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Running title: Ae. aegypti ZIKV sexual transmission

First evidence of Zika virus venereal transmission in *Aedes aegypti* mosquitoes[1]

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BACKGROUND: Zika positive *Ae. aegypti* males collected in the field suggest that vertical and/or venereal transmission of ZIKV may occur.
OBJECTIVES: This study demonstrated that venereal transmission of ZIKV by *Ae. aegypti* can occur under laboratory conditions.
METHODS: *Ae. aegypti* colony was used for the ZIKV venereal transmission. The ZIKV used in this study was isolated from patient presenting symptoms and confirmed by RT-qPCR. Male mosquitoes were intrathoracically inoculated with a ZIKV suspension, and transferred to a cage containing virgin females to copulate. In a second experiment, mosquito females were orally infected with a ZIKV suspension by blood feeding membrane, and then placed in cages with virgin males to copulate. After copulation, all mosquitoes were individually evaluated to viral infection by RT-qPCR.
FINDINGS: Females mated with intrathoracically infected males showed Ct values ranged from 13 to 35, and the mean infection rate in females was 45%. Males mated with orally infected females showed Ct values ranged from 29 to 35, and the mean infection rate in males was 35%.
MAIN CONCLUSION: This study shows that ZIKV infection of *Ae. aegypti* mosquitoes occurs not only during blood feeding, but also during copulation.

Keywords: sexual transmission, mosquito vectors, mating, *Flavivirus*

Zika is a disease caused by an arbovirus (Zika virus, or ZIKV) of the *Flaviviridae* family, *Flavivirus* genus. It is a worldwide public health concern. ZIKV was first described in Africa in 1947 (Mukwaya & Sempala 1977), and for a long time it was thought to cause only a benign illness. However, after its emergence in Brazil in 2015 and its spread throughout most of Latin America and the Caribbean, ZIKV infection has been associated with thousands of cases involving severe complications like microcephaly, Guillain-Barré syndrome and death. This severe and unexpected epidemic led WHO to recognize ZIKV as a Public Health Emergency of International Concern (Azevedo et al. 2016, Santos et al.

2016). Zika symptoms are mild and are characterized by the sudden onset of fever, arthralgia, maculopapular rash or nonpurulent conjunctivitis, arthralgia, myalgia, headache, conjunctivitis, pruritus, joint edema and exanthema (Azevedo et al. 2016, Nunes et al. 2016, Vasconcelos & Calisher 2016). Between 2015 and 2016, a total of 707, 133 cases were recorded across 48 countries (Ikejezie et al. 2017). In 2016 alone, 205,578 cases and 8 deaths were recorded in Brazil. The highest incidence rates in Brazil have been recorded in the Central-West Region (231 cases per 100,000) and the North Region (157 cases per 100,000). As of June 2017, there have been 13,353 confirmed cases of Zika, and 322 cases of microcephaly associated with congenital Zika virus infection (SVS-MS 2017).

ZIKV has been found infecting salivary glands of several species of mosquitoes from the *Aedes* and *Culex* genera, but the infection susceptibility of particular species seems to be strongly associated with specific virus strains (Ferreira-de-Brito et al. 2016, Fernandes et al. 2016, Guedes et al. 2017). *Ae. aegypti* is considered the main ZIKV vector (World Health Organization 2016), and is thought to be responsible for the 2015 outbreak in Brazil (Oliveira et al. 2016). Moreover, ZIKV can infect and be transmitted by American populations of *Ae. aegypti* and *Ae. albopictus* (Chouin-Carneiro et al. 2016, Costa-da-Silva et al. 2017).

In addition to transmission by mosquito bite, ZIKV can be sexually transmitted between humans, ZIKV can generate viremia sufficient to infect competent mosquito vectors when introduced intrarectally or intravaginally in monkeys (Hastings & Fikrig 2017, Haddow et al. 2017, Musso et al. 2015), and ZIKV can be detected in human semen (Musso et al. 2015). Zika positive *Ae. aegypti* males have been collected in the field which suggests that vertical and/or venereal transmission of ZIKV may occur between mosquitoes (Ferreira-de-Brito et al. 2016, Thangamani et al. 2016). The maintenance of

arboviruses in nature is greatly enhanced when venereal transmission occurs in conjunction with other transmission mechanisms. Venereal transmission has been demonstrated in *Ae. aegypti* infected with the Chikungunya virus (Mavale et al. 2010).

This study demonstrates that venereal transmission of ZIKV by *Ae. aegypti* can occur under controlled laboratory conditions. This is an important finding because it may partially explain the high dispersion rate of infected mosquitoes during Zika epidemics, and it highlights the importance of mosquitoes control programs.

