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## **RECOMMENDED CITATION**

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**Title:** Zika infection decreases *Aedes aegypti* locomotor activity but does not influence egg production or viability

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## **ABSTRACT**

Zika has emerged as a new public health threat after the explosive epidemic in Brazil in 2015. It is an arboviroses transmitted mainly by *Ae. aegypti* mosquitoes. The knowledge of physiological, behavioral and biological features in virus-infected vectors may help the understanding of arbovirus transmission dynamics and elucidate their influence in vector capacity. Here, we aimed to investigate the effects of Zika virus (ZIKV) infection in the behavior of *Ae. aegypti* females by analyzing the locomotor activity, egg production and viability. *Ae. aegypti* females were orally infected with ZIKV through an artificial feeder to access egg production, egg viability and locomotor activity. For egg production and viability assays, females were kept in cages containing an artificial site for oviposition and eggs were counted. No significant difference in the number of eggs laid per females neither in their viability were found between ZIKV infected and non-infected females, regardless the tested pair of mosquito population and virus strain and the gonotrophic cycles. Locomotor activity assays were performed in activity monitors, an average of 5<sup>th</sup>, 6<sup>th</sup> and 7<sup>th</sup> days after infective feeding was calculated and a significant decrease in the

locomotor activity in ZIKV infected females was observed. These results suggest that even when mosquitoes are infected with ZIKV, in places where there are many oviposition sites, they are able to maintain the dissemination of the vector population. Besides, the decreased locomotor activity does not seem to influence negatively in ZIKV transmission and may explain case clustering within households reported during Zika outbreaks such as in Rio de Janeiro 2015. High mosquito infestation index and abundant vector breeding sites may also influence this disease transmission.

**Key words:** Zika virus, *Aedes aegypti*, locomotor activity, egg production, egg viability.

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## **INTRODUCTION**

The Zika virus (ZIKV) is an arbovirus belonging to the *Flaviviridae* family, first isolated in 1947 in Uganda, West Africa from a sentinel rhesus monkey (Dick *et al.*, 1952). Since then, sporadic human cases were reported in Asia and Africa. The first large Zika epizooty reported occurred in Micronesia in 2007 (Lanciotti *et al.*, 2008).

ZIKV was previously believed to cause only a mild and self-limiting illness, but it has emerged as a new public health threat after the explosive epidemic in Brazil in 2015, specially due to the increased severe congenital malformations (microcephaly) and neurological complications reported in the country (Possas *et al.*, 2017). This new epidemiological scenario led the World Health Organization to declare the Zika epidemic as a Sanitary Emergency in Public Health in February of 2016.

ZIKV is transmitted by several species of *Aedes* mosquitoes, mainly *Ae. aegypti* (Diallo *et al.*, 2014). Besides being the single confirmed natural ZIKV vector during the Pan-American (Ferreira-De-Brito *et al.*, 2016) *Ae. aegypti* is also the only vector for several other arboviruses circulating in the New World, such as the four Dengue virus (DENV) serotypes (Dos Santos *et al.*, 2017), Chikungunya virus (Vega-Rúa *et al.*, 2014) and Yellow Fever virus (Couto-Lima *et al.*, 2017).

The knowledge of the physiological, behavioral and biological features of the vector, such as preference and frequency of hematophagy in humans, eggs laying, gonotrophic discordance and resistance to desiccation (Consoli & Lourenço-De-Oliveira, 1994) may help the understanding of arbovirus transmission dynamics and elucidate the role of those parameters impacting vector capacity.

The hematophagous and anthropophagic behaviors are crucial to females' egg maturation. A single *Ae. aegypti* female can lay from 100 to 200 eggs per batch, and multiple times throughout her lifetime after each blood meal (Chapman, 1998). *Ae. aegypti* eggs are laid usually on container surfaces near water, preferentially in shaded places, (Consoli & Lourenço-De-Oliveira, 1994) and

their embryonic development and egg viability is directly related to environmental temperature (Farnesi *et al.*, 2009).

