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Masters in the Parasite Biology Post Graduation Program

THE IMPACT OF AGING AND ZIKA VIRUS INFECTION ON *AEDES AEGYPTI* FEMALE FECUNDITY

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ABSTRACT

MASTER THESIS IN THE PARASITE BIOLOGY

Martha Thieme Petersen

The emergence and re-emergence of arboviral diseases, have characterized a Strong global health problematic. Zika virus have reaches the Americas in 2014, gaining more visibility after being associated with microcephaly in newborns and other neurological complications. Its transmission on the continent is mainly carried by females of the species *Aedes aegypti*, a species that lives very closely to the human population in the cities. For other vector-pathogen models, there has already been observed a fitness cost caused by the viral infection, additional to a negative impact caused by ageing over several life-history traits of the mosquito, such as fecundity with a decrease in the number of eggs laid as they grow older. However, the effects of senescence and ZIKV infection on *Ae. aegypti* biology remains very underexploited so far, and it's still unknown whether the decreased fecundity is due to aging itself and an inability to produce eggs, or due to a lesser consumption of blood as they age. Therefore, this experiment aimed to investigate the impact of aging, blood meal size and ZIKV infection over the mosquito fecundity and oviposition success, additionally observing the effects of infection over the mosquito daily survival. For that, we divided the experiment in 2 groups, one that would receive a first infected blood meal and the other a first uninfected blood meal, each of these groups divided into 3 cohorts regarding the age at which they would receive their first meal (7, 14 and 21 days old). These mosquitoes were kept with 10% sugar solution cottons every day and blood fed every week with uninfected blood, they were monitored daily for survival and weekly for fecundity, oviposition success and blood meal size by the quantification of hematin. Using ANOVA, we observed a strong negative impact of ZIKV infection over the mosquito's survival. Effects over oviposition success were analyzed by logistic regression, showing that ZIKV infection has a negative effect over this parameter and even though the age of first blood meal did not affect the uninfected groups, the success from the ZIKV cohorts dropped from 76.1% on the first cohort to 59.3% in the third cohort. Clutch size and size of blood meal were analyzed by using a repeated measure analysis, having number of eggs and hematin amounts as the repeated measures. While the blood meal size did not influence clutch size, it was strongly influenced by age in the uninfected group, becoming less efficient to produce eggs per µL of blood ingested, clutch size sharply decreasing with age. On the other hand, the infected group showed a larger ingestion of blood than the uninfected and a more stable production of eggs per µL of blood ingested, fecundity even increasing over time. Therefore, our results try to elucidate aspects of vectorial capacity by observing the effects of senescence and pathogen infection over *Aedes aegypti* life history traits.

SUMARY

LIST OF IMAGES

LIST OF ABREVIATIONS

DENV	Dengue virus
ECSA	East-Central-South-African genotype
IOL	Indian Ocean Lineage
CHIKV	Chikungunya virus
ZIKV	Zika virus
YFV	Yellow fever virus
PAHO	Pan America Health Organization
VC	Vectorial capacity
EIP	Extrinsic incubation period
WNV	West Nile virus
NIRS	Near infrared spectroscopy

1. Introduction

1.1. Mosquito-borne diseases

Mosquitoes of the genus *Anopheles*, *Culex* and *Aedes* are recognized as the most important pathogen vectors in the world due to its role in the transmission of a high range of parasites and viruses, such as *Plasmodium sp*.; *Wuchereria bancrofti* and West Nile virus; and dengue, yellow fever, chikungunya and Zika virus, respectively (WHO 2017a). Mosquitoes are distributed over the globe but are observed at more dense populations on the tropics, where the most suitable climatic conditions are found. Current estimations point that more than half of the human population lives in tropical and subtropical regions, i.e., at areas with high risk of disease transmission (Hay et al. 2004, Bhatt et al. 2013).

Several of these diseases are caused by arboviruses (arthropod-borne virus) and transmitted through the bite of an infected vector. The genome of most viruses is composed by RNA, with the African swine fever virus being the only known exception (Śmietanka et al. 2016). Generally, arboviruses are transmitted in sylvatic cycles between vertebrate hosts and hematophagous arthropods. Humans tend to be only occasional hosts during activities such as outdoor leisure, extractivism or housing on forest fringe. However, with unplanned urbanization in metropolitan regions of megacities, expansion of transportation network across the world, the adaptation of arthropods to the man-altered environment and the lack of effective long-term strategies to control disease vector populations lead to an increasing number of human cases and outbreaks of different diseases caused by arboviruses (Gould et al. 2017, Lorenz et al. 2017).

Although malaria is considered the disease with the highest mortality rates among them, in the last decades millions of people have experienced intense arbovirus transmission from outbreaks of both emerging and re-emerging diseases. In the 1990's there was a significant outbreak of West Nile Virus (WNV) on North America (Lanciotti et al. 1999), followed by chikungunya (CHIKV) invasion to Caribbean islands, Central and South America (Nunes et al. 2015, Rodrigues et al. 2016). Moreover, in 2014-2015, it was the Zika virus the one to reaching from the Pacific Islands and establishing itself in the Americas (Musso 2015a). Whist its' symptoms are mild, like fever and rash,

Zika virus gained more importance and visibility after being associated with microcephaly in newborns and other neurological conditions, being announced as a public health emergency (WHO 2016). As a more recent addition, there has been the risk of re-urbanization of the Yellow Fever Virus (YFV) with an outbreak occurring by the end of 2016 and beginning of 2017. With the largest number of documented cases since 50 years, from July 2017 through March 2018 there has been 1,131 confirmed cases of yellow fever disease and 338 deaths from the disease were reported in the Southeast region of Brazil. In the same period from the previous year, there were 660 confirmed cases and 210 deaths by yellow fever (Brazilian Ministry of Health 2018). In the meantime, dengue is the one with the highest incidence, having showed an exponential increase on transmission rates, up to 30-fold since the 1970's, periodic outbreaks every 3-5 years and around 400 million new infections every year (Simmons et al. 2012, Bhatt et al. 2013). DENV also has made Brazil the country with the highest number of registered cases in Latin America.

1.1.1. Dengue virus

Dengue is one of the diseases with the greatest impact on the global health scenario with approximate 3.9 billion people living at risk of acquiring infection by at least one of the four circulating serotypes (Brady et al. 2012). With an estimate of 390 million infections by the dengue virus (DENV) every year, it can be found with greater incidence and prevalence around the tropics, where temperature, humidity and cities with chaotic urbanization are favorable to its transmission cycle maintenance (Bhatt et al. 2013).

The virus consists in a positive-sense, monopartite and single stranded RNA virus from the *Flavivirus* genus and Flaviridae family. It can be found as four different serotypes of the dengue virus (DENV 1-4) each divided into several distinct genotypes, which leads to the possibility of a person contracting dengue fever at least four times in a lifetime (Chen & Vasilakis 2011). The occurrence of dengue outbreaks is strongly associated with a low herd immunity on human population. Whenever the ratio of susceptible/resistant hosts surpass a theoretical threshold, the likelihood of a dengue outbreak increases significantly (Focks 2003). The disease caused by the dengue virus will often lead to the presentation of mild and unspecific symptoms, like nausea, fever,

rash and aches throughout the body, some cases even being asymptomatic (Gubler & Kuno 1997). Remarkably, only recently the role of asymptomatic hosts in maintaining infection became clearer (Duong et al. 2015). Nonetheless, a few cases may aggravate from the regular mild clinical presentations to a more severe disease characterized by a hemorrhagic fever and even shock (WHO 2009). Current estimates pose that 2,5% of dengue patients may die due to DENV infection (WHO 2017b)

It is believed that in the past DENV transmission was carried mainly on a sylvatic cycle and was transmitted by arboreal mosquitoes. Later, both the virus and the *Aedes aegypti* mosquito started an irreversible process of adapting to more urbanized centers and to human dwellings, enhancing the transmission rates to human hosts (Weaver & Reisen 2010). The first suspect dengue epidemics (based on the historical description of symptoms) were reported on the XVIII century in Asia and a year later in the Americas (Pontes e Ruffino Neto 1994). Probably, the virus and its vector dispersed passively over the globe during the colonial period with the slave trade between Africa and the Americas. A second wave of passive dispersal might be achieved on last century with international trade of used tires and a complex network of global aquatic, terrestrial and aerial transportation, enabling displacements of thousands of kilometers on a 24-h period (Gubler 2011).

Although it was likely introduced much earlier into the American continent through the slave trading, the first epidemic to happen in Brazil was in 1981, in the city of Boa Vista, Roraima, when it was detected the circulation of both dengue serotypes 1 and 4, its spread rapidly contained (Osanai et al. 1983). Five years later, a new DENV1 outbreak would happen, but this time in Rio de Janeiro and soon reaching the northeastern region, with incidences of 35.2, in 1986 and 65.1, in 1987, per 100,000 habitants, this characterized the first of the three epidemic waves to hit the country till the year of 1999 (Schatzmayr et al. 1986). The second wave happened between 1990 and 1991, affecting specially the states of Ceará with 249.1 cases/100.000 habitants, and Rio de Janeiro, with 613.8 cases per 100,000 habitants, and the third wave happened in 1997-1998 after a great dispersion of the vector (*Ae. aegypti*) through the country allowing the virus circulation in other states and cities (Braga & Valle, 2007). Furthermore, the serotype 2 of the virus was firstly isolated in 1990 in the city of Rio e Janeiro (Nogueira et al. 1990). In 2001, DENV3 was isolated in Nova Iguaçu, RJ, soon detected circulating in the state of Roraima, probably introduced from the large transit

of people between the frontier with Venezuela, and nowadays, DENV can be found circulating in almost all federal units (Nogueira et al. 2001, Braga & Valle 2007, Fares et al. 2015). The presence of all four dengue serotypes circulating in the country increases the endemicity of the disease, and leads to the current high levels of transmission, a total of 1,5 million of cases being reported in 2016 (Brazilian Ministry of Health 2017a).

1.1.2. Chikungunya virus

Chikungunya transmission in urban and peri-urban cycles is mainly associated with mosquitoes of the *Aedes aegypti* and *Aedes albopictus* species. Chikungunya outbreaks are often characterized by high levels of morbidity, and, differently from dengue, may evolve into a chronic phase that can last several months after infection. After a short incubation period, the infected individual presents symptoms like rash, fever, headache, rigors and a severe joint pain that may last for a couple weeks in the acute phase or for years in chronic manifestation (Schwartz & Albert 2010).

The first CHIKV isolation occurred after a large disease outbreak in 1952-1953 in modern Tanzania (Ross 1956). Chikungunya is an Alphavirus from the Togaviridae family, consisting on a positive-sense single stranded RNA genome (Schmaljohn & McClain 1996). Since its discovery, four distinct genotypes have been identified: the East-Central-South-African (ECSA) and the West African genotypes, responsible for the epidemics in that continent; the Asian genotype, circulating mainly through the southeast Asia; and the Indian Ocean lineage (IOL) responsible for outbreaks in the Indian Ocean islands and Asia (Weaver 2014).