Materials and methods

Mosquito strain

Immature *Ae. aegypti* specimens were collected from several breeding sites around the city of Manaus. Specimens were reared through two generations under laboratory conditions. Individual F2 adult females were stored at -80^{o}C and total RNA from each mosquito female was extracted with TRIzol® Reagent (Thermo Fisher, Waltham, MA) following manufacturer's instructions. The F2 adult female progenitors were tested for the presence of ZIKV (Lanciotti et al. 2008), CHIKV (Lanciotti et al. 2007) and DENV (Gurukumar et al. 2009) by Real Time PCR (RT-qPCR), and the individual ovipostures were separated for breeding and assays. Batch eggs from F2 adult females that tested negative for all three viruses were used to establish the virus-free mosquito colony. Mosquitoes were maintained under laboratory conditions at 27^{o}C, in 70% relative humidity. Larvae were reared in plastic containers containing tap water and were fed fish food. Adults were kept in plastic cages and offered a 10% sucrose solution *ad libitum*.

Zika virus strain

The Zika virus used in this study was isolated from a female patient presenting classical symptoms of arbovirus infection. Zika infection was confirmed by RT-qPCR (Lanciotti et al. 2008). This strain was obtained after the second passage on *Aedes albopictus* C6/36 cells kept at 28°C in Leibovitz's L-15 medium supplemented with 2% fetal bovine serum (FBS) and Antibiotic/antimycotic.

After three days of infection on C6/36 cells, viral titer was determined by flow cytometry, based on a previously published protocol for the dengue virus (Medina et al. 2012). Briefly, we used the anti-flavivirus 4G2 monoclonal antibody, followed by anti-mouse IgG Alexa 488-conjugated secondary antibody, diluted at 1: 2,000 and 1: 3,000, respectively. After incubation and washing, cells were analyzed by flow cytometry, counting at least 100,000 events.

Intrathoracic microinjection of mosquitoes

One group containing 50 (two-day-old) male mosquitos was intrathoracically inoculated with a 0.2µL suspension of ZIKV at a titer of 10^7 infectious units/ml (FACS IU/mL), following Rosen & Gubler (1974). Microinjection was made using the Nanoject II injector (Drummond Scientific Company). Mosquitoes were subsequently maintained at 27^{o}C, in 70% relative humidity and offered a 10% sucrose solution *ad libitum*. Four days after injection, the male mosquitoes were transferred to a cage containing 100 (six-day-old) virgin females and left to copulate for five days at a proportion of one male to two females. After the five-day copulation period, all females were individually placed in 1 ml of TRIzol® Reagent and stored at -80ºC prior to viral detection by RT-qPCR (Figure 1A). Two independent biological experimental replicates were made for this assay.

Oral infection of mosquitoes

One group containing 50 (two-day-old) virgin females was fed using a Parafilm® membrane. A suspension of ZIKV at a titer of 10^7 infectious units/ml (FACS IU/mL) was used for blood feeding. Fully engorged females were transferred to plastic cages and maintained at 27ºC, in 70% relative humidity, and offered a 10% sucrose solution *ad libitum*. Nine days after blood feeding, the females were placed in cages with *Ae. aegypti* AaM3V$^-$ virgin two-day-old males and left to copulate for four days at a proportion of one male to two females. After the four-day copulation period, 20 surviving males were placed individually in 1 ml of TRIzol® Reagent and stored at -80ºC (Figure 1B). Two experimental biological replicates were made.

Zika virus detection in mosquitoes

Mosquitoes were individually homogenized in 1 ml of TRIzol® Reagent, and RNA was extracted following manufacturer's instructions. Viral RNA was detected using TaqMan® Fast Virus 1-Step Master Mix in a StepOnePlus Real Time PCR System (Applied Biosystems) using ZIKV primers and probes described previously (Lanciotti et al. 2008). The RT-qPCR conditions were: 50ºC for 5 min, 95ºC for 20 sec, and 45 cycles at 95ºC for 3 sec and 60ºC for 30 sec with fluorescence acquisition. For all RT-qPCR assays, the MS2 RNA bacteriophage was introduced prior to RNA extraction in order to track false-negative reactions due to PCR inhibition, as described previously (Naveca et al. 2017).

RT-qPCR reactions were analyzed by cycle threshold (Ct) values where Ct $\leq$ 38 was considered positive for the presence of ZIKV RNA. Data was analyzed using StepOne V2.3 software (Applied Biosystems). Infection rates were calculated by dividing the number of infected mosquitoes by the number of mosquitoes tested for ZIKV infection.