Concerning activity patterns mosquito species are classified as diurnal, crepuscular and nocturnal (Clements, 1999). *Aedes aegypti* is considered a diurnal and crepuscular species that can modify the activity pattern according to changes in the physiology and viral infection (Clements, 1999). Accordingly, DENV-2 artificially-infected *Ae. aegypti* females showed increased locomotor activity, potentially effecting the infection kinetics and disease transmission (Lima-Camara *et al.*, 2011). Moreover, the number of eggs laid by *Ae. aegypti* females orally challenged with DENV-2 may vary during the mosquito lifetime, with a decrease from one gonotrophic cycle to another (Maciel-De-Freitas *et al.*, 2011) .

Here, we aimed to investigate the effects of ZIKV infection in the behavior of *Ae. aegypti* females by analyzing the locomotor activity, egg production and egg viability.

## **MATERIALS AND METHODS**

### **Mosquito populations and rearing**

Eggs from *Ae. aegypti* mosquitoes (strain PAEA, Tahiti, French Polynesia) were hatched in plastic trays containing 1.5L of Milli-RO water and approximately 1g of yeast (two tablet of Vitalab®, Brazil). Larvae were fed with the same quantity of yeast, every two days, until pupae development according to (Farnesi *et al.*, 2009) The pupae were counted and separated in cages (with approximately 400 each) for adult emergence. Males and females mosquitoes

were kept together in cages with 10% sucrose solution *ad libitum*. In all experiments, rearing of mosquitoes was carried out in an incubator (Precision Scientific Incubator, USA) at 25°C, in a photoperiod of 12 hours of light and dark (LD 12:12) and 60–80% relative humidity.

### **Virus and experimental infection**

The ZIKV strain ZIKV/H.sapiens/Brazil/PE243/201 (GenBank accession number KX197192.1) was used for oral experimental infection of *Ae. aegypti* females as previously described (Oliveira *et al.*, 2017). The virus was previously isolated from a febrile patient in the state of Pernambuco and molecularly characterized (Coelho *et al.*, 2017). Briefly, females were allowed to feed through a membrane attached to an artificial feeder kept at 37°C for approximately 40 min inside a Biosafety level - 2 (BSL-2) insectary facility. The infectious blood meal consisted of 1:1 mix of rabbit red blood cells and L-15 culture medium containing ZIKV at a final concentration of  $10^7$  PFU/mL; ATP pH 7.4 at a final concentration of 1 mM was included as a phagostimulant. Control mosquitoes also fed with a similar blood meal, but with a non-infected L15 culture medium (Oliveira *et al.*, 2017). After blood meal, mosquitoes were cold-anesthetized and only the fully engorged females were considered. Viral detection was done by PCR (see Supporting Information and Fig S1).

### **Egg production assays in ZIKV infected and uninfected mosquitoes**

For gonotrophic cycle assays, approximately 450 females, around two weeks-old, were used per condition (infected or uninfected). We performed three replicas per experiment; each one contained, at least, 50 females that were deprived of sugar prior to one infected or uninfected blood meal (with *Swiss* mice or rabbit blood), for approximately 40 min. In both cases, infected or uninfected blood meal, engorged females mosquitoes were selected (at least, 150 females, in each condition, was used per experiment). After three days, the oviposition were stimulated in cages containing an artificial site for oviposition: plastic container containing 100 mL of filtered water and three strips of rectangular filter paper, 8 cm x 15 cm in size, for two days. In these assays we used the second and third gonotrophic cycles. Were made three independent experiments.

### **Eggs viability assays**

For eggs viability analysis, the eggs obtained as described above were removed from the oviposition site and placed to dry in a humid chamber. After one week drying, eggs were removed carefully from the filter paper using a brush and counted in the Egg Counter Program (©BioAlg Group, Faro, Portugal). Afterwards, randomly selected and tested for viability as described below.

Each replica was set up with 50 counted eggs and placed on filter paper to stimulate hatching in Petri dishes containing 50 mL of industrial yeast extract solution 0.15% (weight/volume) for 24 hours in a Precision Scientific Incubator (Thermo Fischer) under a constant temperature of 25°C and 60–80% relative humidity according to (Farnesi *et al.*, 2009). We analyzed eggs viability from the

second and third gonotrophic cycles. Each viability experiment analyzed contained 600 eggs (300 per gonotrophic cycle, being 150 from uninfected and 150 from ZIKV infected females). This assays was composed by three independent experiments, totalizing 1,800 eggs.