It was mainly part of a sylvatic cycle, circulating in the forests areas of Africa between nonhuman primates and arboreal mosquitoes. The spread of CHIKV beyond Africa probably started during the 18th century while being carried in ships to other countries alongside the mostly competent vector, the mosquito *Aedes aegypti*. Still, from 1879, when the first occurrences of urban transmission is predicted, to 2006, outbreaks were restricted to the African and Asian continents (Weaver & Lecuit 2015). Between the years of 2005-2006, the large epidemic happening on the Reunion island was associated with a mutated chikungunya virus of the ECSA genotype carrying a substitution of an alanine to a valine in the position 226 of the E1 gene (E1-A226V),

which proved to be better transmitted by *Aedes albopictus* (Tsetsarkin et al. 2007). While *Aedes aegypti* is a more competent transmitter of the original ECSA genotype and of the Asian genotype, the wide distribution of *Ae. albopictus* increases the risk of dispersion of the mutated virus through European and American countries (Tsetsarkin et al. 2007, Vega-Rúa et al. 2014). The status of CHIKV as a reemergent global threat changed after an outbreak erupted in the Mediterranean Europe (specially in Italy), with 217 cases of chikungunya confirmed (Liumbruno et al. 2008).

Only by the end of 2013 the first autochthonous cases of the disease caused by the Asian strain of chikungunya virus (CHIKV) was confirmed in the French island of Saint Martin and Martinique island, on the Caribbean. After that, autochthonous cases were soon reported in other 38 regions in the Americas, starting a rapid and unprecedented invasion over Central and South America (Leparc-Goffart et al. 2014). The first autochthonous case of CHIKV caused by the Asian genotype in Brazil was reported in 2014 in the north of the country in Oiapoque, Amapa, a state that makes frontier with the French Guiana, a country delimited by the Caribbean Sea. Closely, another outbreak was reported in Feira de Santana, Bahia, at the Northeast of Brazil, where it was the ECSA genotype the one found circulating. In 2015, the virus begun to spread to areas in the southeast of the country where of approximately 18,000 cases happening in the area during 2016, 13,000 of them were restricted to the city of Rio de Janeiro (Souza et al. 2017). In 2017, the Brazilian Ministry of Health (b) reported an incidence of 63.9 cases/100 thousand inhabitants, most cases found in the northeast of the country followed by the northern region. With two out of four distinct genotypes circulating in the country (Nunes et al. 2015), surveillance of this virus dispersion becomes crucial when assessing the burden over the population.

1.1.3. Zika virus

Zika virus (ZIKV) is a positive-sense, monopartite, single stranded RNA virus of the Flaviridae family and is divided into three main lineages: Asian, East African and West African (Faye et al. 2014). Its polyprotein encodes for a capsid (C), precursor of membrane (prM), envelope (E) and seven non-structural proteins (NS), all flanked by two noncoding regions (59 and 39) in a RNA molecule with the length of 10794 kb: 59-C-prM-E-NS1-NS2A-NS2BNS3-NS4A-NS4B-NS5-39 (Chambers et al. 1990, Kuno &

Chang 2007). Each of these proteins are responsible to different viral functions such as maintenance of the viral cycle, binding and membrane fusion with host cells, RNA polymerase and RNA capping (Lindenbach & Rice 2003).

This virus was firstly isolated in 1947 from a sentinel rhesus monkey (*Macaca mulata*) situated in the Zika forest, Uganda. The area had been picked as one of the yellow fever study areas, having six sentinel platforms stationed near the forest canopy with a rhesus monkey on each of them, having their body temperature assessed daily. When one of the monkeys presented an elevated body temperature, it was brought back to the laboratory having its blood sampled, the serum inoculated intraperitoneally or intracerebrally into different groups of mice and subcutaneously into another rhesus monkey. From the tested animals, only the mice inoculated via intracerebral exhibited symptoms of disease on ten days after infection. The virus was isolated from the brain of that mouse and from the serum of two Rhesus monkeys. Afterwards, this virus lineage was denominated Zika in response to the name of the forest in which it was originally isolated (Dick et al. 1952).

Zika virus natural cycle consists on the transmission between sylvatic mosquitoes, mainly of the *Aedes* genus, and monkeys. ZIKV was described in mosquitoes after capturing *Aedes africanus* also for yellow fever virus (YFV) monitoring in Zika forest (Dick et al. 1952). Later, virus isolation was performed after capturing *Ae. apicoargenteus*, *Ae. luteocephalus*, *Ae. vitattus*, *Ae. aegypti* and *Ae. furcifer* (Hayes, 2009). The first description of *Ae. aegypti* mosquitoes naturally infected with ZIKV was in 1969, in Malaysia (Marchette et al. 1969).

The first report of a human case of ZIKV happened in Uganda 17 years after the virus was isolated on monkeys in 1947 (Simpson, 1964). Additionally, a further serum prevalence study carried out on some regions of Nigeria pointed that 40% of sampled population had the neutralizing antibody for Zika virus, showing a high prevalence of the virus among local inhabitants (Fagbami 1979). Still, there were no reports of outbreaks until April 2007 when there was a reported outbreak at the Yap Islands, Micronesia, of a disease showing symptoms as rash, fever, conjunctivitis and arthralgia, clinically distinct from the ones caused by dengue virus. There was a total of 49 confirmed (tested by RT-PCR) and another 59 probable (tested for IgM antibodies) cases of Zika, but by doing estimates, it was possible that 73% of the Yap Islands residents had been infected by the Zika virus (Duffy et al. 2009).

Thereafter, cases have been reported in other countries. In 2013, a dengue-like fever outbreak have erupted in French Polynesia. Later, laboratory assays could diagnose the pathogenic agent as the ZIKV, which produced an estimate of 19,000 suspected cases throughout the islands (Cao-Lormeau et al. 2014). It is believed that the virus spread overseas through other Pacific Islands until reaching the Americas on 2014. Controversial evidences point that ZIKV may have arrived either during the World Cup soccer competition or more probably during the canoe race Va'a World Sprint Championship taking place in Rio de Janeiro, Brazil, where several Pacific countries were participating (Musso 2015a, Zanluca et al. 2015). On 2015, the first autochthonous case of Zika in Brazil was reported after the virus was isolated from the serum of eight patients from Natal, Rio Grande do Norte (Zanluca et al. 2015).

Zika virus transmission through mosquito bite is massively associated with mosquitoes belonging to the genus of *Aedes*. Remarkably, despite some claims *Culex quinquefasciatus* may act as a natural vector (Guo et al. 2016; Guedes et al. 2017), several evidences have point almost exclusively to *Aedes aegypti* as its single vector worldwide (Lourenço-de-Oliveira et al. 2018; Roundy et al. 2017). Under laboratory conditions, this species have already been tested as a competent vector using both laboratory (Costa-da-Silva et al. 2017) and wild populations (Weger-Lucarelli et al. 2016; Chouin-Carneiro et al. 2016). Regardless the population origin, the presence of ZIKV-infective particles in the saliva fourteen days after infection was a common trend among the above mentioned studies. Moreover, this species have already been detected carrying natural infection by the Zika virus when captured on the field (Guerbois et al. 2015; Ferreira-de-Brito et al. 2016).

Current estimates show that so far 84 countries have reported autochthonous cases of ZIKV. From that, 31 have reported microcephaly on newborns associated with the ZIKV infection and 23 countries have presented an increased incidence of Guillain-Barré syndrome among ZIKV patients (WHO 2017c). The dramatic increase in microcephaly and Guillain-Barré syndrome pushed the World Health Organization (WHO) to declare ZIKV outbreak a global public health emergency (WHO 2016). After a rapid decrease in the number of cases, the state of emergency was ended by November of 2016, the period between the first and 22nd epidemiological weeks of 2017 showing a 14 fold decrease in number of cases when compared to the same period in 2016 (PAHO 2017). However, the number of asymptomatic cases, and the

non-specificity of the clinical manifestations, which may be mistaken with other arboviral diseases, still leave the need to constant surveillance.

1.2. *Aedes (Stegomya) aegypti*

1.2.1. Biology

Mosquitoes are insects from the Diptera order and Culicidae family with more than 3,000 species described (Consoli & Lourenço-de-Oliveira 1994). They are holometabolous insects, going through the stages of egg, four larval states, pupae and adult during its life cycle (Figure 1). It happens when a bloodfed and inseminated female lay its eggs on the walls of a suitable breeding site, which can resist in the environment and may remain viable for more than a year without being in contact with water (Rezende et al. 2008). After 2-3 days of embryonic development and when in contact with water, these eggs will then hatch into the first larval state (L1). The time it takes for a larvae to mature into pupae varies from 7 to 10 days, and is dependable on a series of external factors such as larval density, amounts of food available and temperature or the area the breeding site is located (Consoli & Lourenço-de-Oliveira 1994, Moore & Whitacre. 1972). After approximately two days, a new metamorphosis occur and the pupae gives way to the adult mosquito.

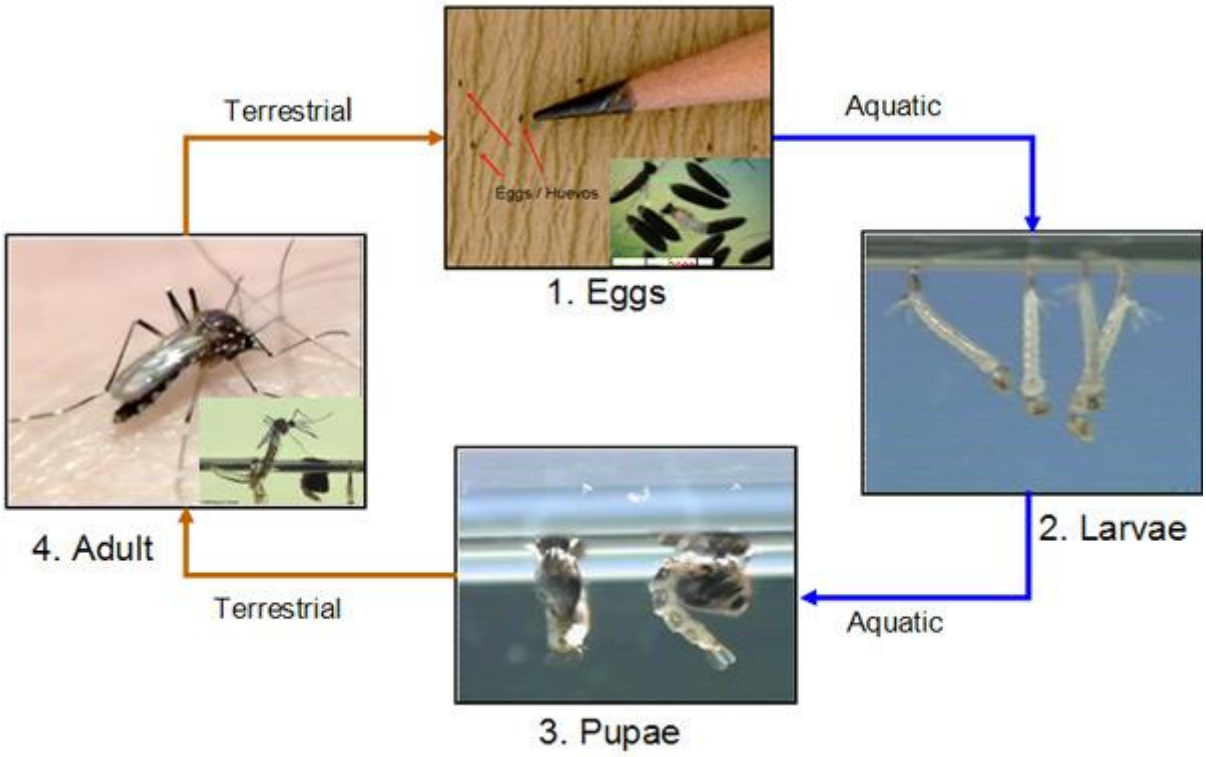

Figure 1: *Aedes aegypti* life cycle (Adapted from CDC. Access in: February 06, 2018).

