mortality study.

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 [34]. 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 [37]. Samples with a Cp value $\leq$ 35 were interpreted as positive, while samples with a Cp value > 35 were interpreted as suspected positive. These samples 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. 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 rank sum test) for treatment over each dpi and, separately, age. Differences in the probabilities of biting was analyzed using logistic regression, again testing for effects of dpi and age separately.

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 capacity (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. All parameters and functions used to fit the data are given in Supplementary Table S1.

Results

Vector competence and EIP_{50} does not vary with age

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 (Figure 1a and 1c). Across each dpi, there were no statistically significant differences among the proportions of infected and disseminated mosquitoes across treatments ($p>0.05$) (Supplementary Table S2).

Again, we compared the proportion of transmitting mosquitoes across all treatments at each dpi and found no significant differences ($p>0.05$). There was no transmission from any of the groups at 5 and 8 dpi. At 11 dpi, 1 mosquito had virus in the saliva from group ZIKV while the M.ZIKV and S.ZIKV groups had none. There was no significant difference among these groups at 11 dpi, however ($p>0.05$).

For illustrative purposes, Fig. 1b and 1d show the infection and dissemination data as a function of age, rather than dpi. Table 1, likewise, has the average transmission proportions per dpi and age. It is intuitive that mosquitoes that are exposed sooner would have higher infection and dissemination rates as older individuals. Given no apparent differences in the vector competence among the three treatments, we tested additional mosquitoes from group ZIKV at older time points (Table 1). From these data, we determined the EIP_{50}, or the time needed for 50% of the mosquitoes to become infectious. The EIP_{50} was determined to be 18.8 dpi.

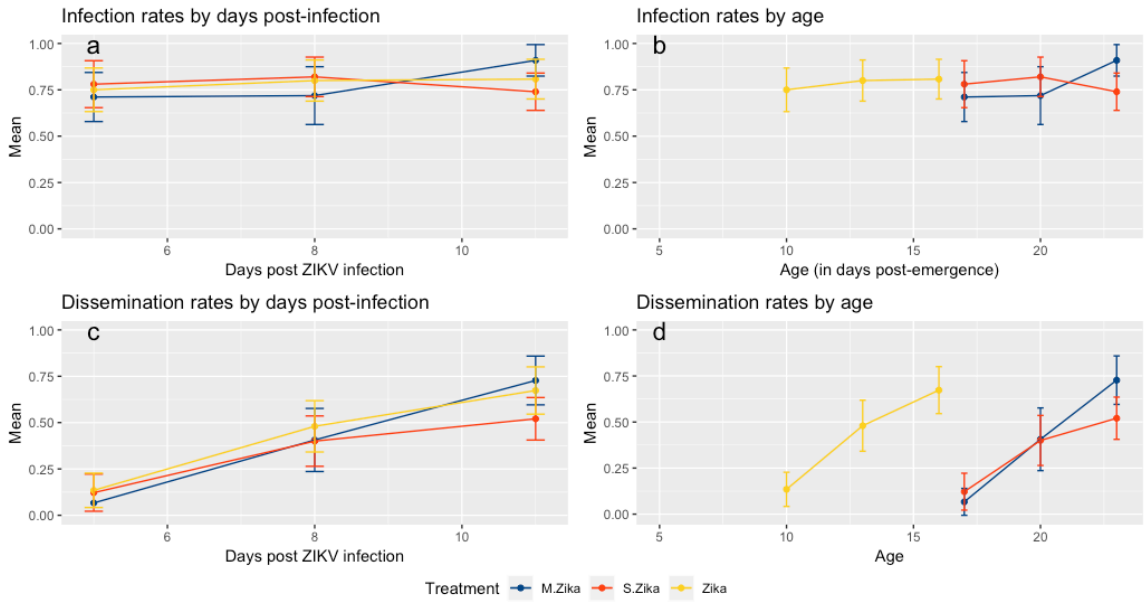

Figure 1. Infection and dissemination rates per treatments.
Each line represents the combined data from three replicates per treatment. Abdomens/legs sampled on 5, 8, and 11 days post-infection (dpi). Rates of infection/dissemination examined in the context of days post-infection (a)(c) and mosquito age (b)(d). No significant differences were noted between treatments at any days post-exposure.