### **Egg production assays in ZIKV infected and uninfected mosquitoes in the other pair vector/virus**

In addition, we verified if egg production or viability records would be similar in other pair vector/virus: *Ae. aegypti* (Urca population) and ZIKV (Rio-S1 strain, GenBank accession number KU926310), both originated from Rio de Janeiro and whose vector competence parameters have been previously described to be considerably high (Fernandes *et al.*, 2016). All mosquito treatments, virus titer in the blood meal and other experimental infection procedures were as described above, except that mosquito female took a second uninfected blood meal 14 days after being orally challenged by ZIKV. Assessments of egg production or viability was limited to the second gonotrophic cycle.

### **Locomotor/flight activity assays**

*Aedes aegypti* females, around 15 days post emergence, were transferred to 4 small circular carton cages (60 per cage) lined with micro tulle (8.5 cm of diameter X 9.5 cm of height). Females were deprived of sucrose solution for approximately 10 hours prior to a blood meal (ZIKV infected or uninfected

blood). Blood feeding followed as described above. ZIKV infected (n= 51) and non-infected *Ae. aegypti* females (n= 54) were individually placed in 25mm glass tubes containing cotton soaked with 10% sucrose solution in locomotor activity monitors (Trikinetics Inc., Waltham, MA, USA) with 32 channels and infrared light beams that capture movement (Araripe *et al.*, 2018). The experiment lasted, at least, 8 days to allow viral dissemination. The monitors were kept inside an incubator (Precision Scientific Incubator, USA) at constant temperature of 25°C, in a LD 12:12 regimen (12 hours of light followed by 12 hours of dark), during seven days and a DD regimen (24 hours of constant dark), for one day. The relative humidity was 60 - 80%. To analyze the locomotor activity, an average of 5<sup>rd</sup>, 6<sup>th</sup> and 7<sup>th</sup> DPI (days post infection), corresponding to LD condition and 8<sup>th</sup> DPI, corresponding to a day in DD condition.

The results were organized and analyzed in Excel (Microsoft Office) with parameters previously established (Gentile *et al.*, 2009) and only mosquitoes that were alive at the end of the experiment and positive for ZIKV were considered (See Fig. S1).

### **Statistical analysis**

The locomotor activity results were analyzed, firstly for Shapiro-Wilk normality test. After, we used the parametric *t*-Student test considering the log (N+1) mean of the individual mosquito data every 30 minutes. Since the mosquito activity data is especially variable, the transformation of the data to logarithm allows their distribution to be more constrained and the average to be less

influenced by very low or very high values. In fact, because we have many zeros in the data series, we must use  $\log(N+1)$  instead of  $\log N$ . The advantage of using this calculation is that it prevents the masking of data by the effect of very high numbers within a single interval (Araripe *et al.*, 2018). In the analysis of egg quantification and viability, we first performed the Shapiro-Wilk normality test. When the data showed a normal distribution, we used the parametric *t-Student*; when data showed a non normal distribution, we used non-parametric Mann-Whitney test. Other specific statistical information's are in the figure legend. All analysis were performed using GraphPad Prism 5 (GraphPad Software, San Diego, California, USA) and p value < 0,05 considered statistically significant.

### **Ethical statement**

All the experiments carried out on this study were approved by the institutional Research Ethics Committees IOC/FIOCRUZ #LW34/14 (for feeding on mice) and CEUA-UFRJ 155/13 (for use of rabbit blood).

## **RESULTS**

### **ZIKV infection has no effect on egg quantity and viability**

All ZIKV orally challenged mosquitoes used in this study tested positive (Fig.S1). To determine whether ZIKV infection affects *Ae. aegypti's* egg production and viability, the second and third gonotrophic cycles were analyzed. The overall results showed that, regardless of the gonotrophic cycle

(Fig. S2) and the pair mosquito-virus strain (Fig. S3), no difference was observed in egg production and viability for ZIKV infected and non-infected *Ae. aegypti* females. Similarly, when we analyzed the second and third gonotrophic cycles comparatively, no significant differences were observed either in quantity ( $p= 0.4091$  and  $p= 0.3496$ , respectively) or viability ( $p= 0.0773$  and  $p= 0.0734$ , respectively) (Fig. 1).