The *Ae. aegypti* mosquito was originally described in sylvatic regions of the African continent. A sub-form named *Aedes aegypti aegypti* became highly adapted to humans and spread over the globe likely during the colonial period on ships used for slave trading (Consoli & Lourenço-de-Oliveira 1994). By the 1950's, the species was considered eradicated in several American countries, including Brazil, after strong vector control campaigns supported by the Pan American Health Organization (PAHO) and Rockefeller Foundation aimed to decrease the incidence of urban YFV. By 1962, 18 Latin American countries, including several Caribbean islands had successfully achieved eradication. However, some countries, namely Cuba, United States of America, Venezuela and a number of Caribbean insular territories failed local eradication. By doing so, during the 1960's, *Ae. aegypti* mosquitoes started to be detected in low densities in the countries where it had been previously considered eliminated (Schatzmayr 2000, Bracco et al. 2007). There are ongoing debate regarding whether *Ae. aegypti* was really eradicated during the 1960's or if the infestation levels had just dropped considerably in a way vector detection would be difficult to accomplish by the available tools at that time (Bracco et al. 2007). Nowadays, this species is

present on all continents, primarily associated with human properties in cities located on tropical and sub-tropical climates (Figure 2) (Kraemer et al. 2015).

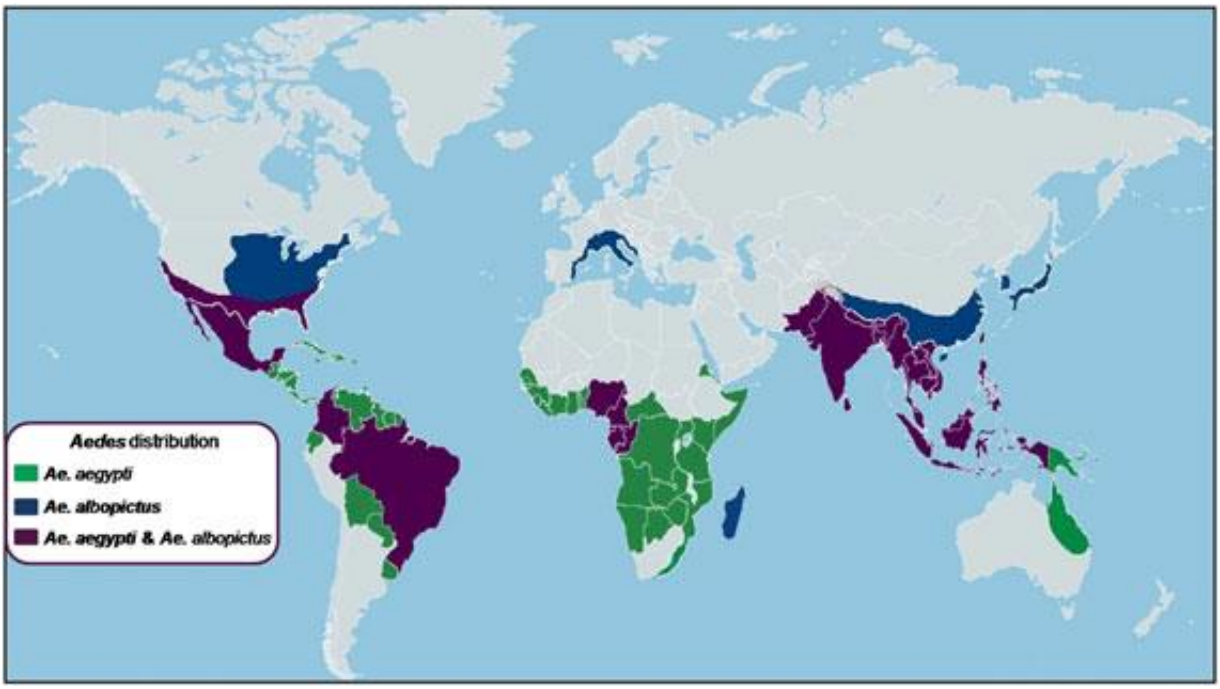

Figure 2: Distribution of the vectors *Ae. aegypti* and *Ae. albopictus* (Adapted from: Agarwal et al. 2017).

Ae. aegypti is an extremely anthropophilic mosquito, that is more likely found in human highly populated and urbanized areas. This predilection to live near human agglomerations allowed the mosquito to lay eggs preferentially on artificial containers holding clean water. Females present an opportunistic oviposition behavior, laying eggs on artificial containers with a high range of size, shape and material, from cans to plant pots or even larger containers like water tanks and pools that are abandoned or taken poor care of, for instance (Peryassu 1908, Consoli & Lourenço-de-Oliveira 1994, Maciel-de-Freitas et al. 2007a).

It's important to notice that the breeding site preference and productivity may vary greatly between different neighborhood disposition and population habits. Studies carried out in three different areas of Rio de Janeiro observed a few of these differences, the areas were a low income region known as Favela do Amorim, a medium income area, Tubiacanga, and a high income area known as Urca. Favela do Amorim is a slum characterized by small homes of one room that usually share walls with other houses, lack of peridomestic area and narrow alleys, with inadequate

sanitation and garbage collection, leading the population to store water in large containers. Tubiacanga is a secluded area, surrounded by the walls of the Tom Jobim International Airport of Rio de Janeiro and Guanabara Bay, most houses have two dorms and a large peridomestic area, and, although it has average sanitization and water supply, inhabitants tend to store water in large containers. Moreover, Urca consists on mainly high standard houses, usually with two floors and three dorms, large and shaded peridomestic areas, sparsely populated and with adequate water provision, garbage collection and sanitation. In 2008, these areas have had 861, 607 and 74 confirmed dengue cases, respectively. The studies show that the areas of low and medium income demonstrated a higher presence of immature forms in water tanks and metal drums, which can be explained by an irregular water supply and need of the population to store water, other productive breeding sites in these areas were kitchen items, plant vases, buckets, plastic plates and covers. Whereas, Urca's most productive breeding sites were domestic drains and discarded plastic pots (Maciel-de-Freitas et al. 2007a, David et al. 2009).

1.2.2. Feeding behavior

Aedes aegypti larvae are detritivorous and will feed indiscriminately by chewing particles from decomposing organic matter, microorganisms or even carcasses of other invertebrates very often from the same species found on the breeding site (Merritt et al. 1992, Clements 1999). However, during pupae stage, the insect tends to remain resting close to the water surface, where it does not feed. This period is characterized by severe chemical, hormonal and physiological changes required to complete mosquito metamorphosis to flying adults (Gilbert 2000).

Upon reaching adulthood, the male *Ae. aegypti* mosquitoes will feed exclusively on sugar prevenient from plant nectar containing fructose and sucrose, from which the energy for metabolism and flight is obtained (Van Handel 1972; Briegel et al. 2001). While females can also feed on sugar, this source of energy might not be the preferred one in all possible scenarios. For instance, *Ae. aegypti* females collected on the rural regions of Thailand seldom had carbohydrates on their digestive system, suggesting mosquitoes on that environment might recur frequently to blood sources to support the basic needs for metabolism (Edman et al. 1992). Besides, data gathered on laboratory

controlled environment revealed that when a female is fed exclusively on sugar, or with sugar and a blood meal, this female will have increased longevity, but will lay fewer eggs than those that has been fed exclusively on blood (Day et al. 1994).

When female mosquito feeds on sugar, most of it will be redirected to a structure called the diverticulum, where it is stored and progressively released to the midgut and just then absorbed into the insect's organism. The continuous release of small portions of sugar assure the provision of the energy required for the basic needs and metabolism. The storage of sugar components on the diverticulum allows the mosquito intestine always to be free to receive and process a blood meal (Consoli & Lourenço-de-Oliveira 1994). The blood ingested by a female mosquito is crucial for the development of the ovaries and the embryonic stage of the eggs. While many species need only one meal to mature their eggs, *Ae. aegypti* often needs to feed more than once in a single gonotrophic cycle, a phenomenon known as gonotrophic discordance (Lea et al. 1956, Scott et al. 1993). This behavior sums up to the epidemiological importance of this species, since the need of numerous bites in order to lay a single batch of eggs has a great impact on the vector capacity by increasing the chances of the mosquito acquiring or transmitting a virus among susceptible hosts on a human population (Scott et al. 1993).

1.3. Transmission Ecology

Arbovirus transmission, maintenance and amplification may be carried by three distinct routes: horizontal, vertical and venereal transmission, that can be carried between a mosquito and a vertebrate host, between mosquitoes (Figure 3) and between humans. The primary route of transmission of arbovirus to the human population regards the direct participation of female mosquitoes, overwhelmingly of the *Aedes*, *Anopheles* and *Culex* genus. First, a susceptible mosquito must bite an infected host. After ingested, the blood containing the infectious particles goes to the midgut where the virus attaches to the epithelial cells and begins its replication, eventually escaping the midgut into the hemocoel and invades other organs and tissues (Hardy et al. 1983). From the hemocoel, the virus will start invading and replicating inside other organs such as the ovaries and salivary glands, so when the mosquito bites a new host the virus may be secreted along the saliva that way,

restating the cycle (Agarwal et al. 2017). It takes from four to seven days for the virus be found disseminated through the head and body of the mosquito. However, only fourteen days after being infected that the virus was found in the mosquito saliva, and now capable of infecting another susceptible host (Salazar et al. 2007, Chouin-Carneiro et al. 2016).

Although not as common, there is some speculation that the transmission interspecies plays an important role on the virus cycle maintenance during interepidemic periods and evidence of vertical and venereal transmission of arboviruses being already documented. A pool of male *Culex pipiens* have already been found positive for West Nile Virus (Anderson et al. 2006). Martins et al (2012), when testing pools of larvae of *Aedes aegypti* and *Aedes albopictus*, found two pools of *Ae. albopictus* larvae to be infected, one with DENV2 and the other with DENV3, while one pool of *Ae. aegypti* larvae was found carrying infection by DENV2. Among other viruses, there were also two reports of ZIKV naturally infected male pools being found in the wild, once in Africa (Diallo et al. 2014) and another time in South America (Ferreira-de-Brito et al, 2016). In laboratory conditions, venereal (Campos et al. 2017, Pereira-Silva et al. 2018) and vertical (Ciota et al. 2017) transmissions have also been observed for Zika virus, supporting the evidence of it happening in natural conditions.

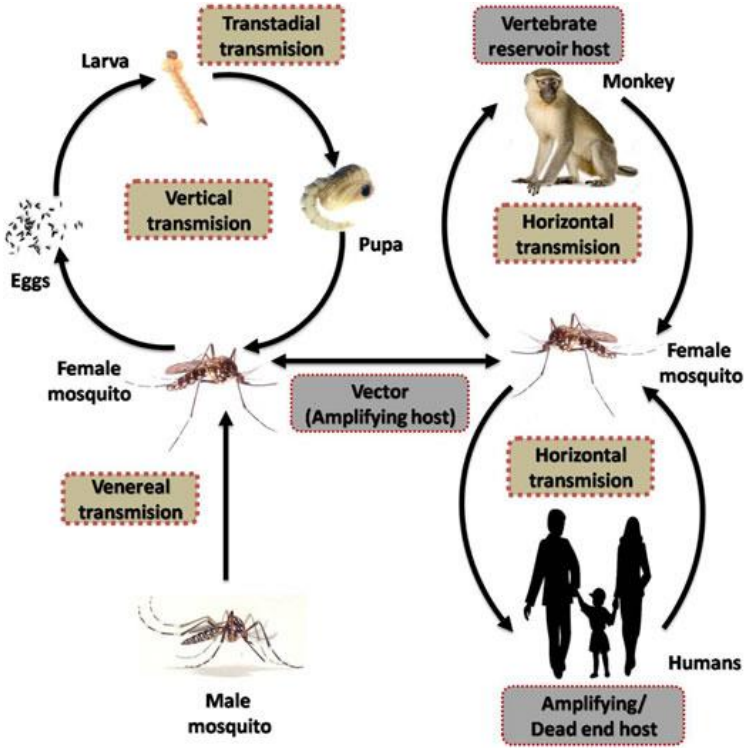

Figure 3: Arboviral horizontal, venereal and vertical modes of transmission regarding the invertebrate host (Adapted from Agarwal et al. 2017).