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

Treatment	dpi (Age)	% Transmission (n)
ZIKV	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.ZIKV	5 (17)	0 (10)
	8 (20)	0 (10)
	11 (23)	0 (10)
	16 (28)	0 (10)
S.ZIKV	5 (17)	0 (10)
	8 (20)	0 (10)
	11 (23)	10 (10)
	16 (28)	0 (10)

Mortality of *Aedes aegypti* is modestly affected by exposure status and timing of bloodmeal

Pairwise comparisons of the ZIKV-infection treatments determined that group ZIKV had a significantly longer average time to death (TTD) when compared to groups M.ZIKV and S.ZIKV, though this difference was modest (Figure 2). The TTD for the Z group was 25.9 days, 25.3 days for the M.ZIKV group, and 24.5 days for the S.ZIKV group. When the corresponding mock-bloodmeal treatment were compared to each infection treatment group, there

was a significant difference between the S.ZIKV and S.M treatments only, with an estimated difference in TTD of two days (Supplementary Table S3). Give these results and an EIP_{50} of 18.8 dpi, and that the two older groups (S.ZIKV and M.ZIKV) were fed at 12 days old, these two groups would not, on average, survive long enough to transmit (12 + EIP_{50} = 30.8 days old for average transmission time). The non-linear fit and predicted daily mortality rate was performed on Treatment ZIKV (Supplementary Figure S2). The infection and dissemination trends for each treatment in the mortality study are given in Supplementary Table S4.

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 (Supplementary Table S3). When all ZIKV infected treatments were pooled into a single group and the uninfected, blood-fed controls were another group, there was no significant difference in mortality ($p<.70$). When we predicted the daily survival rates, we pooled all three ZIKV treatments together (ZIKV, M.ZIKV, S.ZIKV) and obtained the parameter estimates for the non-linear fit (Supplementary Figure S2). Though there was a significant difference between ZIKV and the M.ZIKV and S.ZIKV groups, the differences were quite small (0.6 and 1.4 days, respectively).

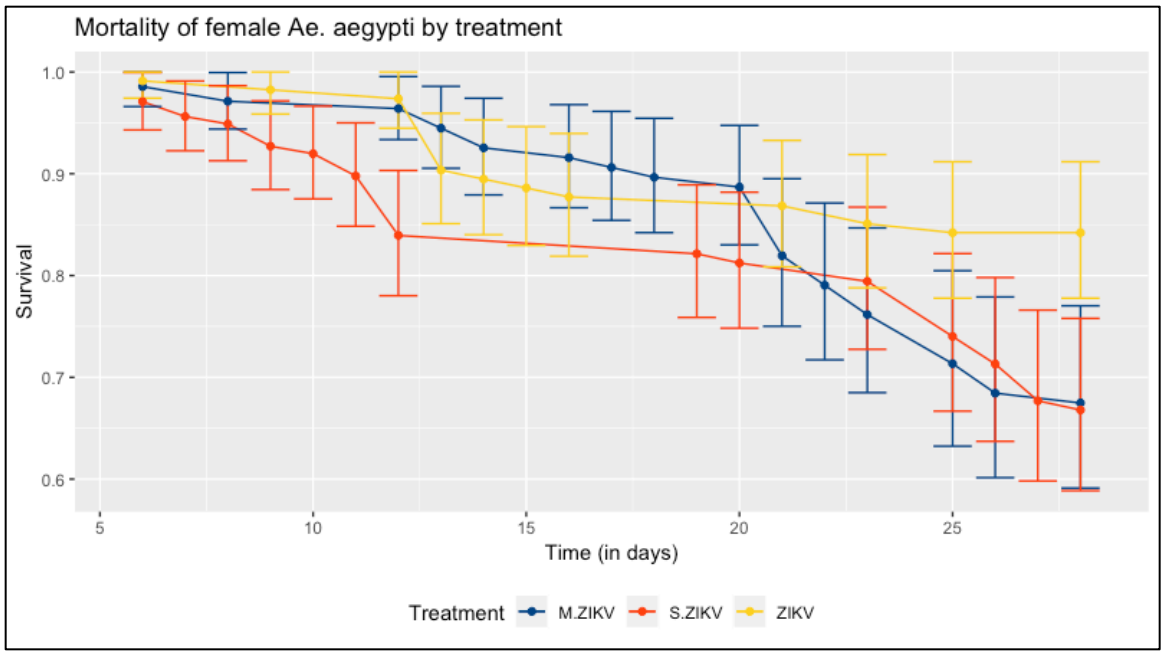

Figure 2. Survival curves of female *Ae. aegypti* by treatment.
Each line represents the combined data from three replicates per treatment: ZIKV (ZIKV, blue line), Mock-ZIKV (M.ZIKV, red line), and sugar-ZIKV (S.ZIKV, yellow line). Average time to death of treatment ZIKV was significantly longer than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

