UNDERSTANDING THE EFFECTS OF ENVIRONMENTAL VARIATION ON VECTOR-VIRUS INTERACTIONS AND TRANSMISSION IN THE ZIKA VIRUS SYSTEM

by

BLANKA TESLA

(Under the Direction of Melinda Brindley and Courtney Murdock)

ABSTRACT

Zika virus (ZIKV) is an arbovirus primarily transmitted by *Aedes* mosquitoes. ZIKV typically causes asymptomatic infections or relatively mild symptoms. However, ZIKV infection during pregnancy can lead to congenital Zika syndrome, a unique pattern of birth defects and disabilities. In 2016, introduction of ZIKV into naïve and susceptible populations quickly reached epidemic levels and spread to more than 65 countries worldwide. As this was the first time that severe disease outcomes were linked to ZIKV infection, therapeutics, vaccines, and even solid diagnostic tools were not available. There was an immediate need for their development, but also an urgent need to control and limit ZIKV transmission, to understand the main drivers for viral spread, and to predict potential spreading patterns. There are multiple factors that affect the transmission dynamics of arboviruses such as Zika. Epidemiological outcome of disease depends on the pathogen-host interactions that are defined by numerous intrinsic factors such as pathogen and host genetics, as well as extrinsic biotic and abiotic factors. Environmental factors can have direct effect on virus replication and infection outcome or indirect effect

due to altered physical barrier, immune response, and overall host fitness. There are also various socioeconomic factors and human behaviors that can shape transmission. We demonstrated that increasing ZIKV dose in the blood-meal significantly increases the probability of mosquitoes becoming infected and infectious. Using these data to parameterize an R_0 model, we showed that increasing viremia from 10^4 to 10^6 PFU/mL increased relative R_0 3.8-fold, demonstrating that variation in viremia substantially affects transmission risk. Temperature is known to be one of the strongest drivers of vector-borne transmission. We used a temperature-dependent model to infer temperature effects on ZIKV transmission and showed that transmission was optimized at 29°C, and had a thermal range of 22.7°C - 34.7°C. We have also demonstrated why ZIKV transmission is ineffective at cool temperatures and how temperature alters ZIKV replication in mosquito cells. Assessing how biotic and abiotic factors alter our standard formulation of vectorial capacity and model predictions is critical in order to predict the seasonality and geography of ZIKV spread so we can deploy effective disease interventions.

INDEX WORDS: Zika virus, arboviruses, mosquito-borne transmission, temperature

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BLANKA TESLA

BS, University of Zagreb, Croatia, 2010

MS, University of Zagreb, Croatia, 2014

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2019

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BLANKA TESLA

Major Professor:	Melinda Brindley
	Courtney Murdock
Committee:	Daniel Mead
	Ted Ross
	Michael Strand

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
December 2019

DEDICATION

To my grandfather.

ACKNOWLEDGEMENTS

I would like to thank my mentors, Dr. Brindley and Dr. Murdock. I could not have wished for better mentors. I will always be grateful for everything I have learned from you. Thank you for your guidance and support, and for always having your doors wide open. You are a true inspiration and my role models. I would like to thank my committee members, Dr. Mead, Dr. Strand, and Dr. Ross for their valuable comments. Special thanks to Marissa, Maria, and Kerri for their friendship and moral support. I share the most beautiful memories of this challenging journey with you. I would like to thank my loving parents, Marina and Sinisa. I know it was not easy having a daughter at the other end of the world, but your unconditional love has helped me follow my dreams. I would like to thank my brother Tomislav for answering every call and listening when I needed him to listen and talking when I needed him to talk. Lastly, I would like to thank my husband Ivan. I would have never finished graduate school without you. Thank you for spending countless hours waiting for me in the lab, for being with me during every time-point in the middle of the night, for holding my hand during most challenging moments, and for never holding back.

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CHAPTER 1

INTRODUCTION

Zika virus (ZIKV) is a positive-sense, single-stranded RNA flavivirus. Despite the high rate of asymptomatic infections and cases presenting with mild symptoms (1), ZIKV was declared a public health emergency of international concern in 2016 (2) due to the increase in loss of pregnancies, microcephaly, and other brain and eye abnormalities in infants whose mothers became infected during pregnancy (3). The primary transmission route of ZIKV is through the bite of *Aedes* mosquitoes, but the virus can also be transmitted sexually (4), from mother to fetus (5), and through blood transfusion (6). Currently, there are no therapeutics nor vaccines for this emerging arbovirus, therefore the only way to mitigate the disease is through vector control and public education campaigns. Due to adverse pregnancy outcomes associated with ZIKV infection, research has been focusing on the development of therapeutics, vaccines, and novel vector control tools. Yet, important ecological and molecular questions remain unanswered, such as what role asymptomatic hosts play in transmission and how key environmental drivers modify the mosquito-virus interaction, both of which are important for successfully predicting virus transmission and implementing control strategies.

People with asymptomatic infections are generally considered a dead-end for transmission, but in some systems asymptomatic carriers produce enough virus to successfully infect mosquitoes (7, 8). Because people without clinical symptoms are likely to be more active and potentially exposed to more mosquitoes than symptomatic

people, asymptomatic carriers may play an important role as a source of virus for mosquito vectors. Understanding how variation in viremia influences vector competence is the first step towards predicting the role host viremia plays in ZIKV transmission. A better characterization of how relevant environmental variation, such as temperature, affects transmission is critical for predicting how the virus might spread geographically and seasonally so that efficient vector control strategies can be implemented. Mosquitoes are small ectotherms, and their physiology, survival, and reproduction are all strongly affected by temperature variation (9-11). Currently, no data exist on how temperature variation affects the extrinsic incubation period (EIP) and vector competence for ZIKV, which are two key parameters in vector-borne pathogen transmission. Although, arboviruses can persist in different hosts across a wide range of temperatures, extreme low and high temperatures can directly affect virus replication by altering virus structure and fluctuation of the surface proteins (12-14), and it can alter cellular metabolism and factors that may enhance or hinder viral replication (15, 16). To provide a better understanding of the effects of viral dose and environmental variation on vector-virus interactions and transmission of ZIKV, we introduce the following specific aims:

Specific aim 1: *Hypothesis: Larger doses of ZIKV will result in higher infection prevalence and vector competence, shorter extrinsic incubation period (EIP) and overall higher transmission of the virus.* To test the hypothesis, we will orally infect field-derived populations of *Ae. aegypti* (Southern Mexico) with four different doses (10^3, 10^4, 10^5, 10^6 PFU/mL) of a Mexican ZIKV isolate. We will use the fourth generation of mosquitoes that is well adapted to membrane feeding. We will titrate mosquito bodies, heads, and saliva to measure the effect of viral dose on infection prevalence, dissemination rates,

vector competence, and EIP. We will also track mosquito mortality rates to determine if the virus dose influences mosquito survival.

Specific aim 2: *Hypothesis: Temperature variation will have a unimodal effect on vector competence, extrinsic incubation period (EIP), and survival of ZIKV-infected Ae. aegypti mosquitoes.* To measure the thermal performance of ZIKV in field-derived populations of *Ae. aegypti*, we will orally infect the F4 generation of mosquitoes with ZIKV and maintain them at eight constant temperatures (16°C, 20°C, 24°C, 28°C, 32°C, 34°C, 36°C, 38°C). We will titrate mosquito bodies, heads, legs, and saliva to measure changes in proportion of infected mosquitoes, vector competence, and EIP. We will track mosquito mortality at each temperature to assess the effect of temperature on survival.

Specific aim 3: *Hypothesis: Sub-optimal temperatures inhibit ZIKV replication by altering cellular environment and host factors necessary for viral replication or/and by preventing a viral function required to complete the viral replication cycle and produce progeny virus.* To determine the mechanism responsible for inhibition of ZIKV replication at sub-optimal temperatures, we will conduct a series of experiments in mosquito cells (C6/36) maintained at optimal temperature or adapted to grow at cool temperature (20°C). We will examine and compare each part of virus replication cycle at different temperature treatments.

References

1. Duffy MR, Chen T-H, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med. 2009;360(24):2536-43.

2. World Health Organization. WHO statement on the first meeting of the International Health Regulations (2005) (IHR 2005) Emergency Committee on Zika virus and observed increase in neurological disorders and neonatal malformations 2016 [Available from: http://www.who.int/mediacentre/news/statements/2016/1st-emergency-committee-zika/en/.

3. Mlakar J, Korva M, Tul N, Popovic M, Poljsak-Prijatelj M, Mraz J, et al. Zika virus associated with microcephaly. N Engl J Med. 2016;374(10):951-8.

4. Musso D, Roche C, Robin E, Nhan T, Teissier A, Cao-Lormeau VM. Potential sexual transmission of Zika virus. Emerg Infect Dis. 2015;21(2):359-61.

5. Besnard M, Lastere S, Teissier A, Cao-Lormeau V, Musso D. Evidence of perinatal transmission of Zika virus, French Polynesia, December 2013 and February 2014. Euro Surveill. 2014;19(13).