### **ZIKV infection decreases females *Aedes aegypti* locomotor activity**

To evaluate the influence of ZIKV infection in the locomotor activity, we tested infected and non-infected females for 7 days under a LD12:12 cycle and one day in DD condition (Fig. 2), but we focused our analysis on the 5<sup>th</sup>, 6<sup>th</sup> and 7<sup>th</sup> DPI to LD and 8<sup>th</sup> DPI to DD. The first response when the lights come on (a startle response not controlled by the endogenous clock), commonly seen in locomotor activity tests, was also detected in our assessments, but in low intensity (Fig. 2A and B). On the other hand, in DD condition this startle response was eliminated, as expected (Fig. 2C). Comparatively, in the two peaks activity, infected *Ae. aegypti* females showed significantly lower locomotor activity than the uninfected ones (ZT5 - ZT13;  $p<0.05$ ) (Fig. 2B). In both infected and control groups, the major activity peak occurs between ZT 6 to 10 in LD condition. On the other hand, in DD condition the major activity peak is between ZT 9 to ZT 11 (Fig. 2C). The anticipation showed in DD condition is expected to *Ae. aegypti* species. Specifically at this time, the infected females presented less activity compared to the control ones. After the peak, the activity is drastically reduced in both groups in LD and DD conditions (Fig. 2).

## DISCUSSION

Since Zika outbreak and all the consequences caused by the virus infection in newborns, mainly in Brazil, the major efforts of Scientific Community were focused on the interaction between virus and the vertebrate vector (humans). However, we believe it also fundamental to investigate in details the effects of this virus in the insect vector. We aimed to contribute to a major understanding of possible changes caused in the *Ae. aegypti* females behavior when infected with Zika virus, which will inevitably influence the success of vector control measures.

Recently, *Ae. aegypti* was reported naturally infected by ZIKV (Ferreira-De-Brito *et al.*, 2016). Field and laboratory studies to assess the transmitting success of such an arbovirus to a new host and their offspring are still scarce. Here, we analyzed egg laying and viability as well as locomotor activity in ZIKV infected *Ae. aegypti*.

There was no significant difference in the number of eggs laid per females neither in egg viability between ZIKV infected and non-infected females, regardless the tested pair of mosquito population and virus strain. However, a borderline P value in the statistical analysis ( $p= 0.054$ ) and the great variation found reflects the trend of a higher average viability in eggs from infected females (Figs. 1, S2 and S3). In fecundity analysis, it was also not found differences between ZIKV infected and uninfected mosquitoes, even using another laboratorial approach (Gaburro *et al.*, 2018). In contrast, a previous study showed that DENV infected *Ae. aegypti* females exhibited lower fecundity (egg quantity) (Maciel-De-Freitas *et al.*, 2011). Furthermore, a lower egg

production and hatching was observed when *Ae. aegypti* females were infected by DENV-1 or DENV-2 (Maciel-De-Freitas *et al.*, 2011; Buckner *et al.*, 2013). Therefore, our data suggest that the ZIKV infection does not cause damage to the fertility and viability in its main vector *Ae. aegypti*. This feature is important to keep the vector population in the nature, causing indirectly impact in the disease transmission.

The locomotor activity has an important role in arboviruses spread and transmission dynamics. It was previously observed that DENV-2 infection causes an increase in locomotor activity in *Ae. aegypti* during the 24-hours period in a LD 12:12 cycle (Gentile *et al.*, 2009; Lima-Camara *et al.*, 2011). On the other hand, different interferences in the physiology, like the insemination and blood feeding, can interfere as well, for instance, decreasing the locomotor activity of *Ae. aegypti* and *Ae. albopictus* (Lima-Camara *et al.*, 2014). However, different from our previous study with locomotor activity (Lima-Camara *et al.*, 2011), we performed the experimental mosquito infection via oral artificial feeding to mimic as close as possible the natural kinetics of ZIKV infection and dissemination.