Another uncommon, yet relevant, way of arbovirus transmission is between humans through contact with contaminated fluids and needles, saliva and sexual intercourse. Studies have shown evidence of the presence of dengue and Zika virus in the saliva of patients (Musso et al. 2015b, Gourinat et al. 2015). Moreover, that viral particles of ZIKV, DENV and WFV can be found in urine for a prolonged period after the acute stage of the disease, increasing the window for detection of infection in these patients (Gourinat et al. 2015, Mizuno et al. 2007, Tonry et al. 2005). Chikungunya virus could also be detected in patients saliva and urine, however, the virus copies in both fluids decreased significantly after the symptomatic phase, not being a good means to prolonged detection as they are for other arbovirus (Musso et al. 2016). Evidence also points to the potential of sexual transmission of these diseases, Zika virus already described on human semen, and a few reports of people who did not live and had not traveled to risk areas and had developed the disease (Barzon et al. 2016, Septfons et al. 2016).

1.3.1. Vectorial capacity (VC)

The concept of vectorial capacity was introduced by Garret-Jones (1964), as a concept defining how many infective bites could a population of vector distribute to man from a single case if all female vectors became infected upon biting it. This definition was firstly used to malaria vectors, but later extrapolated to other models, including vectors for arboviruses and it covers several parameters that can be expressed according the following equation:

$$VC = \frac{mbca^2 P^n}{Ln\,(P)}$$

Each of these parameters represents a different aspect that will directly influence the capacity of a vector acquiring and transmitting a virus:

- *m* represents the density of the vector, meaning how many female mosquitoes exist per person. This variable can be affected by seasonal and climate changes, since mosquito densities are strongly related to more rainy seasons (Consoli & Lourenço-de-Oliveira. 1994, Liu-Helmersson et al. 2014). Moreover, as previously described, areas socioeconomic profile can also influence on vector density, due to availability of productive and unattended breeding sites (Maciel-de-Freitas et al. 2007a, David et al. 2009).

- *b* represents the probability that an infected mosquito has of transmitting the parasite when biting a susceptible host and *c* is the probability that a mosquito has of becoming infected with a parasite when biting an infected host. The vector competence of a species can be directly affected by genetic components and genotype interactions (Tabachnick 2013). Studies infecting different populations of a mosquito species with viruses prevenient of other areas reported varying levels of susceptibility and vector competence of the different populations to a single virus strain (Lourenço-de-Oliveira et al. 2004, Lourenço-de-Oliveira et al. 2013, Vazeille et al. 2013).

- *P* stands for the mosquito survivorship, considering that the longer a vector lives, higher is the chance it enters in contact with a transmittable pathogen and live through its incubation period, allowing the virus to be possibly transmitted to a new host. As the availability of breeding sites, this parameter can also be affected by

socioeconomic profile of the environment, and the daily survival rates are much lower in high income areas than in areas of low income, a difference of approximately 40% (Maciel-de-Freitas et al. 2007b, David et al. 2009).

- *a* is the bite rate of the insect, a parameter that can be influenced by the vector's density and biting behavior, such as the need for feeding multiple times in a single gonotrophic cycle (Scott et al. 1993). Additionally, this parameter is squared in the equation considering it takes, at least, two bites in order for the parasite to be vertically transmitted: a first one to acquire the pathogen and a second one to inoculate it into a new host.

- *n* represents the extrinsic incubation period (EIP), meaning the time it takes from the moment the mosquito takes an infected blood meal for the parasite to reach the vector's salivary glands. Climate have also shown to have an important role on the EIP of certain virus and can shorten the time it takes for the vector become infective. An experiment observing the effect of microclimate over the extrinsic incubation periods of six different pathogens (bluetongue, Schmallenberg, dengue, West Nile Virus, *Dirofilaria* and *Plasmodium sp*), observed reduction of the EIP in all of them (Haider et al. 2017). The effect of temperature also seen, among other studies, when infecting Ae. albopictus with DENV-2, as higher were the temperature, shorter was the EIP, at 32°C it reaching only 5 days, when it usually varies between 10-14 days to the virus reach the salivary glands (Liu et al. 2017).

1.3.2. The effect of a pathogen infection on the mosquito biology

The use of the vectorial capacity formula proved to be a great theoretical tool to estimate how effective a vector population is in transmitting a pathogen. However, getting an accurate measure of all components of the equation on a field setting can become extremely demanding (Dye 1986). Besides, inaccurate estimations might lead to the overlooking of other parameters relevant to epidemiological research such as the effects of the pathogen infection have over the biology of the mosquito. In fact, the vectorial capacity formulae and rational might be revisited if pathogen infection is able to alter dramatically any of its parameters.

If a parasite infection can provoke changes to its host behavior or pose a fitness cost on its life-history traits, then it may even affect the probability of transmission

according to the vectorial capacity formula. There are some reports pointing host manipulation by the pathogen in some model systems. Regarding feeding behavior of the host, it has been seen that a few species of *Plasmodium* may cause the increase of the mosquito bite rate, enhancing the probability of malaria transmission to a new host. When Rossignol et al (1986), observed the behavior of *Ae. aegypti* infected with sporozoites of *Plasmodium gallinaceum*, although the mosquito showed a reduced number of eggs per clutch, the number of attempts at probing before feeding had increased. Similarly, when infected with *P. falciparum* sporozoites, *Anopheles gambiae* also showed an increased bite rate, as well as a larger amount of ingested blood than the uninfected group (Koella et al. 1998).

A similar trend have been observed for dengue virus (DENV) infections in *Ae. aegypti* mosquitoes. Platt et al (1997), observed that DENV-3 infected mosquitoes spend longer periods of time probing as well as blood-feeding than control groups. And more recently, Sylvestre et al (2013), showed that DENV-2 also had the same effect over the invertebrate host, and, when infected with the virus, it would spend a longer time ingesting blood. However, interestingly, these modifications were observed just after the second gonotrophic cycle onward. Authors speculated it was due the extrinsic incubation period of DENV (around 7-12 days (Watts et al. 1987)) and the time virus was spread through mosquito body (Sylvestre et al. 2013).

A critical parameter regarding the interaction among mosquitoes and pathogens is the lifespan of the infected host. From an evolutionary perspective, any reduction in vector survivorship would have negative consequences on pathogen transmission. Some papers show reduced survival when mosquito vectors are infected with protozoa and virus. For example, *Ae. aegypti* mosquitoes infected with DENV-2 as well as *Anopheles stephensi* mosquitoes infected with *Plasmodium yoelli nigeriensis* gametocytes had a reduced lifespan when compared to control groups (Hogg 1995, Maciel-de-Freitas et al. 2011, Sylvestre et al. 2013). Although its relevance, the potential impact of the reduction of vector lifespan due to pathogen infection on transmission remains elusive.

Another recurring observation on several parasite-vector systems regards the effect of ageing over mosquito fecundity. While it is noticed that age itself can negatively influence the vector fecundity (McCann et al. 2009), the effects of ageing over the clutch size is further enhanced when the mosquito is infected with a pathogen.

When monitoring the fecundity of *Culex tarsalis*, the control group laid a larger first clutch, with the number of eggs reducing over time. The same trend was observed on the group infected with West Nile virus (WNV), however, the number of eggs present on the first batch were only half of the number present on the uninfected group (Styer et al. 2007a). Yet another study, shows that the *Anopheles stephensi* when fed on *Plasmodium yoelli nigeriensis* infected mice containing either gametocytes or pre-infective stages of the parasite, would lay a reduced number of eggs than those of the uninfected group. Furthermore, the mosquitoes who had fed on the mice containing gametocytes would have an even further reduced clutch size than those fed on the pre-infective stages (Hogg. 1995).

A similar pattern of reduction was observed by Maciel-de-Freitas et al, (2011) after showing DENV-2 infected *Ae. aegypti* females had a sharper reduction on the oviposition success (represented by the number of females laying at least one egg per gonotrophic cycle) over time than uninfected ones. Egg-laying success decreased over the clutches as the females became older, ranging from a success of 95% on the first clutch to 75% at the fifth clutch, with an even sharper decrease when mosquitoes were blood-fed with a DENV-2 infective blood. The same results of a sharper fecundity reduction when compared to a control group were observed on similar experiment using a different *Ae. aegypti* population, but the same DENV-2 strain (Sylvestre et al. 2013).

1.4. Mosquito senescence

Mortality is one of the many factors with a great impact over a mosquito vectorial capacity, since the longer it lives, more likely it is that female finds and bites an infected host and survives the incubation period of the pathogen becoming competent to transmit it to another susceptible host (Dye 1992; Clements & Paterson 1981). Still, for a long time it was considered that mosquito mortality rates did not vary with age, since it would die from environmental factors or predation rather than by ageing (Styer et al. 2007b). Although it might sound counterintuitive, there were no evidences up to the 2000's to deny this powerful assumption. Upon carrying a large scale experiment, with 29 replicates and a total of over 100.000 *Ae. aegypti* observed, Styer et al (2007b) observed differences in mortality rates as the mosquitoes aged and compared two

vector capacity models, one considering age-dependent mortality and the other not, to assess the effect this parameter may pose to the overall vector capacity of the mosquito. In this experiment, all cohorts demonstrated age-dependent mortality, females living almost double the period males did (31 days versus 16 days). Moreover, the age-dependent VC model returned with a higher contribution of younger mosquitoes to the transmission of a pathogen, since they are more likely to survive the EIP, and have an overall longer life expectancy than old mosquitoes, while age-independent models give equal importance to young and old mosquitoes (Styer et al. 2007b).

Age prediction analyses may be carried through several different manners. Unfortunately, so far, none of the proposed methods and techniques were able to provide a precise estimation of mosquito age. The structure of salivary glands undergoes morphological changes, with structural alterations that became even more pronounced as the mosquito gets older, as well as apparent function reduction like a loss of secretion and a decrease of protein synthesis (Beckett 1990). Morphological differences can also be spotted by observing the ovaries' trachea and the presence of midgut meconium. Both methods are classified as labor intensive and time-consuming, with a considerable risk of misinterpretation due to entomologist skills in observing the adequate target tissue (Hugo et al. 2008).

Other ways to predict age may be by observing changes in the cuticle through gas chromatography analysis of cuticular hydrocarbon peaks pattern due to their role in prevention of water loss being possibly affected by environmental changes and observations on quantitative changes in these profiles in insects with the passing of time (Desena et al. 1999). However, this technique was only accurate on predicting age in insects up to 12 days old, the exploration of new methods being necessary. One of which is by selecting genes with significant differences in age-dependent expressions. The transcriptional profiles of these genes can be assessed in laboratory in order to determine the mosquito approximate age with a difference of ±3.8 days from the mosquito actual age when observing more recently described genes (Cook et al. 2006, Caragata et al. 2011). A more recent approach, and none destructive as the previously mentioned, is the use of near infrared spectroscopy (NIRS) to collect multiple cuticle scans and later differentiate its biochemical composition through the different resulting spectra considering that an insect cuticle is different from other

insects and that it may differentiate with aging. This method demonstrated an age-predicting accuracy of approximately 80% in field-sampled populations (Mayagaya et al. 2009).