6. Musso D, Nhan T, Robin E, Roche C, Bierlaire D, Zisou K, et al. Potential for Zika virus transmission through blood transfusion demonstrated during an outbreak in French Polynesia, November 2013 to February 2014. Euro Surveill. 2014;19(14).

7. Duong V, Lambrechts L, Paul RE, Ly S, Lay RS, Long KC, et al. Asymptomatic humans transmit dengue virus to mosquitoes. Proc Natl Acad Sci U S A. 2015;112(47):14688-93.

8. Nedelman J. Gametocytemia and infectiousness in falciparum malaria: observations and models. In: Harris KF, editor. Advances in Disease Vector Research. New York, NY: Springer New York; 1990. p. 59-89.

9. Murdock CC, Paaijmans KP, Bell AS, King JG, Hillyer JF, Read AF, et al. Complex effects of temperature on mosquito immune function. Proc Biol Sci. 2012;279(1741):3357-66.

10. Catalan TP, Wozniak A, Niemeyer HM, Kalergis AM, Bozinovic F. Interplay between thermal and immune ecology: effect of environmental temperature on insect immune response and energetic costs after an immune challenge. J Insect Physiol. 2012;58(3):310-7.

11. Neven LG. Physiological responses of insects to heat. Postharvest Biology and Technology. 2000;21:1003-111.

12. Lim XN, Shan C, Marzinek JK, Dong H, Ng TS, Ooi JSG, et al. Molecular basis of dengue virus serotype 2 morphological switch from 29 degrees C to 37 degrees C. PLoS Pathog. 2019;15(9):e1007996.

13. Zhang X, Sheng J, Plevka P, Kuhn RJ, Diamond MS, Rossmann MG. Dengue structure differs at the temperatures of its human and mosquito hosts. Proc Natl Acad Sci U S A. 2013;110(17):6795-9.

14. Hasan SS, Sevvana M, Kuhn RJ, Rossmann MG. Structural biology of Zika virus and other flaviviruses. Nature structural & molecular biology. 2018;25(1):13-20.

15. Srisutthisamphan K, Jirakanwisal K, Ramphan S, Tongluan N, Kuadkitkan A, Smith DR. Hsp90 interacts with multiple dengue virus 2 proteins. Sci Rep. 2018;8(1):4308.

16. Reyes-Del Valle J, Chavez-Salinas S, Medina F, Del Angel RM. Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. J Virol. 2005;79(8):4557-67.

CHAPTER 2

LITERATURE REVIEW

This literature review will summarize the current knowledge and contemporary research done to understand the transmission dynamics and control of arboviral diseases. After introducing arboviruses, their importance, history, and ways of transmission, three arboviruses will be discussed in detail: Zika virus (ZIKV), as a focus of this dissertation, dengue virus (DENV), an extensively studied arbovirus with a long history of transmission in humans, and chikungunya virus (CHIKV), an arbovirus with more recent outbreaks. Next, two main vectors (*Aedes aegypti* and *Ae. albopictus*) will be introduced, in particular their history and spread, as well as what makes them such successful vectors. After that, the review will focus on the mechanisms required for the establishment of the infection in mosquito vectors and successful transmission. In particular, which physiological barriers viruses encounter upon infection, what are the main mosquito immune responses against these pathogens, and how can viruses overcome the barriers and suppress or evade the immune responses. The last section will focus on the factors that can affect arboviral transmission described through the concept of a disease triangle. These factors include both intrinsic factors, such as genetic variation across viruses and hosts, extrinsic factors, such as variation in abiotic and biotic factors, as well as their interaction. The emphasis will be put on how variation in environmental temperature, one of the main drivers of vector-borne transmission, affects the virus, vector, their interaction, and transmission. Lastly, the main reasons for increased arboviral emergence

and spread we have witnessed in the past few decades will be summarized. With numerous challenges, vector control remains the main solution to mitigate the disease spread. Modeling efforts that assess the vector control strategies and predict the disease outcome are crucial for successfully controlling arboviral transmission.

Arboviruses

Arboviruses (*ar*thropod-*bo*rne *viruses*) are a specialized group of viruses that are transmitted by arthropod vectors, including mosquitoes, ticks, and sand flies. The pathogen, vector, and susceptible host must be in frequent, close contact to ensure perpetuation of the virus. There are more than 130 arboviruses that are known to cause human diseases (1). Some of the most prevalent human arboviral pathogens are transmitted by mosquitoes and represent a major threat to human health. Historically, these viruses were endemic in tropical and subtropical areas of the world, however in the past few decades, arboviruses have become an emerging problem around the world. The first connection between arthropods and disease was postulated in 1881 when a Cuban scientist suggested that yellow fever was transmitted by mosquitoes. After it was verified in 1900, yellow fever virus became the first identified arbovirus, but also the first known virus to infect humans (2). Yellow fever virus (YFV) and dengue virus (DENV) were the first documented mosquito-borne arboviruses to cause major outbreaks in the Americas in the 17th century, while West Nile (WNV), Japanese encephalitis (JEV), Rift Valley fever (RVFV), chikungunya (CHIKV), and Zika viruses (ZIKV) are just some examples of arboviruses causing major outbreaks around the world today (3).

Transmission of arboviruses in humans can occur in three ways. The first is direct spillover during the sylvatic cycle, when the virus that circulates among the mosquito

vector and the enzootic host gets transmitted to humans (4). Some examples of arboviruses that were transmitted to humans through bridge vectors are CHIKV, DENV, ZIKV, and YFV. The second mechanism includes the amplification of the virus in domestic animals that live in close proximity to people, followed by a spillover. Some examples of epizootic cycle are JEV and RVFV that amplify in domestic livestock. Lastly, in urban epidemic cycles, humans serve as amplification hosts and the virus can be transmitted between humans via anthropophilic mosquitoes, such as *Aedes aegypti* and *Ae. albopictus* (Fig 2.1). In rare occasions, some viruses can be transmitted from human to human via sexual contact or perinatally, as was seen during the ZIKV outbreak (4). There are several factors that affect the transmission dynamics of arboviruses such as climate change, urbanization, and globalization. Increased trade can result in the spread of invasive mosquito species, and increased travel can result in pathogen spread between continents within a day. In places with well-established vector populations, imported cases can result in local transmission, and in worst cases, in explosive epidemics.

Zika virus

Zika virus (ZIKV) belongs to the *Flaviviridae* family and its genome organization resembles those of other flaviviruses, including DENV, WNV, and YFV. The 10.8 kilobases-long positive-sense, single-stranded RNA has one open reading frame encoding for a single polyprotein (5). The polyprotein is cleaved by host or viral proteases into three structural and seven non-structural proteins. The structural proteins (precursor membrane (prM), envelope (E), and capsid (C) proteins) form the virus particle, while non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) play an important role in virus replication, polyprotein processing and manipulation of host

response (5). The ZIKV replication cycle starts with the virus binding to the host cell. One of the most important receptors for ZIKV is the Gas6-AXL tyrosine kinase receptor complex (6). Gas6 interacts with the surface-exposed phosphatidylserine on the ZIKV particle and AXL on the surface of the cell. ZIKV enters the cell through receptor-mediated endocytosis. The low pH in the endosome triggers trimerization of the E proteins, which results in exposure of the fusion domain (7). Once viral and cell membranes fuse, the RNA genome is released in the cytoplasm. The incoming genome serves as mRNA and produces viral proteins. The nonstructural proteins form a replication complex and synthesize negative-sense RNA, which serves as a template for more positive-sense RNA. Virus replication takes place in the endoplasmic reticulum (ER) and immature particles are released in the ER lumen. Noninfectious immature particles consist of the nucleocapsid and a lipid membrane with 60 irregular spikes of three prM-E heterodimers. The immature particles are then glycosylated and transported to the Golgi. The low pH in the Golgi triggers a conformational E protein change from 60 trimers to 90 dimers. During this process, prM acts as a chaperone and covers the fusion loop at the spike. Once the E proteins rearrange and form a smooth surface of dimers parallel to the membrane, the prM cleavage site is unveiled. Host protease furin cleaves the pr peptide and forms mature particles that are released by exocytosis (8) (Fig 2.2).

Zika virus is primarily transmitted through the bite of *Aedes* mosquitoes, but the virus can also be transmitted from mother to fetus, sexually, and through blood transfusion (9-11). Symptoms of ZIKV infection are relatively mild and usually start 3-14 days after the infection. Common symptoms include low-grade fever, skin rash, conjunctivitis, headache, and arthralgia, usually lasting up to 1 week; however, 70% of

people exposed to ZIKV will have no symptoms at all (12). Despite the high rate of asymptomatic infections, ZIKV was widely feared and declared a "public health emergency of international concern" in 2016, owing to the 20-fold increase of microcephaly in newborns, an increase in the probability of pregnancy loss, and brain and eye abnormalities in infants born from infected mothers (12). Additionally, there was a 19% average increase in autoimmune neurological complications (Guillain-Barré syndrome) associated with ZIKV infection in Brazil (13).