Recently, Ryckebusch *et al.*, 2017 showed in the *Ae. aegypti* PAEA strain that the dissemination of the ZIKV occurs between 6<sup>th</sup> DPI and 14<sup>th</sup> DPI. In our analysis, we consider the interval between 5<sup>th</sup> and 8<sup>th</sup> DPI to test the locomotor activity, to assure the virus spreading throughout the body and the mosquito survival.

Our results corroborated the anticipation peak of activity, being even this main peak of the locomotor activity, which is characteristic of the PAEA strain of *Ae. aegypti* (Lima-Camara *et al.*, 2011). However, ZIKV infection in the *Ae. aegypti*

PAEA strain caused a decrease in the activity pattern during the whole light phase in comparison to the control group throughout the analyzed days in LD12:12 condition (Fig. 2A and 2B). Furthermore, in DD condition, which means an absence of environmental conditions, both ZIKV infected and uninfected females presented a clear anticipation of the major peak activity (Fig. 2C). The maintenance of the activity rhythm in DD indicates that the decrease in locomotor activity caused by the viral infection is not a simple response to light, but rather there is an interaction between ZIKV infection and the circadian clock. Our results are quite different from those found recently by Gaburro *et al.*, 2018. It is worthy to mention that we used a validated approach in behavior studies (Gentile *et al.*, 2009; Lima-Camara *et al.*, 2011; Araripe *et al.*, 2018) in which the individual insects are isolated from host odors and inter-specimens communication. These cues may cause a bias and interfere in the overall activity of mosquitoes.

It is worth to notice that Zika virus shows very different characteristics from those seen in other viruses of the same family, such as its high tropism by human and mosquito brain tissues (Gaburro *et al.*, 2018). It is important to note when the transcriptome of Zika infected mosquito midguts is compared with the one of DENV infected, most of the regulated genes (61%) are uniquely regulated by Zika infection, showing a remarkable difference in the mosquito response to these viruses, which could lead to very different physiological and behavioral responses (Angleró-Rodríguez *et al.*, 2017).

The occurrence of Zika epidemics in recent years may be due to several factors, and the non-vector borne forms of transmission cannot be excluded, like in different humans fluids (Paz-Bailey *et al.*, 2017). In Brazil, another

determinant may greatly influence the success of arboviruses spread and sustainability. The behavioral, climatic and environmental risk factors for ZIKV transmission have been addressed recently (Fuller *et al.*, 2017). Moreover, the high mosquitoes infestation index (Maciel-De-Freitas *et al.*, 2008), abundant vector breeding sites and deteriorated infrastructure may also be influencing the transmission of diseases, such as Dengue and Zika (Brasil *et al.*, 2016). Our data show that Zika infection does not affect neither egg production nor viability and decreases mosquito's locomotor activity. These alterations do not seem to influence negatively in Zika transmission, once the majority of positive cases tested in the 2015 outbreak in Rio de Janeiro clustered within households (Brasil *et al.*, 2016). A similar pattern was observed in Cali, Colombia, where the transmission radius of Zika was lower when compared to other arboviruses transmitted by *Ae. aegypti* (Krystosik *et al.*, 2017). Thus, lower mosquitoes locomotion would not interfere significantly in this pathogen spread.

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## **CONFLICTS OF INTERESTS**

The authors declare that there is no conflict of interest.

## **AUTHOR CONTRIBUTIONS**

Conceptualization, KPP, RVB, LCF, RLO; Methodology, RVB, MHOS, RLO, LCF; Validation, KPP, MEBR, LCF, OATC, RTF, SSC; Formal analysis, RVB, KPP, RTF, LCF, MEBR, RLO, SSC; Investigation, KPP, MEBR, SSC; Resources, RVB, MHFS, RLO; Writing - original draft, KPP, MEBR, LCF; Writing - review & editing, RVB, LCF, RLO, MHFS; Supervision, RVB, LCF, RLO, MHFS; Project administration, RVB, MHFS, RLO; Funding acquisition, RVB, MHFS, RLO.