The effects of ageing on pathogen transmission goes beyond surviving to the extrinsic incubation period. Ageing may also alter morphology and even downregulate other vital functions of the insect. Detoxification and biosynthetic functions also showed to be impaired by aging, with a decrease in the levels of enzymes involved in the process (Hazelton & Lang 1984). Therefore, the lower detoxification functions of older mosquitoes, together with an increased cuticle permeability, could be the main reason to explain why older individuals have greater susceptibility to insecticides, particularly considering *Anopheles* mosquitoes challenged with DDT, permethrin, deltamethrin, malathion and propoxur (Lines & Nassor 1991, Chouaibou et al. 2012). Regarding another aspect of mosquito-virus interaction, the vector immune functions is also negatively affected by the aging process. When 14 days old mosquitoes are inoculated with microfilariae, for instance, a significantly decrease on the melanization response is observed if compared with those inoculated before on day old post-emergence, that showed a similar response to those of 4 to 6 days old (Christensen et al. 1986).

Additionally, an experiment assessing the flight performance and usage of energy reserves in different species of mosquitoes as they aged showed that older mosquitoes had a substantial decrease in speed and distance covered as well as in the utilization of their energy reserves. An increase in glycogen levels were detected as the mosquito suffered from a reduction in its flight ability (Nayar & Sauerman 1973). Moreover, an experiment with *Culex quinquefasciatus* females demonstrated that, while the size of blood meal and body size of the mosquito had a positive influence over the number of eggs laid per gonotrophic cycle, the number of eggs per clutch reduced significantly over time (McCann et al. 2009).

1.5. Justification

Parasite and vector interaction have often been overlooked, neglecting that the pathogen may modify or even manipulate its invertebrate's host biology. By that, several parameters of vector life-history traits might be modified across different stages of its life cycle. The late outcome is that pathogen infection itself may influence each

of the parameters of vectorial capacity and thus affect transmission in either a positive or negative way. Several studies have pointed a significant effect of ageing over fecundity, parameter that influences one of the many important concepts involved on the vector dynamics and vectorial capacity (Dye 1986). It has been seen that *Cx. quinquefasciatus* has its fecundity reduced as ageing (McCann et al. 2009). The same pattern of reduction was seen on *Cx. tarsalis* (Styer et al. 2007a), *Ae. aegypti* (Maciel-de-Freitas et al. 2011, Sylvestre et al. 2013) and *Anopheles stephensi* (Hogg 1995), where the presence of a parasite infection only further reduced the egg clutch sizes, when compared to the uninfected groups, as the mosquitoes aged.

However, it is still unclear whether the reduction in the number of eggs laid (fecundity) is due to a smaller ingestion of blood as the female ages, or if female ability to produce eggs production gets naturally reduced by ageing. A recent study with *Drosophila melanogaster* showed that both male mating capacity and the number of females laying eggs after mating with them present a significant decrease. A lower amount of sperm is stored in the female's seminal receptacle shortly after mating and induce fewer post-mating traits on the females suggesting that the males capacity to produce high quality seminal fluid particles was impaired (Ruhmann et al. 2018). Taking into account the published results of mosquitoes and other insects, we hypothesize that biological changes of similar nature may also occur to female mosquitoes as they age, leading to a smaller production of eggs. Therefore, we intend to assess the correlation of aging, blood meal size and fecundity and whether ZIKV infection can enhance the effects of ageing on *Aedes aegypti* females.

2. Objective

Investigate the effects of aging and blood meal size on the fecundity of *Aedes aegypti* females infected or uninfected with ZIKV.

2.1. Specific objectives

1) Evaluate the impact of aging over the size of blood meal and fecundity of a population of *Ae. aegypti*.

2) Evaluate the impact of blood meal size on fecundity.

3) Evaluate the impact of ZIKV infection over longevity, blood meal size and fecundity of a population of *Ae. aegypti*.

3. Submitted Paper

The following results were originally submitted on March 1, 2018 to Emerging Microbes and Infection as a Research Article.

“Dear Mrs Petersen,

This is to acknowledge that your manuscript EMI2018164 with title "The impact of the age of first blood meal and Zika virus infection on *Aedes aegypti* egg production" by Martha Petersen, Isabella D da Silveira, Aline Ferreira, Mariana David, Thais Chouin-Carneiro, Liesbeth Van der Wouwer, Louis Maes, and Rafael Maciel-de-Freitas has been submitted to Emerging Microbes & Infections.

Editorial Office shall contact you when Initial Checking is complete.

Thank you very much for your contribution.

http://mts-emi.nature.com/cgi-bin/main.plex?el=A3CU4jU4A5EsC5F6A9ftdjVtEAM0i2jhWrdTZ7GEuuwZ

Emerging Microbes & Infections
Editorial Office”

And later submitted to Plos One.

The impact of the age of first blood meal and Zika virus infection on *Aedes aegypti* egg production

Running title: Aging and ZIKV effects on mosquito egg production

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Abstract

The impact of senescence and pathogen infection on *Aedes aegypti* life-history traits remains poorly understood. Herein, we focused on the impact of Zika virus (ZIKV) infection and the age of first blood intake over female's blood meal and clutches sizes and, mostly important, on the egg production ratio per µL of blood. For that, we monitored three groups of ZIKV-infected and uninfected groups that received their first blood meal at 7 (Young feeders), 14 (Mature feeders) and 21 days old (old feeders). Afterwards, mosquitoes were daily monitored for survival, received a blood meal free of ZIKV once a week and the number of eggs laid per female were registered 3-4 days after blood feeding. Infection by ZIKV and age of feeding produced a strong negative impact on *Ae. aegypti* survivorship and on oviposition success (the likelihood of laying at least one egg per gonotrophic cycle). Interestingly, clutch size presented a dramatic reduction on uninfected mosquitoes but raised from 36.5 in clutch1 to 55.1 in clutch3. Blood meal size remained stable for uninfected females, but a slight increase was observed for the infected counterparts. Egg production was strongly affected by age of feeding on uninfected *Ae. aegypti*, with younger females laying three times more eggs than when older. On the other hand, ZIKV-infected mosquitoes had a constant but low egg production.

Introduction

In the last decades, mosquito-borne arboviruses have emerged in different regions of the globe causing severe outbreaks on human population. Since the 1970's, dengue virus (DENV) transmission has shown a 30-fold increase in its worldwide incidence with estimates of around 400 million new infections every year [1,2]. During the late 2000's, chikungunya virus (CHIKV) became pandemic after reaching the Americas with at least two distinct genotypes: the Asian genotype probably arrived through the Caribbean while the East-Central-South African (ECSA) genotype was first detected in central Brazil [3,4]. In 2014, Zika virus (ZIKV) emerged in Pacific islands and later invaded the Americas, bringing a public health emergency due to its association with microcephaly in newborns [5,6]. Between December 2016 and April 2017, an

outbreak outside the endemic region of Brazil resulted in the largest epizootic of jungle Yellow Fever virus (YFV) with 209 deaths and a case-fatality superior to 30% [7].

With the exception of the sylvatic cycle of YFV, which is maintained by New World primates and sylvatic mosquitoes, DENV, CHIK and ZIKV have *Aedes* mosquitoes as their primary vectors [8,9]. The dominant role of *Ae. aegypti* as the primary vector of such arboviruses is partially explained by the fact such species lives in close association with human dwellings. Females are more likely to obtain energy for their metabolism by blood feeding on human hosts rather than in other vertebrate or from sugar feeding. Around 3-4 days later, females preferentially lay their eggs on a variety of man-made breeding sites on the surroundings of human properties [10-12].

The intensity of disease transmission is partially shaped by alterations in mosquito vectorial capacity, which is defined as the total number of potentially infectious bites on humans on a single day [13,14]. For example, dengue transmission intensity is governed by local variations of *Ae. aegypti* vectorial capacity parameters[15]. An accurate estimate of the components of *Ae. aegypti* vectorial capacity in endemic field settings has proven to be extremely difficult due to the complex and multifactorial effects of clime, landscape, mosquito and host densities and breeding sites availability [16].

Although of paramount relevance, the effects of pathogen infection on the biology of mosquitoes have been receiving low attention so far. Some arboviruses are able to invade several tissues including mosquito's brain and are likely to modify its physiology, metabolism and behavior. Therefore, arboviruses are prone to affect the vectorial capacity and thus the pattern of disease transmission [17,18]. A reoccurring observation that have been noticed in several studies is the effect of senescence and pathogen infection over fecundity (i.e. the number of eggs laid per clutch). Older *Culex quinquefasciatus* females laid less eggs over time, especially after 10-days post-eclosion [19]. A similar pattern was also observed for *Cx. tarsalis* [20]. The infection with pathogens worsens this fecundity reduction. The number of eggs laid by *Ae. aegypti* females decreased more than two-fold within the first five clutches, and dengue-infected individuals presented a sharper reduction on fecundity over time [21,22]. *Culex tarsalis* infected with West Nile Virus (WNV) presented a harsher reduction in fecundity than females belonging to the uninfected control group [20]. Moreover, a smaller first

clutch was observed in *Anopheles stephensi* when mosquitoes were fed with a blood meal infected with *Plasmodium yoelii nigeriensis* [23].

The present study investigated i) the effects of the age of first feeding and blood meal size on the fecundity of *Ae. aegypti* and ii) whether Zika virus infection produced an additional loss on the mosquito life history traits while females aged.

Results

ZIKV oral infection. We used a total of 500 F1 *Ae. aegypti* from a colony established from field-caught mosquitoes from Urca, Rio de Janeiro, Brazil. Mosquitoes were divided into three groups according to the age when they received their first blood meal: YF (first blood meal at 6-7 days old, N=300), MF (first blood meal at 13-14 days old, N=100) and OF (first blood meal at 20-21 days old, N=100). In every group, half of the mosquitoes received a ZIKV-infected first blood meal, meanwhile the other half served as uninfected controls receiving only blood and cell culture media free of ZIKV and followed the same feeding procedure. A sample of 18 mosquitoes was indiviadually tested at 14 (N=10) and 21 (N=8) days post infection (dpi) for the presence of ZIKV RNA copies with RT-PCR. All analysed mosquitoes were positive and showed high numbers of ZIKV RNA copies in their bodies (2,6 x 10^7 PFU in average), assuring infection. Mosquitoes sampled at 14 and 21 dpi had a similar amount of ZIKV (t-test = -1.28; df = 11.003; p = 0.228; Figure 1). From this results, it was assumed that the mosquitoes used in fecundity experiments were also ZIKV positive.

ZIKV effects on survival. The highest longevities were observed for mosquitoes belonging to the uninfected treatment. Regardless the age group, uninfected mosquitoes survived longer than the ZIKV-infected counterparts (YF: χ^2 = 46.7, df = 1, P < 0.001; MF: χ^2 = 6.3, df = 1, P = 0.014; OF: χ^2 = 8.5, df = 1, P = 0.003). Survival curves indicate a sharp decrease in survival immediately after blood feeding, irrespective of the age group and treatment (Figure 2). As expected, survival was also affected by the age of first feeding, since mortality was higher when older mosquitoes were blood fed (Table 1). The ANOVA corroborated the survival data: ZIKV-Infected mosquitoes survived less than the uninfected and the age of infection negatively

affected survival, as expected (Table 2). A strong interaction between treatment and age group was observed: the negative effects on mosquito survival were more evident when older mosquitoes were infected with ZIKV.