Zika virus was first detected in 1947 in a sentinel Rhesus monkey from the Ziika Forest of Uganda and was isolated from *Ae. africanus* mosquitos in 1948 (14). The first human cases of Zika were detected in 1952 during a serological study of Ugandan and Tanzanian residents, and 2 years later the first human ZIKV isolate was obtained from a 10-year-old girl in Nigeria (15, 16). In the following decades, the virus spread across Africa and tropical Asia, occasionally causing dengue-like fever outbreaks. During this period, ZIKV was not considered a major public health concern and there were no reported links to microcephaly or other complications. That changed in 2007, when the first outbreak outside of Africa and Asia occurred on the island of Yap (17). This key epidemiological event was viewed as an isolated oddity by many scientists at the time, but it was quickly followed by the spread of ZIKV across Oceania and the Pacific islands between 2013 and 2014 (18). The following year, ZIKV was detected in Brazil, and was spreading throughout the Americas (19). Recent research on ZIKV evolution supports the hypothesis that two lineages of ZIKV diverged into an African group and an Asian group; with the Asian genotype recently introduced to the Americas (20).

Dengue virus

Dengue virus (DENV) is another positive-sense single-stranded flavivirus. Like ZIKV, it has an icosahedral structure with the pseudo T=3 symmetry. However, DENV surface proteins are less compact, so the particle is less stable compared to a ZIKV particle (8). The DENV replication cycle also starts with receptor-mediated clathrin-dependent endocytosis. Low pH in the endosome causes DENV conformational changes that lead to viral-cell membrane fusion. Polyprotein translation and virus replication take place in invaginated membrane vesicles on the ER. The nucleocapsid buds into the ER lumen and acquires a lipid membrane envelope containing prM and E proteins. After cleavage of the pr peptide, the spiky immature particle becomes smooth (5). The glycoproteins on the surface can move and expose the membrane underneath the E and M proteins. This "breathing" process is particularly important during secondary infection, when preexisting antibodies bind to the viral envelope and enter the cell via antibody-dependent enhancement (21).

There are four genetically distinct DENV serotypes, DENV 1-4, with DENV 2 being the most virulent strain (22). The virus maintains human transmission primarily through *Ae. aegypti* mosquitoes in urban environments, and *Ae. albopictus* in suburban, rural, and sylvatic environments (1). DENV can also be transmitted vertically from mother to fetus (23), and through blood transfusion (24). About 50% of people exposed to DENV will develop dengue fever 3-14 days after the infection. Dengue fever is a debilitating disease characterized by high fever, severe headache, retro-orbital pain, maculopapular rash, myalgia, and arthralgia (25, 26). In most cases, the disease is self-limiting, resulting in long immunity against that particular serotype. A secondary

infection to another serotype or primary infections in infants born to DENV-immune mothers increases the risk of developing dengue hemorrhagic fever (DHF) (22). DHF is a severe disease characterized by high fever, increased vascular permeability, and thrombocytopenia leading to bleeding, hypotension, intense abdominal pain, and circulatory failure and shock that leads to 20% mortality if left untreated. With proper treatment, the mortality rate can be reduced to less than 1% (22).

DENV, which was known to cause sporadic diseases in humans, has since become a major public health problem. The earliest evidence of dengue-like illness dates back to 265-420 AD in China. The first dengue-like disease in the Americas was reported in the French West Indies in 1635 and later in Panama in 1669 (27). With the increase in naval commerce between 1779 and 1788, epidemics of dengue-like diseases were further described in Indonesia, Egypt, Spain, and the USA (1). A second series of dengue-like epidemics from 1823-1916 resulted in viral spread from Africa to India, Oceania, and the Americas; and after the World War II, dengue epidemics became pandemics (1). Dengue fever was formally described for the first time in 1943 in Japan, while the first cases of dengue hemorrhagic fever were described during a large outbreak in Bangkok in 1958 (25, 28). Today, with an estimated 390 million infections and 3.9 billion people in 128 countries at risk, dengue virus is the most prevalent and dangerous arbovirus causing more illness and death in humans than any other arboviral disease (29).

Chikungunya virus

Chikungunya virus (CHIKV) is a positive-sense single stranded RNA virus in the *Togaviridae* family. Unlike ZIKV and DENV, the 11.8 kb genome has two open reading frames coding for four non-structural proteins (nsP1, nsP2, nsP3, nsP4) and five

structural proteins (C, E3, E2, 6K, E1) (30). The nucleocapsid is surrounded by a host-derived lipid membrane envelope with 240 copies of E1 and E2 glycoproteins organized in a T=4 icosahedral symmetry. E1 (fusion peptide) and E2 (receptor binding sites) proteins form heterodimers arranged in 80 trimeric spikes (30). After binding to the receptor, CHIKV enters the host cell through receptor-mediated clathrin-dependent endocytosis. Mxra8 was recently discovered to be one of the main receptors for CHIKV and many other arthritogenic alphaviruses (31). The acidic environment within the endosome triggers conformational changes in the virus envelope, resulting in the exposure of the E1 peptide and membrane fusion (30). Once the viral genome is released in the cytoplasm, the first viral polyprotein is translated and cleaved into nsP1-nsP4. Non-structural proteins form a viral replication complex that generates the full-length negative-strand RNA intermediate. The RNA intermediate serves as a template for both 26S subgenomic mRNA and 49S genomic RNA (32). Subgenomic RNA encodes for the C-pE2-6K-E1 polyprotein that is further processed by autoproteolytic serine proteases (32). Once the capsid is released in the cytoplasm, the remaining proteins are directed to the ER. In the Golgi, pE2 and E1 form heterodimers and traffic to the plasma membrane. During this maturation step, pE2 is cleaved by a cellular furin to form E2 and E3. Although E3 in most cases is not incorporated in the virus particle, it plays an important role in the proper folding of pE2 and its association with E1. Assembled particles with icosahedral cores bud at the cell membrane (30) (Fig 2.3).

The name chikungunya is originally from the Makonde language and means “that which contorts or bends up,” which describes the posture of patients afflicted by symptoms of severe arthritis and joint pain (33). Approximately 85% of infected people

develop symptoms that include rash, high fever, headache, photophobia, and severe joint pain. A significant percentage (30–40%) of patients will suffer from chronic joint disease that can last weeks, months, or even years after initial infection (33-35). While chikungunya-like illness has been documented for centuries (36), it was first recognized as an endemic disease in 1952 in East Africa (33). Shortly thereafter, with the aid of newly developed viral diagnostic tools, CHIKV was isolated from human sera in Tanzania, and *Ae. aegypti* was identified as the main vector (37, 38). From 2005 to 2006, CHIKV spread to several islands in the Indian Ocean, with the French island, La Réunion, experiencing one of the largest outbreaks on record for the time, with approximately 40% of the island's population infected and 273 deaths (33). Interestingly, *Ae. albopictus* was the predominant vector for the La Réunion outbreak. Subsequent research demonstrated that this primary vector switch coincided with the selection for a single amino acid change from alanine to valine at position 226 in the CHIKV E1 envelope glycoprotein (E1-A226V) that increased midgut infection, replication, dissemination, and transmission in *Ae. albopictus* (39, 40). After the La Réunion outbreak, CHIKV continued to spread and cause large outbreaks. In 2006 and 2007, CHIKV reached India, with more than 1.5 million estimated cases (38, 41). At the same time, the first locally acquired cases in temperate regions occurred, with outbreaks in Italy and France (42, 43). These outbreaks were also largely driven by *Ae. albopictus*. In 2013, CHIKV was introduced to the Caribbean island system, likely via infected travelers, and quickly spread throughout the Americas (44).

Mosquito vectors

The global spread of two important mosquito vectors, *Ae. aegypti* and *Ae. albopictus*, resulted in the explosive spread of many arboviruses around the world, including ZIKV, DENV, and CHIKV. *Ae. aegypti*, also known as the yellow fever mosquito, is the primary vector for some of the most important arboviruses in humans. *Ae. aegypti* diverged from the generalist zoophilic tree-hole breeder *Ae. aegypti formosus* into a domestic anthropophilic form (45). The mosquito was introduced to the new world from Africa during human migration and trade between the 15th and 19th centuries (46). Today, *Ae. aegypti* is well-established in tropical and subtropical regions around the world. Outside of Africa, *Ae. aegypti* is almost exclusively found in close association with humans in domestic environments, where it breeds in artificial water storage containers. There are several reasons why *Ae. aegypti* is one of the most capable vectors of the aforementioned arboviruses. *Ae. aegypti* has evolved to live in close proximity to humans throughout its entire lifecycle. They prefer artificial manmade water containers as a larval habitat, they prefer to rest indoors close to humans, and they strongly prefer human blood and tend to feed on multiple humans during a single gonotrophic cycle. This high human exposure increases the mosquito's probability of becoming infected with an arbovirus and increases the frequency with which they can transmit the virus to another host (47). Although *Ae. aegypti* has a stronger host preference for humans in comparison to *Ae. albopictus*, they are more geographically restricted because they do not diapause in colder climates (48).