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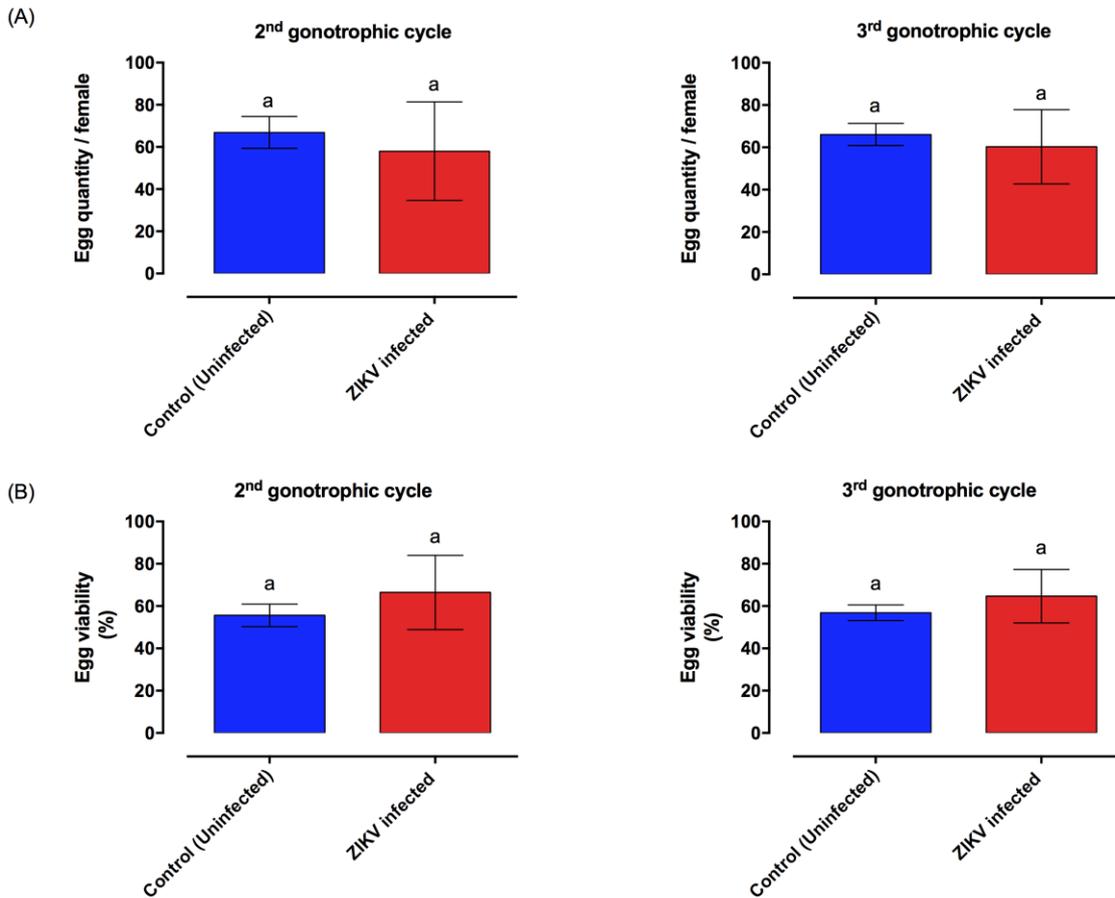
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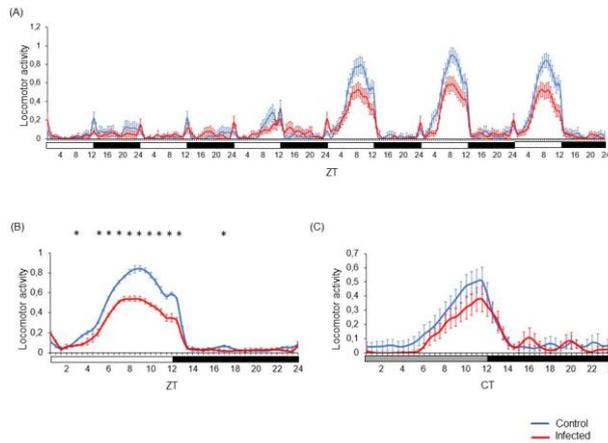
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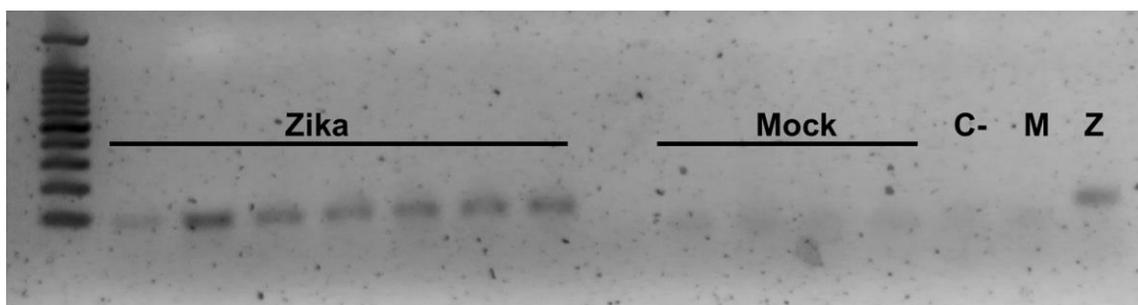