Biting rate is not affected by infection or dissemination status, but is age-dependent

Using LBLR, we were able to estimate the biting rates as a function of age. There were no differences among the three treatments with regard to biting rates across dpi ($p>.05$) (Figure 3a). In addition, when the same data was tested on a day-by-day basis but as a function of age rather than dpi, there was no significant different among the treatment groups ($p>.05$) (Figure 3b). We then combined all three treatments and analyzed the biting rate among those mosquitoes found to have been infected and/or disseminated versus those that

were not. We found that there were no significant differences in biting rates based on infection or dissemination status ($p>.05$), though an effect of infection has been shown for DENV-2 and DENV-3 [38-40]. Due to no statistical differences among groups or across infection/dissemination status, we combined all groups into a single cohort and determine that there was an overall effect of age ($p<.0001$) based on a non-linear least squares fit, with a lower asymptote enforced at 0 (Figure 3c). This shows the general trend of a decrease in biting rate as either dpi or age increase.

In addition to biting rate, we used LBLR to determine whether the proportion of mosquitoes that fed per dpi was affected by age at infectious blood meal. There was no significant effect of treatment, but time was significant both when assessed as dpi and age where the probability of a mosquito biting decreased with an increase in either dpi or age ($p>.05$). Again, pooling the three treatments and with the additional age time points from the ZIKV group, we predicted 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 (Supplementary Figure 3).

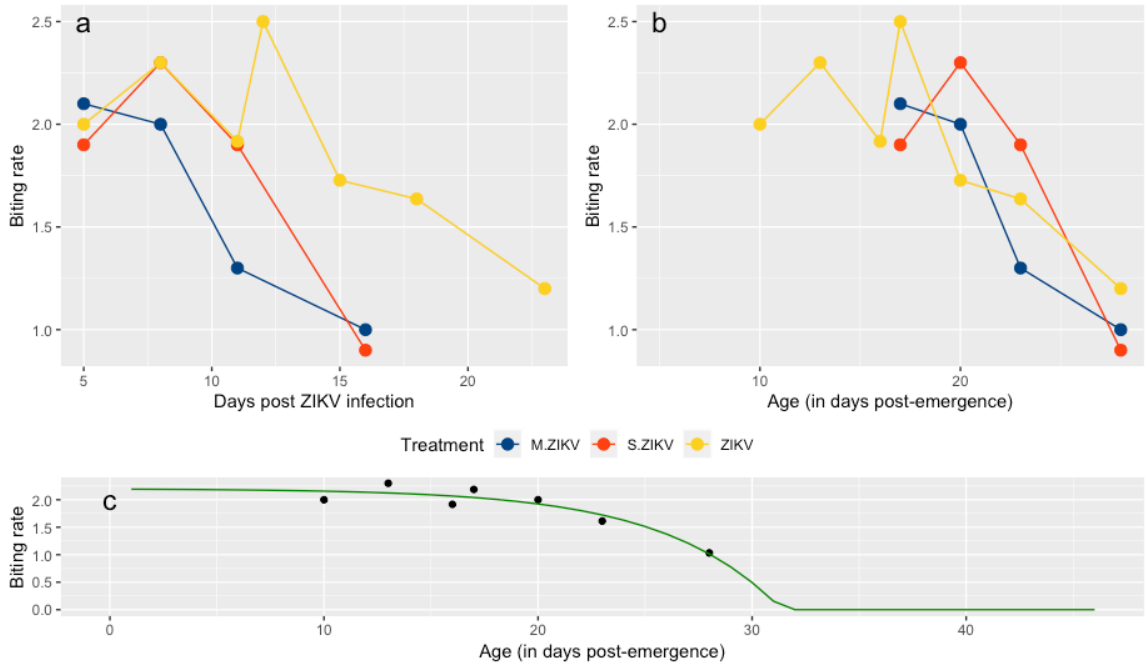

Figure 3. Biting rates per treatment. (a) Estimated biting rate per each treatment as a function of day post-infection (dpi). (b) Estimated biting rates per treatment as a function of mosquito age. (c) Collapsed biting rates as a function of age across all three treatments (dots) and daily biting rate predictions from fitted model (green line).