The Asian tiger mosquito, *Ae. albopictus*, has been a driving force in the worldwide emergence of chikungunya virus. In central Africa, *Ae. albopictus* played a

key role in the 2007 emergence of DENV, CHIKV, and possibly ZIKV. This mosquito originated from a zoophilic forest species in Asia, however its range was rapidly expanded during 1980s. *Ae. albopictus* was introduced to Europe, the USA, and Brazil probably via international trade of used tires and lucky bamboo plants, and it invaded tropical and temperate regions around the world (49). Unlike *Ae. aegypti*, it lacks preferential coexistence with humans and feeds opportunistically on humans and animals. *Ae. albopictus* lives in more varied environments and breeds in both natural and artificial containers (50). The mosquito adapted to moderate climatic conditions, and in temperate areas it produces eggs that are resistant to desiccation and can remain viable for more than a year (48). This greater tolerance of *Ae. albopictus* for cold environments poses a threat to larger geographic areas and could lead to enhanced arboviral transmission in temperate regions of the world. In addition to a wider geographical distribution, this resilient and aggressive mosquito can survive both in rural and urban environments, which makes it such an important arbovirus vector (51).

Tissue barriers and immune responses to arbovirus infection in mosquitoes

Arboviruses need to overcome different tissue barriers and the innate immune response in order to establish persistent infection in the mosquito vector and ensure transmission to another host. Female mosquitoes ingest virus through the viremic blood from an infected vertebrate host. Once the virus reaches the midgut lumen, it comes in close proximity with epithelial cells and initiates infection. A mosquito midgut consists of a single layer of epithelial cells surrounded by basal lamina. Once the virus enters cells, it replicates and spreads within the midgut epithelium. “Midgut infection barrier” is a term for all the obstacles a virus needs to overcome before exiting the midgut (52). The

virus can get diverted into the diverticulum or peritrophic matrix, or it can be inactivated by digestive enzymes. Virus replication could also be inhibited by the absence of the receptor or by the intracellular immune response. After successful replication inside the midgut epithelium, the virus needs to pass through the basal lamina and overcome the second barrier, the 'midgut escape barrier'. This is most likely accomplished through the tracheal system surrounding the midgut or cardia/intussuscepted foregut, which has disordered basal lamina. Before it can infect the salivary glands, the virus needs to undergo a second round of amplification in various tissues such as hemocytes, fat bodies, tracheas, and muscles. Finally, to be successfully transmitted, the virus needs to get into the saliva of the mosquito, which is then injected into the host when the mosquito takes a subsequent blood meal. Similar to the midgut, the virus needs to overcome two tissue barriers associated with the salivary glands, the 'salivary gland infection barrier' and 'salivary gland escape barrier' however molecular mechanisms of these barriers have not been well understood so far (53) (Fig 2.4).

Throughout this whole process, the virus encounters numerous immune responses that are actively interfering with virus replication. Major immune signaling pathways involved in antiviral defenses are the Toll pathway, the immune deficiency (IMD) pathway, and the Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway. RNA interference (RNAi), although not considered a classical immune pathway, also plays an important role in antiviral defense. The immune response is initiated when pattern recognition receptors (PRRs) recognize microbial pathogen associated molecular patterns (PAMPs). PAMP recognition can trigger different immune signaling pathways either through the proteolytic cleavage of a cytokine in Toll pathway

or through various caspases and kinases in IMD and JAK-STAT pathways (54). This cascade will result in activation of different transcription factors and expression of effector genes that encode for antimicrobial peptides (AMPs) such as defensins, cecropins, diptericin, etc (55). Most of the mosquito antiviral responses have been studied in DENV system. DENV infection has been shown to activate the transcription of main Toll components such as Späetzle, Toll, Rel1A and multiple AMPs (56). Although silencing the IMD pathway had no effect on DENV, DENV and Sindbis virus (SINV) have been shown to upregulate the IMD response (56), while expression of JAK-STAT effector genes decreased DENV titers in the *Ae. aegypti* midgut (57) and in salivary glands. The most well-characterized invertebrate antiviral defense system is the RNAi. During replication, the virus creates long double-stranded RNA (dsRNA) intermediates that are recognized and cleaved by Dicer-2 (Dcr2) into 21-nucleotide small-interfering RNA (siRNA). Activated siRNA is loaded on to an RNA-induced silencing complex (RISC) and degrades one strand while the other strand is used for targeted degradation of complementary viral single-stranded RNA (ssRNA) (56). Knockdown of Dcr2 in *Ae. aegypti* results in more efficient DENV replication, shorter extrinsic incubation period (EIP; time it takes for mosquito to become infectious after ingesting infectious blood), and increased DENV transmission efficiency, therefore it is considered a key mediator of vector competence (58). Virus infection can also induce humoral immune response such as extracellular phenoloxidase (PO) cascade that can directly kill viruses in mosquitoes and other insects (53, 54), and complement-like proteins that can induce AMPs and limit flavivirus infection in *Ae. aegypti* (53).

Besides tissue barriers and immune responses, other systems can interact and have direct or indirect effects on the infection outcomes. Blood meal digestion and hemoglobin degradation cause the release of pro-oxidant molecules, such as heme, that can be toxic to mosquitoes. Blood ingestion therefore triggers antioxidant response which suppresses antiviral innate immunity and can contribute to arboviral infection (59). Studies have shown that antioxidant responses can facilitate the establishment of DENV, but not ZIKV, in *Ae. aegypti* midgut (60). Reactive oxygen species can also alter midgut microbiome which can further affect infection outcome (61)

Arboviruses, on the other hand, have numerous strategies to escape tissue barriers and evade or suppress mosquito immune responses. It has been shown that arboviruses can manipulate mosquito genes to facilitate infection, dissemination, and transmission in the vector host (62). Further, DENV was shown to downregulate immunity-related genes and cause impaired production of AMPs in *Ae. aegypti* cells (63). Semliki Forest virus (SFV), CHIKV, and JEV were shown to suppress Toll, Imd, and JAK/STAT signaling pathways in mosquito cells (64-66), however few studies have investigated those mechanisms *in vivo*. ZIKV, DENV and many other arboviruses, replicate within vesicular compartments of the ER. This was suggested to be an important mechanism of immune evasion as sequestered replication intermediates become inaccessible to the RNAi machinery (67). Arboviruses can also evade immunity through apoptosis and autophagy. Since arboviruses have to replicate at levels high enough to ensure transmission yet low enough to minimize the effects on mosquito fitness (62), the balance between the virus replication and mosquito immunity allow them to coexist long enough to transmit the virus (68).

Genetic and environmental factors for arboviral transmission

Epidemiological outcome of the disease depends on the pathogen-host interactions that are defined by numerous intrinsic factors such as pathogen and host genetics, as well as extrinsic biotic and abiotic factors. A conceptual illustration that demonstrates the interactions between the pathogen, host and the environment is called the disease triangle. Pathogen intrinsic factors and genetics (G_P) determine its virulence, invasion, and persistence in the population. Host intrinsic factors and genetics (G_H) determine its susceptibility or resistance, and whether the host will clear, tolerate, or succumb to infection. Any evolutionary changes in the pathogen or host and how they interact (G_P x G_H) can lead to disease emergence. Environmental or extrinsic factors (E), which can be biotic (competition, parasitism, predation) or abiotic (temperature, humidity, photoperiod), can affect pathogens (G_P x E), the host (G_H x E), or their interaction (G_P x G_H x E) (Fig 5).

Virus and vector genotype

Arboviruses need to alternate replication in disparate hosts and changing environments. In order to succeed in such a dynamic system, they require significant plasticity. All arboviruses, with the exception of African swine fever virus, have an RNA genome with error-prone replication, lack of a proofreading mechanism, short virus generation times, and high levels of viral progeny. RNA-dependent RNA-polymerase (RdRp) has low fidelity with an average error frequency up to 10,000 times greater than the host genome (69). Arboviruses often exist as a collection of variable genomes within a host, and this mixed population of genomic variants is called mutant swarm. Genetic diversity of a swarm is governed by a dynamic balance between mutation and selection

towards viruses with increased virulence in vertebrate host and/or increased vector competence in invertebrate vector (70). Despite the potential for greater mutation rates and rapid adaptation inherent in RNA viruses, the consensus sequence of most arboviruses is highly conserved in nature. This could be due to requirement for replication in two distinct hosts. Only mutations that are beneficial or neutral for both can become fixed, while adaptation to either hosts alone would impose a fitness cost. Several studies have shown that passaging the virus in mosquitoes or mosquito cells enhanced mosquito infectivity, but reduced viremia or virulence in vertebrates or vertebrate cells, and vice versa. Alternating between vector and host, on the other hand, does not affect infectivity nor viremia (70, 71).