**Fig. 1: Effect of ZIKV infection on the eggs quantity (A) and viability (B) of PAEA *Ae. aegypti* females infected with ZIKV PE243, according to the second and third gonotrophic cycles.** The lack of significance is represented by p values > 0.05 obtained by using the parametric t-Student and non-parametric Mann-Whitney tests, respectively. Error bars represent mean ± s.d of three independent experiments.

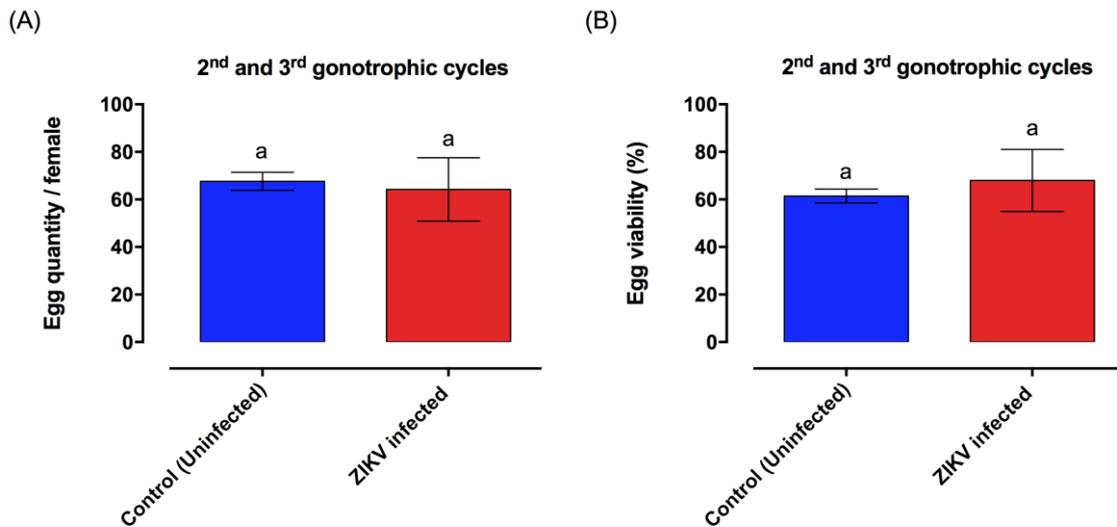


**Fig. 2: Locomotor activity of *Ae. aegypti* females infected by ZIKV.**

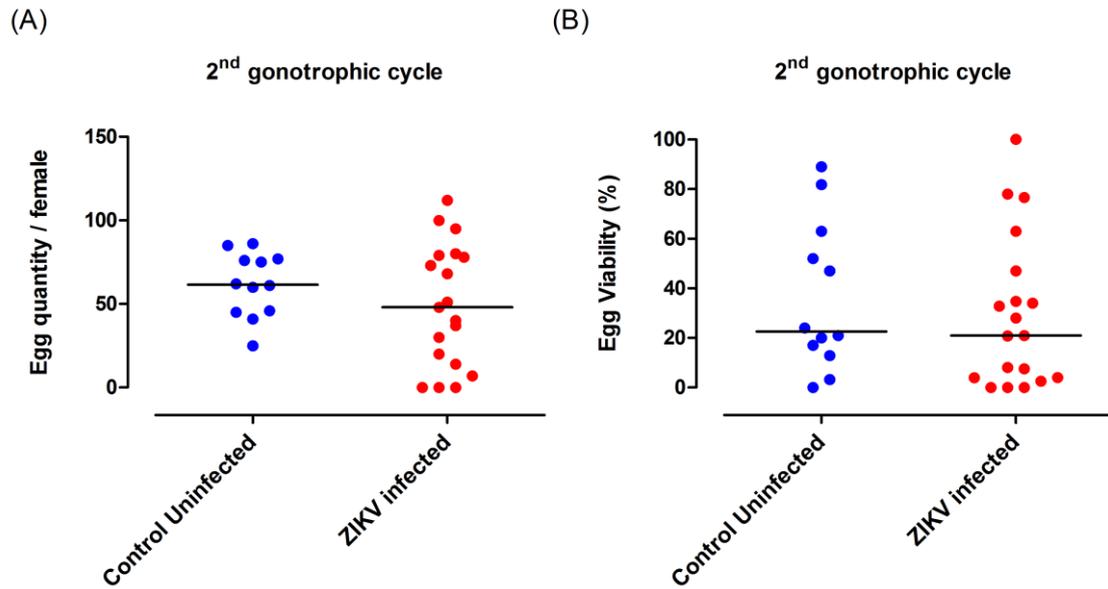
Locomotor activity of control (blue line,  $n = 51$ ) and infected *Ae. aegypti* females (red line,  $n = 54$ ). The mosquitoes were observed from the second to the seventh DPI in LD12:12 (A). The first DPI was not included because we considered them to be still adapting to the system. We also show an average profile in LD (the graph referred only to 5<sup>th</sup>, 6<sup>th</sup> and 7<sup>th</sup> DPI) (B). On the 8<sup>th</sup> DPI the mosquitoes were kept in constant darkness (DD) (C). Bars below the graphics indicate the light regime: white = lights on in LD cycles, grey = lights off in DD (“subjective day”), black = lights off in LD or DD (“subjective night”). ZT: *Zeitgeber* time within a light/dark cycle experiment; ZT0: time the light turns on; ZT12: time the light turns off. CT: Circadian time in DD. Error bars were shown for each 30 min interval. Asterisks represent the significance of the t-Student test, where  $p < 0.05$ .