Oviposition success. Oviposition success was not affected by the age group, i.e. the age mosquitoes received their first blood meal, in the uninfected group. On the other hand, in ZIKV-infected *Ae. aegypti,* the likelihood of females laying at least one egg per gonotrophic cycle was strongly influenced by the age of infection, dropping from a success of 76.1% in YF to 59.3% in OF (Table 3). Regardless of the age group, ZIKV-infected mosquitoes were significantly less likely to lay eggs than the uninfected group.

Fecundity. This analysis consisted on the number of eggs laid by the females who had laid at least one egg. Overall, ZIKV-infected mosquitoes laid less eggs than those uninfected, with the exception of YF, which uninfected dropped from 63.3 to 42.3 eggs from clutch 1 to clutch 3, meanwhile clutch sizes of those infected surprisingly raised from 36.5 eggs in clutch 1 to 55.1 eggs in clutch 3 (Figure 3). The age of the first feeding apparently not produced any relevant influence on the number of eggs laid per gonotrophic cycle. Wing size had no significant effect over clutch sizes (Table 4).

Blood meal size. Blood meal size was weekly measured by quantifying the hematin from mosquito feces on the filter paper in the bottom of vials (Table 5). ZIKV-infected females ingested significantly more blood than their uninfected counterparts in the first (F = 9.386, df=1, P = 0.002) and second blood meals (F = 14.91, df=1, P = 0.0002). There were no significant effects of the age of infection and wing size. The blood meal size of uninfected mosquitoes remained stable over the two first gonotrophic cycles, however, the amount of eggs produced with a roughly similar amount of blood dropped with ageing (Figure 3; Figure 4). The ratio of egg production per blood volume ingested slightly increased for ZIKV-infected mosquitoes, while the uninfected presented an intense reduction of egg production over time. Infected individuals are less effective in producing eggs from a blood meal than uninfected ones in the first two gonotrophic cycles (Figure 5).

Discussion

This study investigated in greater detail the potential impact of aging, blood meal size and ZIKV infection on *Ae. aegypti* life-history traits including fecundity, namely oviposition success, clutch size and egg production per unit of blood ingested. Mosquitoes received their first blood meal (ZIKV-infected or uninfected) at the ages of 7, 14 and 21 days-old. ZIKV infection produced a negative effect on female lifespan and oviposition success but increased the number of eggs laid per female at later clutches. Furthermore, egg production presented a sharp decrease over time in uninfected mosquitoes, while ZIKV-infected individuals presented a slight increase in the production of eggs per µL of blood ingested.

Mosquito survival is one of many parameters that can influence vectorial capacity, since the mosquito must live for at least 5 days to support ZIKV transmission [29]. Our data show that ZIKV consistently caused a negative effect on *Ae. aegypti* survival. All three age groups that had received a first infective blood meal presented lower survival rates than their uninfected counterparts. The differences in survival rates between the age groups, infected or not, showed a strong age-dependent factor on mosquito mortality [30] that was further enhanced by the presence of ZIKV infection. The likelihood of younger mosquitoes presenting a longer lifespan after ZIKV-infection alludes arbovirus transmission models might consider different mortality distribution for infected individuals [30]. More details regarding the age-dependent mortality, particularly in the scenario where disease vectors are infected with their natural pathogens, would incorporate a more comprehensive knowledge on disease transmission [30-33].

ZIKV infection had a significant impact on fecundity. The likelihood of infected individuals laying at least one egg was statistically lower than for their uninfected counterparts. On the other hand, ZIKV-infected females laid bigger 3rd compared to those uninfected. As far as we are aware, there are yet no papers pointing to any modification in mosquito fecundity due to ZIKV infection. DENV infection is able to reduce fertility and fecundity in vertically infected batches [34] as well as the oviposition success and clutch size in orally challenged individuals [21,22]. The age of the first blood meal negatively affected oviposition success but presented no significant effect on clutch size. The number of eggs laid often decreases over time, but seems to reduce faster if mosquitoes are infected with pathogens. In *Cx. quinquefasciatus*, the number

of eggs per clutch reduced significantly as the mosquitoes senesce [19]. A sharper reduction on clutch sizes was detected when *Cx. tarsalis* and *An. stephensi* were infected with WNV and *P. yoelli nigeriensis*, [20,23]. *Ae. aegypti* females infected with a DENV-2 strain had lowered fecundity , with the main impact occurring 2-3 weeks post-infection [22]. These findings of age-dependent effects on life-history was thought to be a consequence of the dynamics and tropism of DENV, since it is disseminated over the *Ae. aegypti* body after ~10-14 days [35]. The biological relevance of the reduction of oviposition success and late increase on clutch size in ZIKV-infected mosquitoes is still unknown.

So far, the fitness cost due to ZIKV infection on *Ae. aegypti* mosquitoes largely remains unknown. A cost of arbovirus infection on vector survival was likely present in DENV-2 infected *Ae. aegypti*, since infected groups also showed increased mortality rates compared to uninfected [21,22]. One important consideration regarding the fitness cost of arbovirus is the natural history of both virus and vectors. Vector competence to a same virus strain often presents great variation among mosquito populations, showing a strong geographical component [36-39]. Therefore, more realistic results regarding vector competence are expected if mosquito population and virus strain are collected in the same study area or in the immediate vicinity [36]. Remarkably, the need of testing mosquitoes and viruses from the same geographical location to evaluate the fitness cost of infection has not been addressed so far. Despite the abundant evidence showing that vector competence varies across geographic distinct mosquito populations, no proof of impact on life-history traits is available. Despite observing a strong cost of DENV-2 infection on *Ae. aegypti* mosquitoes. Co-evolution of a DENV-2 strain that circulated in the 1970s in Bangkok and Rio mosquitoes did not occur [21,22]. Here, we used *Ae. aegypti* mosquitoes from Rio de Janeiro city and a ZIKV from Pernambuco, a Northeast State distant ~1,800Km. Lastly, it is possible that the cost of ZIKV observed in *Ae. aegypti* mosquitoes is partially due to the recent introduction of the arbovirus in the country [25] or the use of a virus strain isolated in a distant city.

Ae. aegypti is highly adapted to densely urbanized areas, feeding mostly on human hosts and laying eggs 3-4 days later on man-made breeding sites [10,12,40]. The number of eggs laid per gonotrophic cycle is dependent on the amount of blood ingested [19]. Our results show that uninfected mosquitoes ingested a stable amount of blood on the first three gonotrophic cycles, but the number of eggs produced

decreased from 63,3 to 42,3 in average from clutch 1 to 3 (Figure 3). This data suggest that older mosquitoes become less effective in producing eggs with the blood ingested. On the other hand, the blood meal sizes varied in a similar trend over the first three gonotrophic cycles but there was an increase in the number of eggs for the ZIKV-infected mosquitoes over time. As a consequence, the ratio of eggs produced per µL of blood ingested exhibit a slight increase for ZIKV-infected individuals and presented a sharp decrease for those uninfected (Figure 5). Although there are still no other studies with observations on feeding behavior for ZIKV infected individuals, studies regarding other vector-parasite systems have reported changes on feeding behavior. For example, *Ae. aegypti* and *An. gambiae* showed an increased bite rate and probing time when infected with *P. gallinaceum* and *P. falciparum,* respectively [41,42]. Similar results were seen for *Ae. aegypti* mosquitoes infected with DENV, which displayed increased probing time as well as a larger blood intake [17,22] and also presented higher avidity to start a second blood meal [43]. Studies with *Cx. tarsalis* infected with WNV also showed that the infected group would ingest a larger amount of blood than the uninfected group [20].

Although the ZIKV-infected group showed an increase in the egg production during their lifespan, it was on a lower ratio than the uninfected group (most evidently in clutches 1 and 2). Not much is known about the effects of immune response in ZIKV-infected *Ae. aegypti* models. The data presented suggests discrepant effects of infection, since it negatively affected mosquito survival rates and oviposition success but surprisingly increased clutch sizes over time. Perhaps, the lower egg production per µL of blood ingested in ZIKV-infected versus uninfected mosquitoes is a manifestation of the fitness cost associated to infection. The presence of ZIKV is likely to stimulate mosquitoes to mount an immune response to clear infection. The knowledge of cellular and humoral immunity responses of *Ae. aegypti* to arboviruses is still growing. The presence of midgut infection barriers seems to be the most efficient way mosquitoes can avoid virus dissemination [44]. RNA interference, for instance, may modulate infection by producing molecules to inhibit virus replication [45]. So, eliciting an immune response may have caused a trade-off with clutch size, resulting in a lower egg production per µL of blood ingested [46]. Despite interesting, we must recognize that our experimental design does not support such conclusions.

Our exploration of the effects of ageing and ZIKV infection over the fitness of *Ae. aegypti* mosquitoes revealed a strong age-dependent effect in the survival of both groups, in the clutch size of the uninfected mosquitoes and in the oviposition success of the infected group. Additionally, ZIKV showed a negative impact on oviposition success and clutch size over the first two gonotrophic cycles. The biological relevance of these results may be limited for two reasons. Firstly, by considering the probability of daily survival of the species that ranges around 0.83-0.87 in suburban areas and 0.60-0.70 in higher income localities [24,47], which makes that few mosquitoes on a field scenario live long enough to really suffer from the negative effects of ageing and infection. Secondly, very few mosquitoes in a natural population are found carrying an infection with ZIKV [9], making it unlikely that these populations suffer strong selective pressures from the fitness cost caused by the virus. However, we also showed that ZIKV infected mosquitoes seem to ingest a larger amount of blood during the first two meals, which may nevertheless increase the infected mosquito potential to transmit the virus [48].

Methods

Mosquitoes. The mosquito population used in this study was the F1 from a field population previously collected in Urca, a high-income area with high infestation at Rio de Janeiro city, Brazil (-22°57'10.29" S -43°09'35.76" W) [24] and reared to the first generation under laboratory conditions. A total of 80 ovitraps were distributed ~50m apart from each other as a way to guarantee larger genetic variability of mosquitoes. Eggs were hatched in plastic basins containing 3L of water and yeast extract, after which the larvae were fed fish food every day until pupation. Following emergence, adults were kept under insectary conditions (80 ± 5% humidity and 25 ± 3ºC) in cylindrical cages and fed *ad libitum* with 10% sucrose solution up to 36h before the first blood meal. Adults were allowed to mate until the day females were offered their first blood meal.

Virus strain. The infections were performed using one of the circulating strains of Zika virus (BRPE243/2015) obtained from a patient's blood in Pernambuco during

the 2015 Brazilian outbreak and since then maintained in cell culture [25]. Viral titers were quantified via plaque-forming assay prior to experimental infection. The virus sample contained 3.55 x 10^6 PFU/mL and stored at -80ºC until use.

Experimental design and oral infection with ZIKV. To better understand the effects of the age of first feeding on fecundity, adult *Ae. aegypti* females were separated into three different groups. Each group received the first blood-meal (infected or uninfected) on a specific day: either 6-7 (young feeders, YF), 13-14 (mature feeders, MF) or 20-21 days-old (old feeders, OF). At 36h before the first blood meal, mosquitoes were deprived from the sucrose solution and at this moment divided into two distinct sub-groups: one half received ZIKV-infected blood in a proportion of 1mL of virus to 2mL of washed human erythrocytes (infected), while the other half got the blood mixed with 1mL of cell culture (uninfected). The oral infection was conducted through a membrane feeding system (Hemotek, Great Harwood, UK), adapted with a pig-gut covering. After feeding for 30 minutes, fully engorged females were placed in individual plastic vials containing a piece of humid cotton covered with filter paper as oviposition substrate and covered with mosquito net on the top. A cotton soaked in 10% sucrose solution was provided to every female as a carbohydrate source. The same procedure was repeated with MF and OF, which received their first blood meal at 13-14 and at 20-21 days-old, respectively. A sample ~30 of infected mosquitoes was kept in small cylindrical cages until 14 and 21 days post-infection (dpi) and then stored at -80ºC to confirm ZIKV infection. When a dead mosquito was observed, it was removed from the plastic vials, and wing lengths were measured as the distance from the axillary incision to the apical margin, excluding the fringe [26].