Arboviruses typically cause persistent infection in mosquito vectors since increased virulence could affect mosquito fitness and reduce transmission. However, viruses tend to be more virulent in vertebrate hosts as higher virulence and viremia could increase transmission. Many studies have shown that mosquitoes feeding on blood with high viral titers are more likely to become infected and infectious than mosquitoes feeding on low titers (72). However, increased pathogen loads can also have negative effects on transmission potential. High viremia often correlates with the severity of the symptoms (29, 73) which can decrease patients' mobility and chances of encountering mosquitoes. Moreover, high viremia often elicits stronger immune response which can affect host blood quality and reduce infectivity in mosquitoes (29, 74, 75).

There are several examples of virus adaptation and selection resulting in increased vector competence in mosquitoes. Vector competence is defined as the ability of a particular arthropod to become infected and transmit a given virus to a susceptible host.

Vector competence of a mosquito population may vary based on different viruses and even different strains of the same virus (76). The best-known example of vector-driven adaptation in an arbovirus is a single A226V amino acid substitution in E1 glycoprotein of CHIKV that led to a rapid adaptation and switch to a more abundant vector (40). Another example is an outbreak of Venezuelan equine encephalitis virus (VEEV) that was associated with a single mutation in the E2 gene which caused an increased vector competence (77). Explosive expansion of WNV in the US in early 2000s was also the result of a new WN02 genotype that was transmitted earlier and more efficiently by *Culex* mosquito and fully displaced NY99 genotype (78).

Besides arboviral genetic variation, vector competence is determined by vector intrinsic factors which can vary between mosquito species, local mosquito population and even between individual mosquitoes (76). Many studies demonstrated genetic variability in vector competence within and between geographically distinct populations of *Ae. aegypti* (79-82). Genetic variation defines physical barriers and immune response and determines whether mosquito will clear, tolerate or succumb the infection. A lot of effort has been put into identifying and characterizing genes that affect mosquito vector competence with the goal to generate pathogen-resistant mosquitoes. Variability in vector competence is usually driven by joint action of several genes. Such traits are called quantitative traits and the individual gene locations are called quantitative trait loci (QTL) (79). Several QTL affecting vector competence have been identified in mosquitoes (83) however, the specific nature of the genes controlling susceptibility or refractoriness to pathogen infection has yet to be determined. While most studies focus on vector competence, transmission potential includes other traits that also vary across mosquito

populations, such as biting rate, fecundity, and lifespan. Since energetic resources are limited, there might be physiological trade-offs associated with the investment into reproduction versus immunity, however, these relationships are yet to be explored.

The outcome of infection is governed by genotype-genotype (G_P x G_H) interactions and it depends on specific pairing of vector and pathogen genotypes. These interactions have been well characterized between DENV and *Ae. aegypti* (84-87). As hosts induce a selective pressure on viruses, viruses can evolve in response to local host genotypes. By doing so, viruses can further induce selective pressure on host to diversify host defense. This concept where arbovirus selection may drive genome evolution in the invertebrate host and vice versa is called evolutionary "arms race" (88). Most studies demonstrating the evolutionary "arms race" were done in *Drosophila* (89-91) and there is less evidence on arboviruses driving mosquito evolution. One study proposed the polymorphism in the RNAi pathway as an underlying mechanism for resistance of some *Ae. aegypti* mosquitoes to DENV (92). However, with only a small proportion of vector populations actually carrying arboviruses, it is questionable whether arboviruses can induce a strong selective pressure.

Environment effect on viruses and vectors

The vector-borne transmission cycle is a dynamic system shaped by constant environmental changes. Various biotic and abiotic environmental factors can have direct effects on transmission via changes in virus replication and transmission probabilities or indirect effects due to altered physical barriers, nature and magnitude of immune responses, and changes in overall host fitness (e.g. survival and reproduction). Some examples of biotic factors that can affect virus replication and vector competence are gut

microbiota, coinfections with insect-specific virus or with another arbovirus, competition, and predation. Gut microbiota is a part of vector physiology and it can affect key processes related to pathogen transmission. Bacterial communities, which are mosquito-specific (93), may alter vector competence directly by impeding virus replication or indirectly by modulating host immune system. There is evidence that antibiotic depletion of mosquito gut bacteria makes them less susceptible to arboviral infection while introduction of some bacteria can result in enhanced vector competence (94). On the other hand, coinfection with one arbovirus was shown to only mildly affect infection with another arbovirus (95-97). Some examples of abiotic factors that can affect virus replication and/or vector competence are temperature, humidity and photoperiod. Temperature is one of the most important drivers of vector-borne transmission due to its profound effect on the ectothermic mosquito vector, the virus, and their interaction, and will be the focus in some of the following chapters of this thesis.

Arboviruses have evolved to replicate across a wide temperature ranges, from the invertebrate vectors to the febrile mammalian or avian hosts. However, ambient temperature plays an important role in virus replication. It can directly affect virus replication by altering virus structure and fluctuation of the surface proteins which can affect virus entry in the host cell (5, 21, 98). Cryo-electron microscopy showed that DENV structure differs depending on the host temperature, displaying “bumpy” surface in human hosts and “smooth” surface in mosquito hosts (21). Destabilization in the multiple regions of the E protein can cause large scale changes on the surface of the particle and the ability to switch from “smooth” to “bumpy” (98). ZIKV is known to have tighter packing of E-proteins, resulting in higher thermal stability compared to DENV

(8). Furthermore, temperature can change virus replication rate, which typically positively correlates with the temperature. Temperature can also indirectly affect virus replication by altering cellular membranes, metabolism, protein processing in the ER, and by inducing heat-shock responses. Heat-shock proteins (HSP) are molecular chaperons that play an important role in protein folding and protection during stress. HSPs can be associated with the cell membrane and are part of a receptor complex for multiple viruses, including DENV (99, 100), but can also be involved in virus replication. Studies have shown that the heat shock response significantly increased DENV infectivity in human U937 cells (101), and both DENV and CHIKV can upregulate HSPs in mosquito cells under optimal conditions (102, 103).

The ambient temperature shapes every aspect of mosquito life, from development and size, to its physiology, immunity, metabolism, as well as behavior, distribution, and abundance, all of which affect the virus transmission potential (104, 105). Warmer temperatures expedite mosquito development and result in smaller adults. Fluctuating temperatures can cause even faster growth and allow development outside the temperature range where it would normally occur (106). Larval environment will further have carry-over effects on mosquito physiology and immunity. Many studies suggest that temperature conditions during immature stages affect vector competence in the adult stage. For example, it has been reported that *Aedes* reared at low temperature are more susceptible to RVFV, VEEV, and CHIKV (107, 108). Other studies investigated this phenomenon and found that cool rearing temperatures destabilize the RNAi pathway in mosquitoes resulting in increased susceptibility to CHIKV and YFV (109). Warmer temperatures can have positive effect on insect immunology due to accelerated

biochemical processes, such as protein synthesis and increased enzymatic activity. Increased levels of AMPs and hemolymph protein levels, increased activity of lysozyme-like enzymes, and PO activity result in overall better immune performance (110, 111). Metabolic rate of mosquitoes is extremely dependent upon environmental temperatures and has exponential increase with increasing temperatures and gradual decrease with decreasing temperatures. Temperature can also affect the central nervous system, which will ultimately affect the activity of the endocrine system. Hormone production can further affect development and reproduction (112). Reproductive output is often considered a central output of insect fitness. Stressful temperatures can impair oocyte development, sperm production and viability, decrease mating success and decrease overall reproductive output. Lifetime fecundity and number of eggs produced also have unimodal thermal performance. They reach a maximum at a certain optimal temperature and more or less symmetrically decrease toward both lower and upper limits of tolerance (113). Lastly, low temperatures can impair neural and muscular activity and gradually decrease motility and even cause chill coma.

Effects of environment on virus-vector interactions

While numerous studies have investigated the effect of constant and fluctuating temperatures on vector competence for many arboviruses including DENV, WNV, CHIKV (114-120), limited work has explored how environmental variation shapes pathogen-host interactions. One of the first studies exploring G_P x G_H x E interactions in mosquito-borne arboviral transmission was conducted for CHIKV (121). This study demonstrated that the transmission potential of CHIKV depends on a complex interaction between viral strain, mosquito population, and ambient temperatures. In other words, the

probability of a viral strain emergence in a mosquito species or population changes with environmental temperature. More recent work in the DENV-*Ae. aegypti* system reinforce these insights (122), suggesting that these effects are likely important across a wider range of mosquito-transmitted viruses. With increasing evidence indicating the importance of pathogen and mosquito genetics and environmental variability, laboratory and predictive mathematical models should not ignore the complexity of natural situations, especially when assessing the risk of vector-borne emergence.