**Fig. S1: ZIKV detection by RT-PCR.** Whole body of infected mosquitoes were collected 7 days after the feeding and a PCR reaction was performed to confirm the Zika infection. The first 7 lanes showed the infected mosquitoes and the following 4 lanes showed the control (mock). The “C” represents the reaction without any cDNA template, while “M” and “Z” are mock and zika C6/36 supernatant controls, respectively.



**Fig. S2: Overall effect of ZIKV infection on the eggs quantity (A) and viability (B) of *Ae. aegypti* females.** The lack of significance is represented by p values > 0.05 obtained by the parametric t-Student and non-parametric Mann-Whitney tests, respectively. Error bars represent mean  $\pm$  s.d of three independent experiments. No significant differences were found neither in eggs production (p=0.3699) nor viability (p= 0.5757).



**Fig S3: Effect of ZIKV infection on the eggs quantity (A) and viability (B) of *Ae. aegypti* females (Urca population) in the second gonotrophic cycle.** Females took a second uninfected bloodmeal 14 days after being orally challenged by the Rio-S1 ZIKV strain. The horizontal lines represent the median of the data.

### Supporting Information

#### Fig. S1: Molecular detection of ZIKV in mosquitoes

The viral RNA was extracted from the whole body using QIAmp Viral Mini Kit (Qiagen, Germany) according to manufacturer's protocol for molecular detection of ZIKV in the experimentally infected *Ae. aegypti* females. The RT-PCR for detecting ZIKV RNA was conducted using the Zika 4481-5'CTGTGGCATGAACCCAATAG3' and Zika 4552c - 3' ATCCCATAGAGCACCCTCC5' primers [1] in a Veriti 96 well Thermocycler model 9902 (Applied Biosystems, Foster City, California, USA), as previously

described [2], except for the pair Urca *Ae. aegypti* – Rio S-1 ZIKV strain where we used a RT-qPCR which details are available elsewhere [3].

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