Blood feeding and fecundity After the first blood meal, both infected and uninfected females received once a week an uninfected blood meal for 30 minutes. On 4-5 days after every blood meal, the filter papers from the vials were removed and the number of eggs laid per *Ae. aegypti* female was recorded. A new filter paper was added as oviposition substrate for the following clutch. These procedures were repeated every week until mosquitoes from all age groups had died.

Blood meal size quantification. After recording the number of eggs per female, the filter papers were added to 1.5mL tubes containing 1mL of a 1% lithium carbonate solution as a way to dilute the feces there present. A standard curve was prepared by diluting known amounts of blood and measuring the corresponding absorbance (0; 0.8; 1.6; 2.4; and 3.2 µL) producing a R^2 = 0.97. The samples were analyzed in a spectrophotometer with an absorbance at 387 nM [27]. The ratio of eggs produced per blood meal was calculated on the first four gonotrophic cycles, by dividing the number of eggs per female that laid at least one egg by the hematin estimation.

ZIKV-infection confirmation. A total of 18 individuals (10 of which were collected at 14dpi and the other 8 at 21dpi) were randomly selected to ascertain ZIKV-infection. Viral RNA was extracted from the mosquito whole body using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Detection and quantification of viral RNA in mosquitoes was performed using qRT-PCR with SuperScriptTM III PlatinumTM One-Step qRT-PCR Kit (Thermo Fisher Scientific, Invitrogen) in QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Each mix reaction was made with the use of 600 nM forward primer (5'-CTTGGAGTGCTTGTGATT-3', genome position 3451–3468), 600 nM reverse primer (5'-CTCCTCCAGTGTTCATTT-3', genome position 3637–3620) and 800 nM probe (5'FAM- AGAAGAGAATGACCACAAAGATCA-3'TAMRA, genome position 3494–3517). The cycling conditions were such: 95 °C for 2 minutes, 40 amplification cycles of 95°C for 15s, 58°C for 5s and 60°C for 30s. Virus copy numbers on each sample was calculated by interpolation onto a standard curve made up of a 7-point dilution series of an *in vitro* transcribed ZIKV RNA [28].

Statistical analysis. *Ae. aegypti* longevity presented non-normal distribution, but the logarithm of longevity satisfied the assumption of normality (Shapiro-Wilk W = 0.9915, P = 0.0592). Day zero was set as the day in which the YF received their first blood meal, (see experimental design section for further details). Daily survival for MF and OF started to be monitored on the day mosquitoes fed. We analyzed the effect of treatment (infected or uninfected), age on the day of infection (YF, MF, OF) and wing length on the log10 of mosquito longevity with ANOVA. A log-rank test compared a two-sample basis the survival distribution of *Ae. aegypti* females from the interaction

of treatment and age of first feeding. Here, survival rate is defined as the number of individuals still alive as a function of time.

Fecundity and blood meal size were analyzed by considering the first three clutches of eggs laid, as only a small number of females (especially OF) blood fed and later laid eggs at later clutches, precluding adequate numbers for analysis. Two aspects of fecundity were analyzed: oviposition success and clutch size. The oviposition success, *i.e.* the likelihood that a mosquito laid at least one egg (at a given clutch) was analyzed with a logistic analysis that included treatment, age of first feeding, wing length and clutch-number (*i.e.* age). Next, we analyzed the number of eggs per clutch from those mosquitoes that laid at least one egg, using a repeated measures analysis and square-root transformed the number of eggs to satisfy the assumptions of normality. We included clutch-number as the variable repeatedly measured and estimated the effects of treatment, age of first feeding, wing length and ageing on clutch size. Blood meal size in the first three blood meals was analyzed by a repeated measure analysis. Blood meal was included as the variable repeatedly measured and we estimated the effects of treatment, age of first feeding, wing length and ageing on the amount of blood ingested over time. All analyses were carried out with the statistical software JMP 9 (http://www.jmp.com/).t

Ethical statement. Human blood was obtained from anonymous donors whose blood bags would be discarded due to small volume. Blood was derived from the blood bank of the Rio de Janeiro State University. We have no information on donors, including sex, age and clinical condition. The use of human blood was approved by the Fiocruz Ethical Committee (process CAAE 53419815.9.0000.5248).

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Author Contributions

M.T.P., M.R.D, L.V.W and R.M.F conceived the study; M.T.P., M.R.D, T.C.C., L.V.W and R.M.F designed the experiments; M.T.P, I.D.S., A.T.F., T.C.C. and L.V.W performed the experiments; R.M.F. carried out the analysis; and M.T.P., L.M. and R.M.F. wrote the manuscript. All authors commented on the manuscript and contributed to it.

Competing Financial Interests Statement

The authors declare no competing financial interests.

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Figure 1: The viral load on the *Ae. aegypti* mosquitoes body infected with ZIKV.

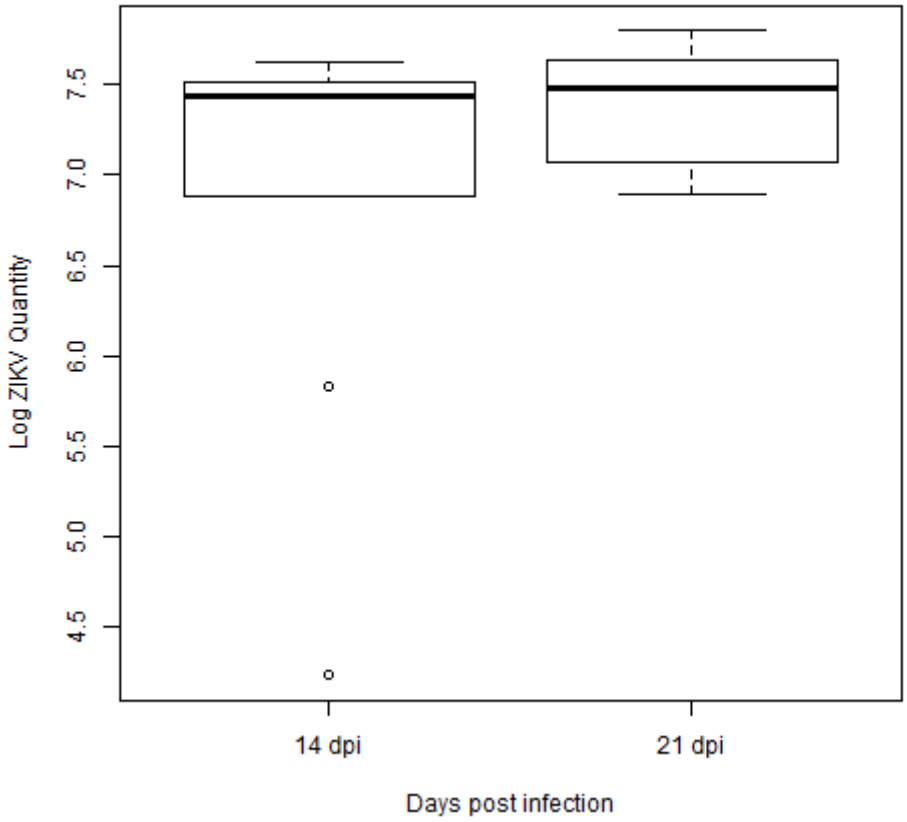

Figure 2: Survival curves of three cohorts of *Ae. aegypti* females infected with ZIKV and uninfected counterparts.

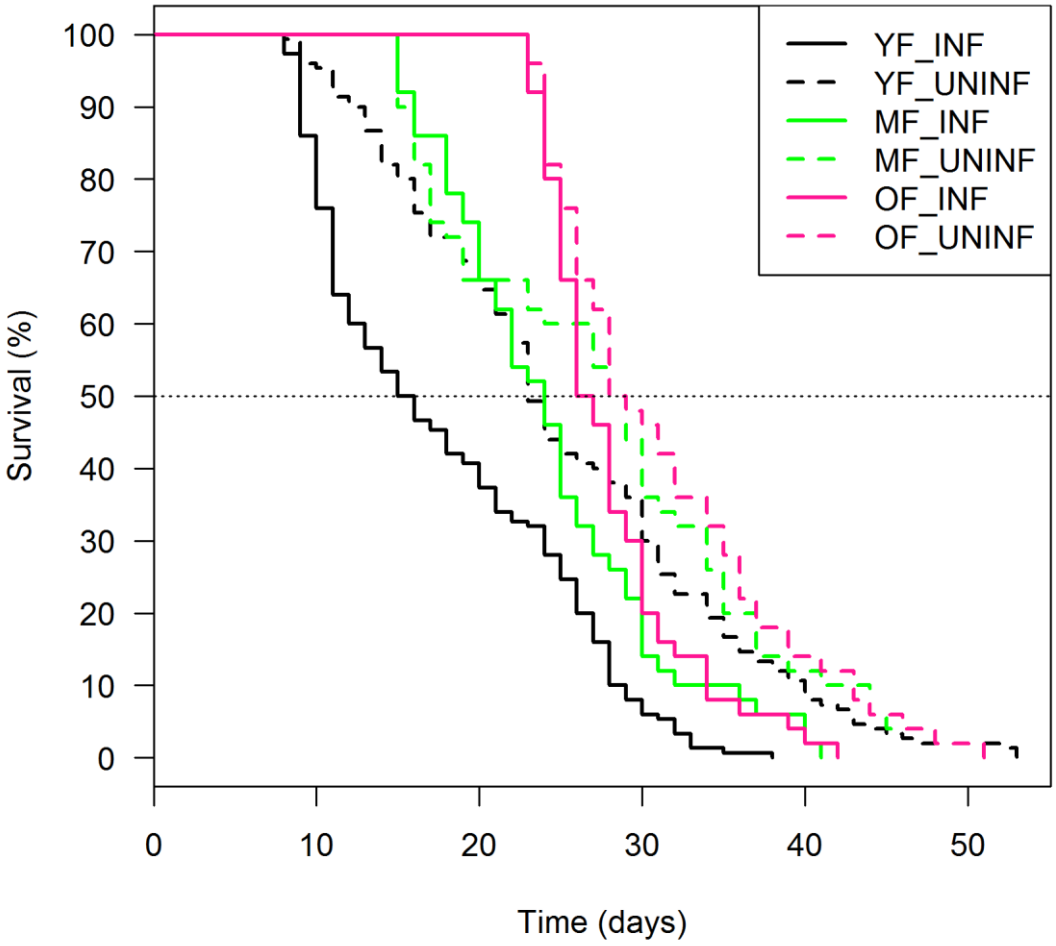

Figure 3: Weekly average of number of eggs laid by mosquitoes from the different treatment and age groups.