Virus emergence

There are several factors contributing to such a widespread distribution of arboviruses. Changes in viral genetics can result in new strains with increased virulence or cause vector switching to peridomestic vectors (123). While some viruses require adaptation in order to amplify to epidemic levels, for others, the adaptation is not necessary. Introducing viruses to new geographical areas with naïve and susceptible vertebrate and arthropod hosts can be sufficient to cause epidemics. Invasion of arboviruses is often a consequence of an increased vector range. Increased transcontinental commerce, adaptation of mosquitoes to cities, development of insecticide resistance, and climate change contributed to mosquito expansions in the past (3). Furthermore, geographical expansion of the human population has also facilitated the appearance of some emerging viruses. Increased urbanization causes extreme concentrations of susceptible hosts but also creates urban heat islands that facilitate transmission, while intensification of agriculture and deforestation bring humans in close contact with zoonotic reservoirs (3). Increased international travel enables movement of

viremic humans across the globe within hours. These factors not only contributed to arboviral emergence into new environments but also made the epidemics hard to control.

Current challenges and vector control strategies

Determining the proportion of the human population infected (prevalence) and the number of new cases (incidence) is an important step in controlling an epidemic and assessing the burden of a disease. These counts are required in order to appropriately plan countermeasures and provide health care needs. Recent epidemics of ZIKV, CHIKV, and DENV were extremely difficult to quantify and control for various reasons. An estimated 80% of people infected with ZIKV, 50% infected with DENV, and 15% with CHIKV, are asymptomatic and never seek treatment, even though they may still be infectious to biting mosquito vectors (17). However, even if people develop symptoms, it can be difficult to accurately diagnose the disease since the clinical presentation of these diseases is nonspecific and overlaps across all three viruses (51). Diagnosis without unique symptoms is particularly challenging in resource-poor settings where expensive testing is not available. Serological testing may not always be a reliable technique since neutralizing antibodies often cross-react with other closely related viruses which leads to inconclusive results. RT-PCR is the main test for detection of viral nucleic acid in the serum. Although more specific, RT-PCR can often lead to a false-negative diagnosis due to the relatively short viremic phases in ZIKV and DENV infected individuals (51). ELISA and plaque-reduction neutralization also have some disadvantages and there is still no ideal diagnostic tool that could be used on a large scale. In addition to being transmitted by infectious mosquito, some arboviruses may have multiple transmission routes and can be transmitted maternally, through blood transfusions, and with ZIKV,

through sexual contact (9, 13, 124). Finally, there are currently no available therapeutics or effective vaccines for most arboviral diseases. The biggest challenge in vaccine development is that the antibodies generated can be weak or cross-reactive, or in sub-neutralizing concentrations that can lead to antibody-mediated enhancement and even more severe manifestation of the disease. Taken together, this leaves public health experts with only two options - vector control and public education - in the battle against these infections.

Vector control, although remains the main solution for transmission control, still presents a huge challenge. Currently, the most efficient method to control mosquito-borne diseases is to reduce mosquito-human contact through reduction or elimination of mosquitoes. Insecticides were considered to be the most important vector-control tool for a long time. Dichlorodiphenyltrichloroethane (DDT) was heavily used during mid-twentieth century campaigns to eliminate *Ae. aegypti* mosquitoes (4). Not only have mosquitoes developed resistance against DDT, but also this highly toxic insecticide is now considered environmentally unacceptable and its use is banned in almost all countries. Urban centers continue to grow, and efficient deployment of vector control strategies is particularly difficult in urban settings due to abundant habitats for the immature stages in close proximity to people, overcrowding, and poverty. Considering the recent arboviral epidemics, it is clear that new pathogens are likely to emerge. Therefore, it is critical to prepare for current and new epidemics with new, efficient, and environmentally acceptable strategies. Public education and increased awareness through community engagement and outreach are the first steps. Relatively cheap and efficient strategies include elimination of household oviposition and larval sites as well as

application of larvicides and adulticide fumigation inside households (4). Innovative, environmentally friendly, and species-specific methods that could be used for control are the release of genetically modified male mosquitoes that carry a lethal gene or sterile males that are not able to produce offspring (125). Another approach is the release of *Wolbachia*-infected mosquitoes. It has been shown that this endosymbiont bacterium interferes with virus replication in mosquitoes reducing arboviral transmission (4). However, lack of deeper understanding of mosquito biology and ecology is one of the limiting factors for this approach. While stopping current and preventing future emergence and spread remain a challenge, increased knowledge of the virus biology, disease ecology, human behavior and socioeconomic factors allow more effective surveillance and implementation of control strategies.

Implications for modeling

To predict the epidemiological outcome of the disease and assess the effectiveness of vector control strategies, mathematical model that links mosquito biology and behavior to pathogen transmission was proposes in the early 1900s. The elements of the model were first conceptualized in the Ross-Macdonald "vectorial capacity" equation (126). Vectorial capacity defines the ability of a mosquito population to spread disease among humans taking into account virus, vector, and host interaction.

Vectorial capacity

$$Vc = \frac{ma^2 \; x \; Vc \; x \; p^n}{-loge^p}$$

In this model, *m* represents vector density in relation to the host, *a* represents daily probability that the vector feeds on a host, *Vc* is vector competence, *p* is the probability of

daily survival, and *n* is the extrinsic incubation period. Vector competence and vector abundance are liner components and weak contributors to the overall vectorial capacity. However, longevity and EIP are exponential terms, and the most powerful components because long-lived infectious mosquitoes contribute the most to the transmission of vector-borne pathogens.

The basic reproductive number of a pathogen (R_0) is used to express the number of secondary infections from a single infected human in a susceptible population. R_0 model consists of a key pathogen, vector, and host parameters that are relevant for transmission and clearance. R_0 model provides the necessary information for estimating the epidemic spread of pathogens. If R_0 is greater than 1, we expect the infection to spread, and if R_0 is smaller than 1, infection will disappear. R_0 works under the assumptions that the pathogen is introduced onto virgin soil where all people at risk are susceptible, that humans mix homogeneously, that the population is so large that accretion of immune individuals is negligible, and that R_0 is measured only during the initial phase of the epidemic (127).

Basic reproductive number of a pathogen

$$R_0 = \sqrt{\frac{a^2\ bc\ exp(-\mu/EIR)\ EFD\ p_{EA}\ MDR}{N\ r\ \mu^3}}$$

In this model, *a* is the biting rate, *bc* is the vector competence, μ is the adult mosquito mortality rate, *EIR* is the extrinsic incubation rate (the inverse of the EIP), *EFD* is fecundity, p_{EA} is the survival probability, *MDR* is the egg-to-adult development rate, *N* is the density of humans, and *r* is the human recovery rate (128).

Key parameters in vectorial capacity and R_0 models are not static, as they are assumed to be in many statistical and mechanistic models, yet they are influenced by genetic variation in both the pathogen and the vector, as well as by abiotic and biotic environmental factors. There is substantial evidence demonstrating the effects temperature has on virus replication and dissemination inside of mosquitoes as well as transmission rates and the extrinsic incubation period (EIP). Increased temperatures accelerate virus replication and dissemination resulting in shorter EIP and increased transmission efficiency. Temperature also has a strong effect on mosquito survival, which is very important from an epidemiological perspective, because only mosquitoes that live beyond the EIP can be vectors (129). Temperature affects all mosquito parameters of the equation, in a nonlinear way which can be incorporated in the model to predict the temperature effects, seasonality and climate change on vector-borne pathogen transmission.

Conclusion

Emerging and re-emerging arboviral diseases represent a major threat to human health and well-being. The recent ZIKV outbreak is an example of how an arbovirus known to cause sporadic and mild infections can turn into a pandemic and a public health emergency. With no therapeutics and vaccines, mitigating ZIKV transmission relies on vector control and public education. However, in order to assess the vector-control measures, and predict the potential spread, we need to understand ZIKV transmission dynamics, characterize the factors that can alter transmission, and use that knowledge to generate mechanistic models. Some of the important knowledge gaps are how is ZIKV transmission potentially affected by variation in viremia among symptomatic and

asymptomatic hosts, and temperature, a key environmental driver of vector-borne diseases. Current modeling efforts ignore the variation in viral concentration on transmission and rely on the assumption that ZIKV responds to temperature similarly to DENV. In order to fill these knowledge gaps, we need to characterize how these factors affect some of key parameters of transmission risk models, such as vector competence, extrinsic incubation rate, and mortality rate. Taken together, this knowledge will enhance our ability to predict the number of people at risk and how that can change with seasons, geographic range, climate change and land use, but also enhance the efficacy of current and future intervention strategies.