Ethics statement

Mosquito field collections were approved by SisBio (Sistema de Autorização e Informação em Biodiversidade – Permission and Information in Biodiversity System) number 12186. This study was approved by the Brazilian National Ethics Committee (CONEP, 3726).

Results

Venereal transmission of ZIKV by *Ae. aegypti* males infected via intrathoracic microinjection

To determine if infected *Ae. aegypti* males can transmit ZIKV to uninfected females, 100 adult females were made available for copulation with infected males. Ct values ranged from 13 to 35 in females mated with intrathoracically infected males (Figure 2A). The mean infection rate in females was 45%, with 40% infected in the first experiment and 50% infected in the replicate (t Test, p = 0,21) (Table 1).

Venereal transmission of ZIKV by orally infected *Ae. aegypti* females

To determine if infected *Ae. aegypti* females can transmit ZIKV to uninfected males, 20 surviving males (10 from each experimental replicate) were tested for ZIKV transmitted venerally by orally infected females. Ct values ranged from 29 to 35 in males mated with orally infected females (Figure 2B). The mean infection rate in males was 35%, with 50% infected in the first experiment and 20% infected in the second experiment (t Test, p = 0,34).

Discussion

To complete the life-cycle in nature the virus must replicate in a mosquito's tissues. Virus particles then spread throughout the mosquito's body, and once they reach the salivary glands they can be transmitted to vertebrate hosts. Details of the ZIKV replication process in *Ae. aegypti* are still unknown. However, some studies have been conducted to ascertain the steps involved in the replication process of DENV, a closely related *Flavivirus.* (Pang et al. 2001, Salazar et al. 2007). In this process, DENV-2 spreads from the midgut at 2 days post infection (dpi), it disseminates to the salivary glands and other organs at 4 dpi, and it can still be detected up to 21 dpi (Salazar et al. 2007). It is not known whether other flaviviruses share these infection dynamics, but we have used this infection time-course as the model for the present study.

This study assessed the ability of *Ae. aegypti* mosquitoes to transmit ZIKV during copulation. Four days (4 dpi)was judged sufficient time for ZIKV to disseminate throughout the body and into the seminal fluid of the male mosquitoes, and five days (9 dpi) was judged sufficient time for the arbovirus to be transmitted venereally and be detectable in the female mosquitoes.

Until now, there has been no strong evidence that ZIKV can be transmitted sexually among mosquitos. However, it is known that female mosquitoes can become infected with arboviruses during haematophagy, and that females can then vertically transmit viruses to their eggs. Vertical transmission of ZIKV has been observed in experimentally infected *Ae. aegypti* specimens (Ciota et al. 2017, Thangamani et al. 2016). The detection of ZIKV in *Ae. furcifer* males (Diallo et al. 2014) and *Ae. aegypti* males (Ferreira-de-Brito et al. 2016) suggests, but does not prove, that vertical and/or venereal transmission of Zika can occur in these two species.

Our findings showed the presence of ZIKV RNA in previously uninfected mosquitoes of both sexes following copulation with ZIKV infected mates. This data strongly supports the possibility that ZIKV is transmitted in the sexual fluids of mating mosquitoes. This poses a concern to public health because the venereal transmission of Zika among *Ae. aegypti* mosquitoes could potentially increase mosquito infection rates and thereby increase the spread of the virus. Furthermore, if venereal infection occurs in natural mosquito populations, this mode of transmission may be an important mechanism of ZIKV maintenance in nature.

In this study, male mosquitoes were infected with ZIKV via intrathoracic microinjection and ZIKV was transmitted to females during copulation. This result indicates that ZIKV systemically disseminates throughout the mosquito body, as it does in other arboviruses from the *Flaviviridae* family. This study shows that ZIKV infection of *Ae. aegypti* mosquitoes occurs not only during blood feeding on infected vertebrates, but also during copulation. To the best of our knowledge, this is the first strong evidence that ZIKV can be transmitted venereally among *Ae. aegypti* mosquitoes.

Experts at the World Health Organization have recently declared that "ZIKV and its associated consequences remain a significant and enduring public health challenge that require intense action, but ZIKV no longer represents a Public Health Emergency of International Concern" (WHO 2016). In the global context, the dissemination of *Aedes* vectors is of such scope that future outbreaks of ZIKV and other flaviviruses will be hard to foresee. Moreover, in the absence of a vaccine, our ability to block the spread of ZIKV relies solely on vector control measures. For this reason, studies that increase our understanding of viral-host biological interactions are of great importance and should be encouraged.