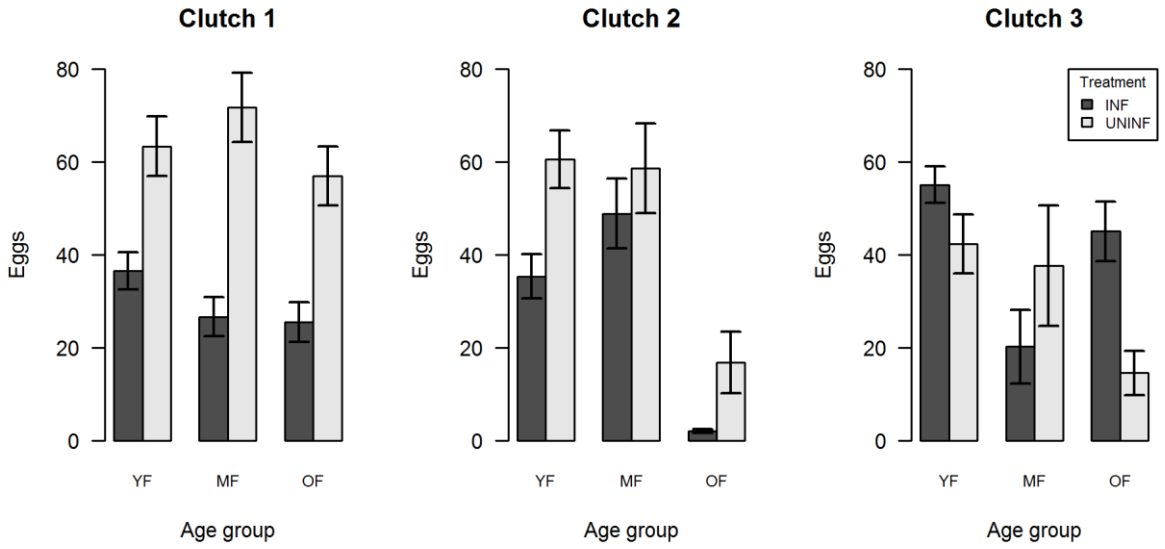

Figure 4: Weekly average of blood meal size of the different treatment and age groups.

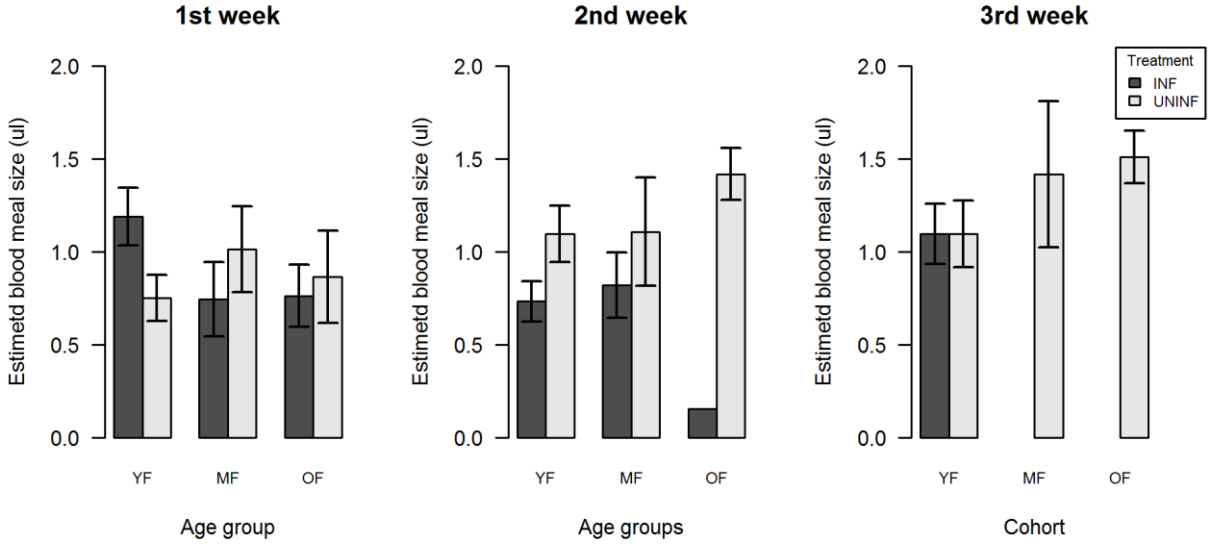

Figure 5: Ratio of egg production per µL of blood of infected and uninfected *Ae aegypti* females.

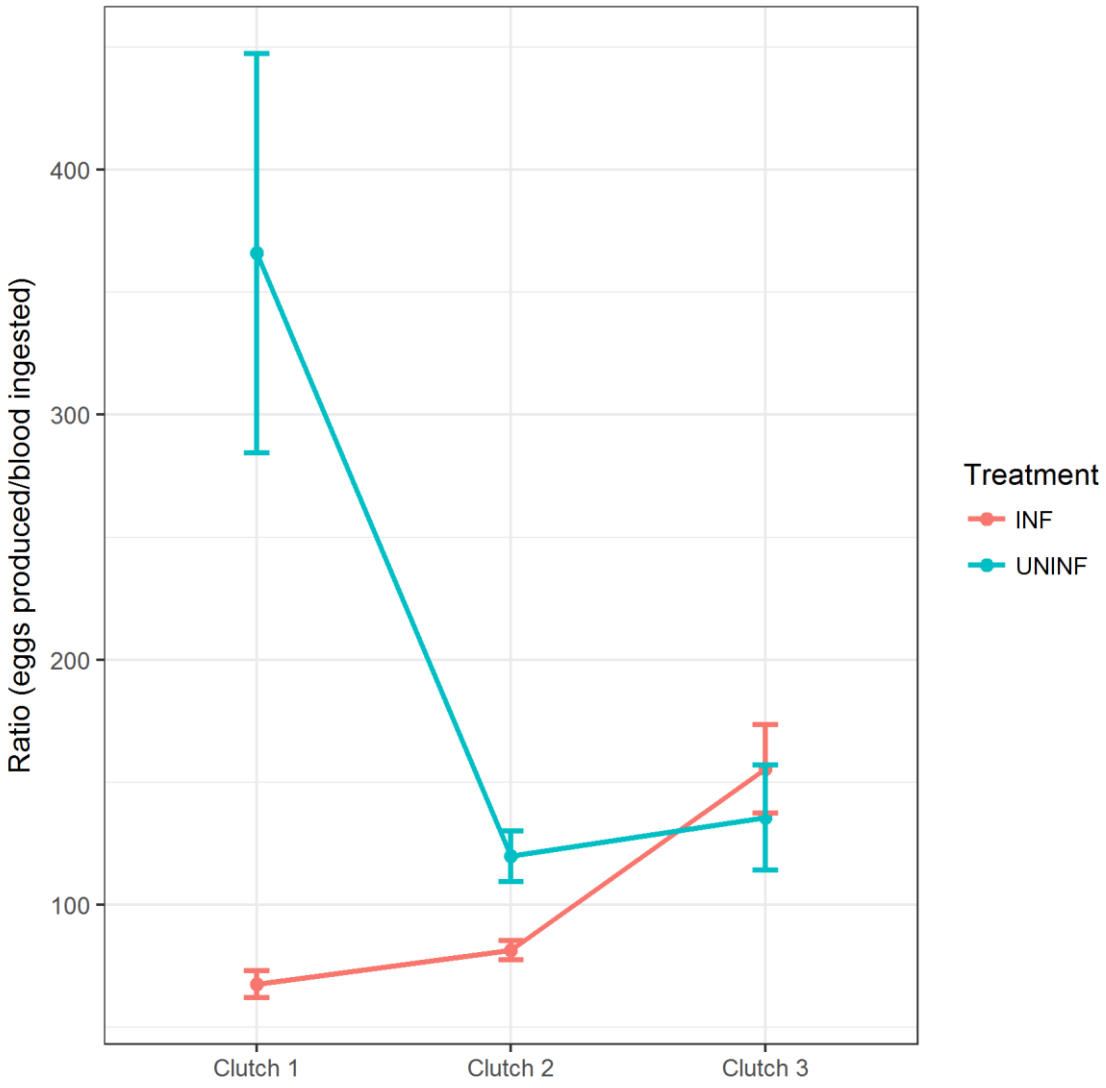

Table 1: Log-rank p-values of the paired comparison of survival curves of infected and uninfected *Ae. aegypti* females from YF (fed with 6-7 days old), MF (13-14 days old) and OF (20-21 days old) groups.

	Uninf_YF	Uninf_MF	Uninf_OF	Inf_YF	Inf_MF	Inf_OF
Uninf_YF						
Uninf_MF	**0.012**					
Uninf_OF	**<0.001**	**0.035**				
Inf_YF	**<0.001**	**0.017**	0.081			
Inf_MF	**<0.001**	**0.014**	0.062	0.575		
Inf_OF	**<0.001**	**<0.001**	**0.003**	**<0.001**	**0.002**	

Table 2: Analysis of variance (ANOVA) of the logarithm of survival of ZIKV-infected and uninfected *Ae. aegypti* mosquitoes.

Source	d.f.	Sum of squares	*F*	*P-value*
Treatment	1	4.883	36.76	**<0.0001**
Age group	2	1.931	7.27	**0.0008**
Wing size	1	1.315	9.90	**0.0018**
Treatment*Age group	2	1.081	4.07	**0.0177**
Treatment*Wing size	1	0.069	0.52	0.4701
Age group*Wing size	2	0.972	3.66	**0.0265**

Table 3: Logistic regression analysis of the clutch, treatment, wing size and cohort on the success of oviposition of *Ae. aegypti* females.

Source	**Nparm**	**d.f.**	χ^2	**P-value**
Clutch	6	6	8.702	0.1910
Age group	4	4	15.079	**0.0045**
Wing	2	2	2.752	0.2525
Treatment	2	2	54.868	**<.0001**
Wing*Treatment	2	2	0.683	0.7104
Age group*Treatment	4	4	0.382	0.9838

Table 4: Repeated measures analysis (with clutch size as the repeatedly measured variable) of the square-root of the number of eggs laid by *Ae. aegypti* females.

Source	**Num df**	**Den df**	***F***	***P-value***
Clutch AND Treatment	2	53	3.374	**0.041**
Clutch AND Age group	4	106	0.634	0.638
Clutch AND Wing	2	53	0.287	0.751

Table 5: Repeated measures analysis (with amounts of hematin as the repeatedly measured variable) of the square-root of the blood meal size taken by *Ae. aegypti* females.

Source	**Num df**	**Den df**	***F***	***P-value***
Clutch AND Treatment	2	65	4.016	**0.022**
Clutch AND Age group	4	130	1.203	0.312
Clutch AND Wing	2	65	1.421	0.248

4. Conclusions

The effects of ageing on *Aedes aegypti* fecundity is still very sparsely known. Even less studied is the effects of Zika, an arbovirus that only recently became worldwide distributed. Therefore, through observation of mosquito groups receiving their first blood meal at different ages and monitoring biological parameters through their lifespan, we were able to scrutinize potential effects of ageing and ZIKV infection on the blood meal and clutch sizes. We showed a strong age-dependent factor over mosquito survival and that infection by ZIKV had an additional negative impact, since infected mosquitoes had shortened lifespan than uninfected.

Observing oviposition success and fecundity, we showed that the control group had a greater influence of aging on fecundity. Even though the amount of blood ingested was stable through its lifespan, the number of eggs produced continuously decreased during every gonotrophic cycle. On the other hand, ZIKV infected individuals showed a constant and low egg production, but concordant to the amount of blood ingested. The oviposition success of infected mosquitoes was significantly reduced when compared to its uninfected counterpart and by the age when the first blood meal was received. Despite this work limitations, our results contribute to a better understanding of the effects of senescence and pathogen infection over the vectorial capacity of *Aedes aegypti*.

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