Comparison of traditional calculation of vectorial capacity and VC_{age}

Only one of our treatments (Treatment ZIKV) was a viable transmission risk, given that the other two treatments did not, on average, survive long enough to reach the EIP_{50}. Thus, we calculated VC_{age} using the mortality function from treatment ZIKV and the pooled non-linear fits for biting rate and probability of biting. For comparison, we first calculated VC with all average values (and assuming a constant density of 1) and determined that the average VC of ZIKV in our mosquitoes was 0.32. As this value is less than 1, this would indicate that there is a low likelihood of an outbreak [41]. However, when we take into

consideration the effects of age and calculate VC_{age}, this risk assessment changes drastically and in an age-dependent manner. During the first 10 days post emergence, VC_{age} was greater than 1, indicative of outbreak potential and in direct contrast to the interpretation of the traditional, average-based calculation. Indeed, 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 Fig. 4, where we demarcate the window of opportunity for biting based on age.

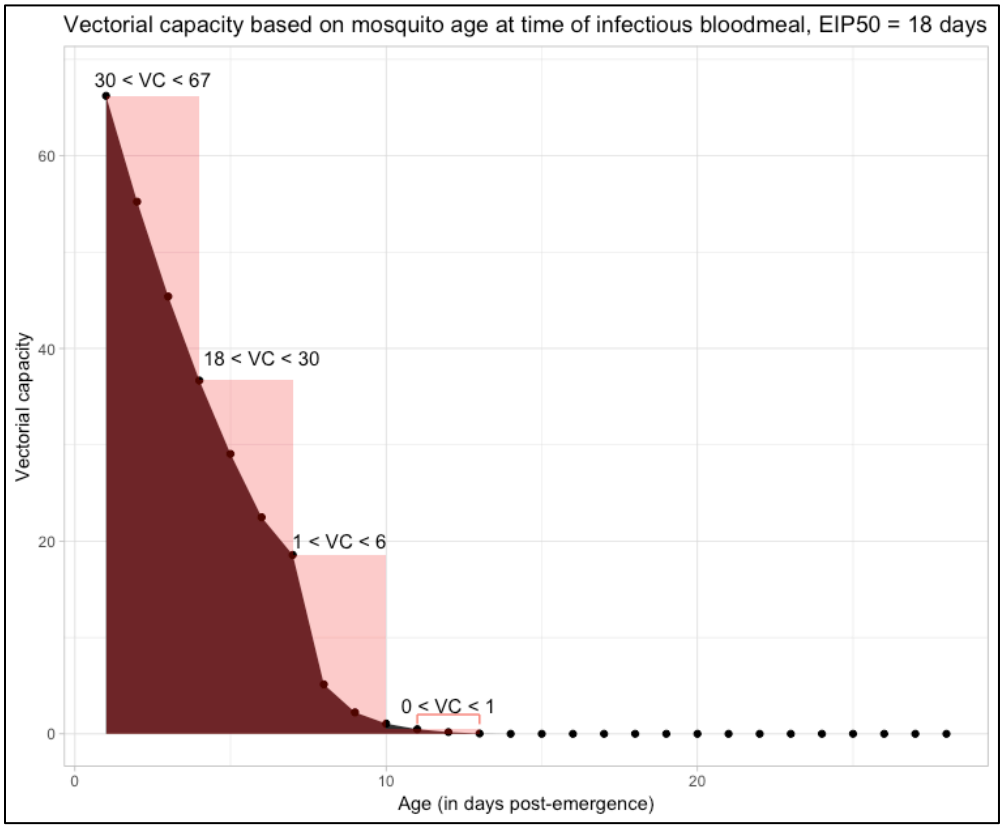

Figure 4. Age-structured vectorial capacity (VC_{age}) based on mosquito age at time of infectious bloodmeal. Dark red area under the curve corresponds to the VC_{age} (y-axis) if a mosquito were to acquire a ZIKV infection at that day post emergence (age, x-axis) based on the parameterization from the experimental data. The light red bars show VC binned across the days equal to the width of the bar.