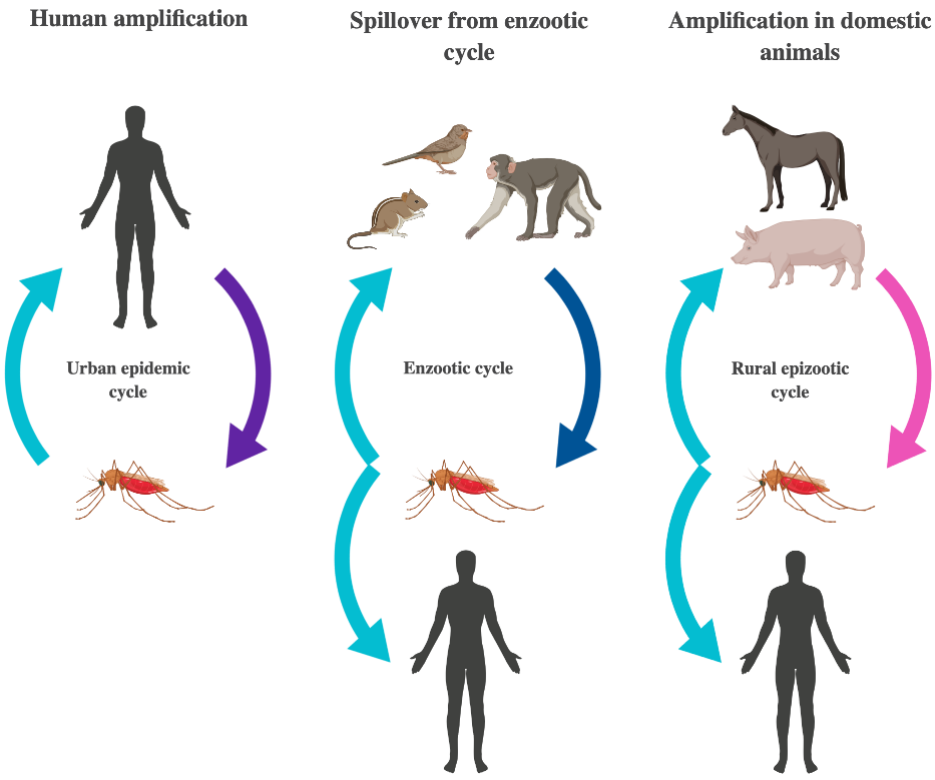


Fig 2.1. Arbovirus transmission cycles. Transmission of arboviruses in humans can occur in three ways. The first is direct spillover during the sylvatic cycle, when the virus that circulates among the mosquito vector and the enzootic host accidentally gets transmitted to humans. The second mechanism includes the amplification of the virus in domestic animals that live in close proximity to people, followed by a spillover. Lastly, in urban epidemic cycle, humans serve as amplification hosts and the virus can be transmitted between humans via anthropophilic mosquitoes.

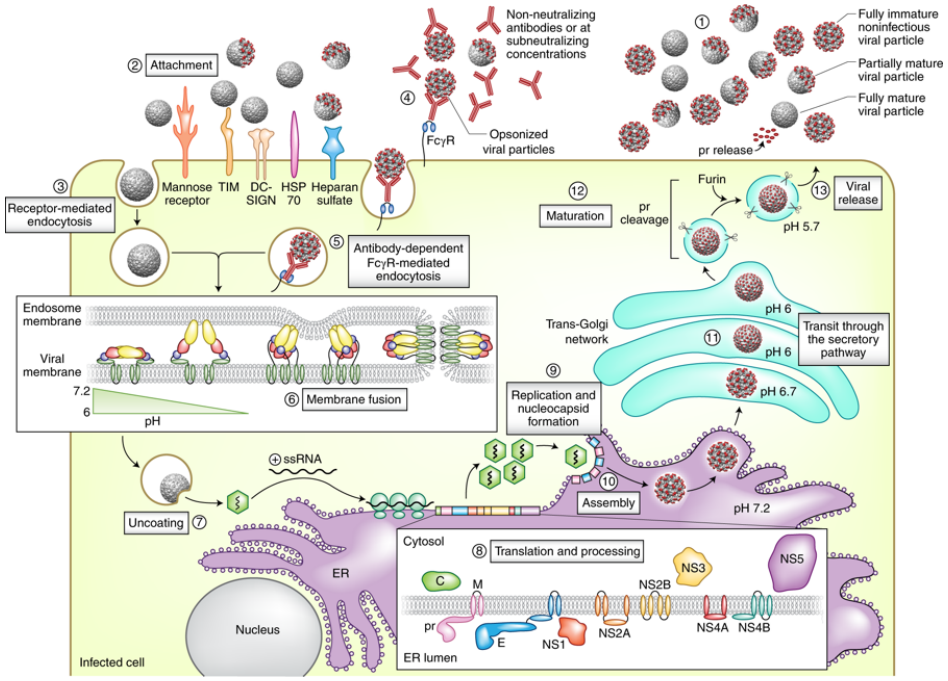


Figure adapted from: The immune response against flaviviruses (130)

Fig 2.2. Flavivirus replication cycle. Flaviviruses exist in a mixed population of fully or partially matured, and fully immature noninfectious particles (1). The virus replication starts when fully or partially matured particle attaches to the host cell (2) and undergoes receptor-mediated endocytosis (3). Alternatively, non-neutralizing antibodies or antibodies at subneutralizing concentrations can opsonize virus particle (4) and virus can undergo antibody-dependent FcγR-mediated endocytosis (5). The low pH in the endosome triggers trimerization of the E proteins, which results in the membrane fusion (6). Once the RNA genome is released in the cytoplasm (7), it serves as mRNA and produces viral proteins (8). Virus replication takes place in the ER vesicles (9) and assembled immature particles are released in the ER lumen (10) and transported through the Golgi (11). During this process, host protease furin cleaves the pr peptide and forms mature particles (12) that are released by exocytosis (13).

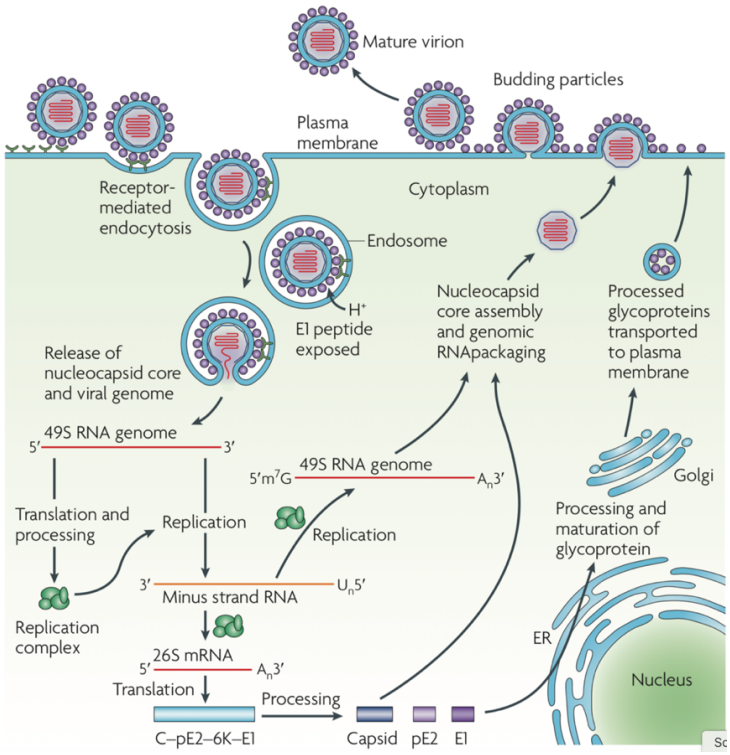


Figure adapted from: Biology and pathogenesis of chikungunya virus (33)

Fig 2.3. Alphavirus replication cycle. The virus enters the cell through receptor-mediated endocytosis. The low pH within the endosome triggers conformational changes in the virus envelope, resulting in the membrane fusion. Once the viral genome is released in the cytoplasm, the first viral polyprotein is translated and cleaved into nsP1-nsP4. Non-structural proteins form a viral replication complex that generates the full-length negative-strand RNA intermediate. The RNA intermediate serves as a template for both 26S subgenomic mRNA and 49S genomic RNA. Subgenomic RNA encodes for the C-pE2-6K-E1 polyprotein that is further processed by autoproteolytic serine proteases. Once the capsid is released in the cytoplasm, the remaining proteins are directed to the ER. In the Golgi, pE2 and E1 form heterodimers and traffic to the plasma membrane. Assembled particles with icosahedral core bud at the cell membrane.