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Author contributions

Experiment conception and design: CMRV, JWPS, FGN, FACP, VAN. Experiments conducted by: CMRV, JWPS, FGN, FACP, VAN, HCMB, JFA. Data analysis: CMRV, JWPS, FGN, FACP, VAN. Paper written by: CMRV, JWPS, FGN, FACP, VAN. All authors read and approved the final version of the manuscript.

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Table. Infection rates in *Ae. aegypti* mosquitoes infected with Zika virus following copulation with infected mates.

Experiments	Copula	N	Infection rate (%)	Mean infection rate (%)
A1	(+) Males X Females (-)	50	40	45
A2	(+) Males X Females (-)	50	50	
B1	(+) Females X Males (-)	10	50	35
B2	(+) Females X Males (-)	10	20	

(+): ZIKV infected, (-): ZIKV free, N: number of mosquitoes tested for ZIKV infection.

Figure 1. ZIKV venereal transmission in *Ae. aegypti* mosquitoes. (A) ZIKV infected males mating with ZIKV free females; **(B)** ZIKV infected females mating with ZIKV free males.

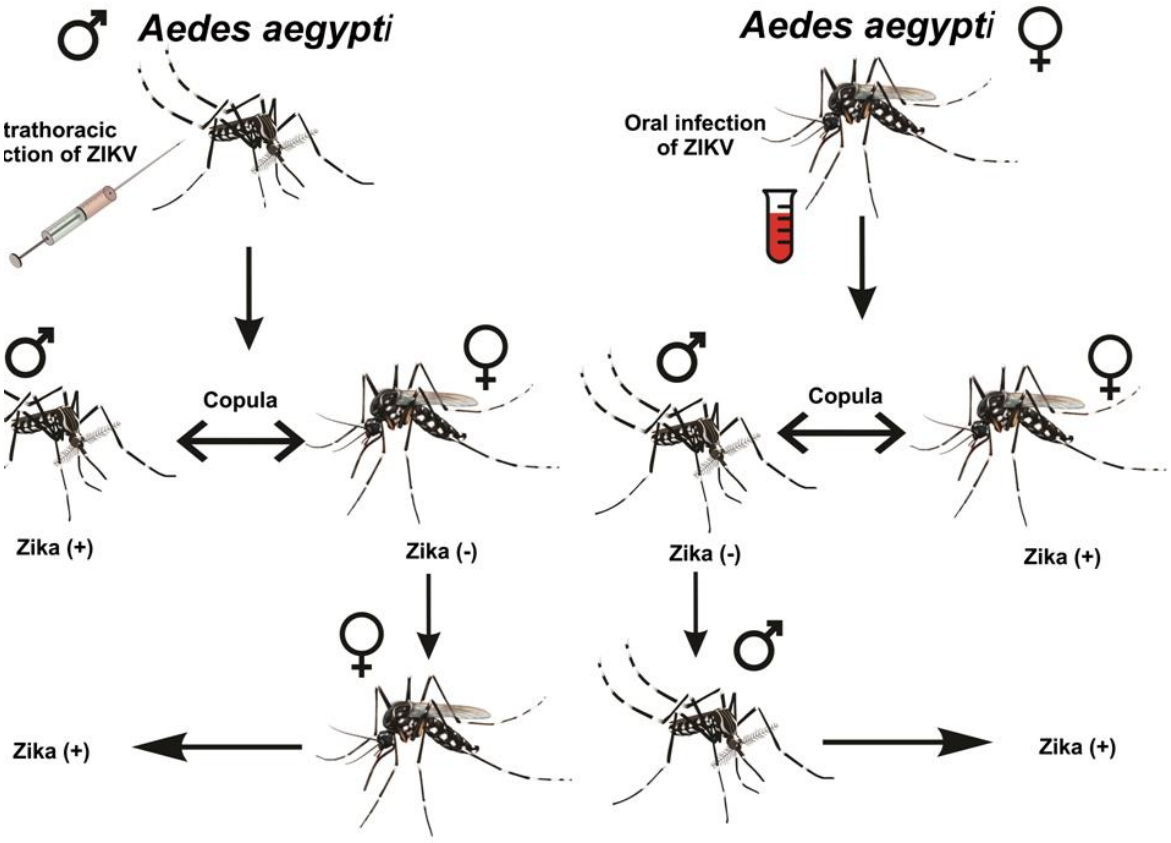

Figure 2. Real-Time Reverse Transcription PCR Cycle Threshold (Ct) values for *Ae. aegypti* mosquitoes infected with Zika virus following copulation with infected mates. **(A)** Negative females that copulated with males infected with ZIKV by microinjection; **(B)** Negative males that copulated with females infected with ZIKV orally.