Discussion

Heterogeneity in vector-borne disease transmission systems has been shown to alter predictions of disease spread [5,6,35]. Temperature, for example, has been shown to be a critical modifier of vector competence and mosquito life traits [19,20,42]. Accounting for heterogeneity of the infectiousness of the human host and the subsequent acquisition of virus by a mosquito has also been shown to lead to differential estimates of transmission compared to the same risk quantification using average measures [43]. Likewise, we demonstrate that mosquito age is a powerful driver of transmission potential due, in large part, to the age-dependence of daily mortality and biting habits. Further, these drivers lead to large differences in the quantification of outbreak potential. In this case, it was the difference between declaring a risk for outbreak or not (Figure 4).

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 compared to field populations [44-46]. It has been shown, however, that heat alone is sufficient to attract mosquitoes and at least initiate host seeking behaviors [47]. We also demonstrate that the inclusion of a new parameter of biting probability and the LBLR method can help quantify age-dependence of daily biting behavior and rates.

Modifications to VC can be used to inform hypotheses regarding the effective density of mosquito vectors, defined as that proportion of mosquitoes

that are transmitting. We demonstrate how this proportion is affected, in part, by the effects of age. These results are intuitive. The earlier a mosquito acquires an infection, the more chances it has to transmit that infection. However, our VC_{age} model, like other models with age-structured vector populations, can be used to put this intuition into a quantitative framework and our experimental findings offer more insights into the importance of the age-dependence of virus:vector interactions. Indeed, at first glance, an EIP_{50} of 18.8 days would suggest that ZIKV transmission is an inefficient process given an average lifespan of ~25 days and considering that laboratory mosquitoes likely live longer than populations in the field. The traditional calculation of VC would seemingly back up this appearance of inefficiency. But our results demonstrate and quantify the scenarios in which this seemingly inefficient phenotype leads to quite robust transmission potential when the mosquito acquires an infection between 1- and 10-days post emergence (Figure 4).

Our results also suggest that determining the age-structure of *Ae. aegypti* populations could further provide insight into the identifying the subset of mosquitoes that actually transmit [22]. Field mosquito infection detection rates remain quite low in surveillance settings for several *Aedes*-borne viruses, which suggests that a small subset of mosquitoes may be responsible for most of the transmission [48-50]. The results herein and the adaptation of VC_{age} may begin to offer some preliminary identification of this subset.

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Author contributions: RCC and EHM conceived of and designed the experiments. RCC, EHM, and ART performed the experiments. RCC performed statistical analyses and VC_{age} derivation. All authors were involved in the manuscript drafting and final approvals.

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Supplemental Table and Figure Legends:

Supplementary Figure S1. Illustration of main treatment design for vector competence experimentation. Three treatments were applied to assess our hypothesis. Treatment ZIKV (ZIKV) received an infectious bloodmeal at 5 days post-emergence (dpe); Treatment M.ZIKV (Mock-ZIKV) received a mock infectious bloodmeal at 5 days dpe, followed by an infectious bloodmeal at 12 dpe; and Treatment S.ZIKV (sugar-ZIKV) received an infectious bloodmeal at 12 dpe.

Supplementary Figure S2. Observed and predicted daily probabilities of survival. Combined observed daily survival rates of all three treatments (dots) and the predicted daily survival rates (green curve).

Supplementary Figure S3. Observed and predicted probabilities of daily biting. The observed daily biting rates (dots) from the "blood-meal" landing experiments and the fitted predictions (green curve) with a force lower asymptote of 0.

Supplementary Table S1. 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.

Supplementary Table S2. Infection and dissemination rates for each day post-infection (dpi) 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.

Supplementary Table S3. 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: ZIKV -- ZIKV at 5 days post emergence (dpe); M.ZIKV – mock bloodmeal at 5 dpe and ZIKV bloodmeal at 12 dpe; S.ZIKV – 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.

Supplementary Table S4. 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).