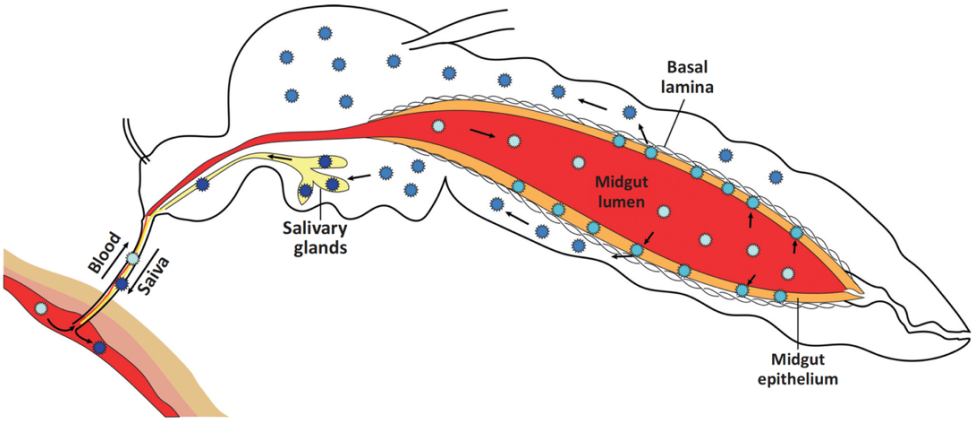


Figure adapted from: How Do Virus–Mosquito Interactions Lead to Viral Emergence(76)

Fig 2.4. Mosquito tissue barriers. Female mosquito ingests virus through the viremic blood from an infected vertebrate host. Once the virus reaches the midgut lumen, it comes in close proximity with the midgut epithelial cells and initiates infection. A mosquito midgut consists of a single layer of epithelial cells surrounded by basal lamina. After successful replication inside the midgut epithelium, the virus needs to pass through the basal and undergo a second round of amplification in various tissues. Finally, to be successfully transmitted, the virus needs to get into the saliva of the mosquito, which is then injected into the host when the mosquito takes a subsequent blood meal.

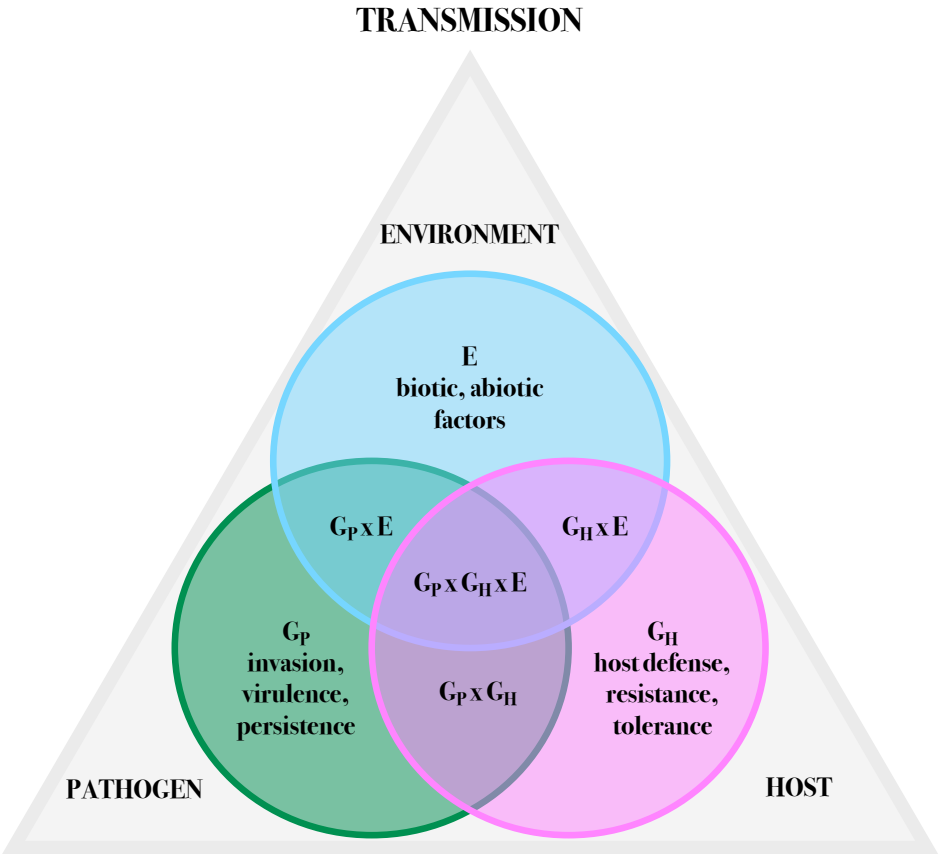


Fig 2.5. Disease triangle. Pathogen intrinsic factors and genetics (G_P) determine its invasion, virulence, and persistence in the population. Host intrinsic factors and genetics (G_H) determine its susceptibility or resistance, and whether the host will clear, tolerate or succumb to infection. Any evolutionary changes in the pathogen or host and how they interact (G_P x G_H) can lead to disease emergence. Environmental or extrinsic factors (E), which can be biotic (competition, parasitism, predation) or abiotic (temperature, humidity, photoperiod), can affect pathogens (G_P x E), the host (G_H x E), or their interaction (G_P x G_H x E).

References

1. Mayer SV, Tesh RB, Vasilakis N. The emergence of arthropod-borne viral diseases: A global prospective on dengue, chikungunya and zika fevers. Acta Trop. 2017;166:155-63.

2. Huang Y-JS, Higgs S, Vanlandingham DL. Arbovirus-Mosquito Vector-Host Interactions and the Impact on Transmission and Disease Pathogenesis of Arboviruses. Frontiers in Microbiology. 2019;10(22).

3. Weaver SC, Reisen WK. Present and future arboviral threats. Antiviral research. 2010;85(2):328-45.

4. Weaver SC, Charlier C, Vasilakis N, Lecuit M. Zika, Chikungunya, and Other Emerging Vector-Borne Viral Diseases. Annual review of medicine. 2018;69:395-408.

5. Hasan SS, Sevvana M, Kuhn RJ, Rossmann MG. Structural biology of Zika virus and other flaviviruses. Nature structural & molecular biology. 2018;25(1):13-20.

6. Persaud M, Martinez-Lopez A, Buffone C, Porcelli SA, Diaz-Griffero F. Infection by Zika viruses requires the transmembrane protein AXL, endocytosis and low pH. Virology. 2018;518:301-12.

7. Harrison SC. Viral membrane fusion. Nature structural & molecular biology. 2008;15(7):690-8.

8. Sirohi D, Kuhn RJ. Zika Virus Structure, Maturation, and Receptors. J Infect Dis. 2017;216(suppl_10):S935-s44.

9. Musso D, Nhan T, Robin E, Roche C, Bierlaire D, Zisou K, et al. Potential for Zika virus transmission through blood transfusion demonstrated during an outbreak in French Polynesia, November 2013 to February 2014. Euro Surveill. 2014;19(14).

10. Musso D, Roche C, Robin E, Nhan T, Teissier A, Cao-Lormeau VM. Potential sexual transmission of Zika virus. Emerg Infect Dis. 2015;21(2):359-61.

11. Mlakar J, Korva M, Tul N, Popovic M, Poljsak-Prijatelj M, Mraz J, et al. Zika virus associated with microcephaly. N Engl J Med. 2016;374(10):951-8.

12. Lazear HM, Diamond MS. Zika Virus: New Clinical Syndromes and Its Emergence in the Western Hemisphere. J Virol. 2016;90(10):4864-75.

13. Centers for Disease Control and Prevention. Zika symptoms 2017 [Available from: https://www.cdc.gov/zika/symptoms/symptoms.html.

14. Dick GWA, Kitchen SF, Haddow AJ. Zika Virus (I). Isolations and serological specificity. Trans R Soc Trop Med Hyg. 1952;46(5):509-20.

15. Smithburn KC. Neutralizing antibodies against certain recently isolated viruses in the sera of human beings residing in East Africa. J Immunol. 1952;69(2):223-34.

16. MacNamara FN. Zika virus : A report on three cases of human infection during an epidemic of jaundice in Nigeria. Trans R Soc Trop Med Hyg. 1954;48(2):139-45.

17. Duffy MR, Chen T-H, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med. 2009;360(24):2536-43.

18. Roth A, Mercier A, Lepers C, Hoy D, Duituturaga S, Benyon E, et al. Concurrent outbreaks of dengue, chikungunya and Zika virus infections - an unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012-2014. Euro Surveill. 2014;19(41).

19. PAHO. Pan American Health Organisation 2018 [Available from: http://www.paho.org/.

20. Enfissi A, Codrington J, Roosblad J, Kazanji M, Rousset D. Zika virus genome from the Americas. Lancet. 2016;387(10015):227-8.

21. Zhang X, Sheng J, Plevka P, Kuhn RJ, Diamond MS, Rossmann MG. Dengue structure differs at the temperatures of its human and mosquito hosts. Proc Natl Acad Sci U S A. 2013;110(17):6795-9.

22. Guzman MG, Harris E. Dengue. Lancet. 2015;385(9966):453-65.

23. Chye JK, Lim CT, Ng KB, Lim JM, George R, Lam SK. Vertical transmission of dengue. Clin Infect Dis. 1997;25(6):1374-7.

24. Mohammed H, Linnen JM, Munoz-Jordan JL, Tomashek K, Foster G, Broulik AS, et al. Dengue virus in blood donations, Puerto Rico, 2005. Transfusion. 2008;48(7):1348-54.

25. Braack L, Gouveia de Almeida AP, Cornel AJ, Swanepoel R, de Jager C. Mosquito-borne arboviruses of African origin: review of key viruses and vectors. Parasit Vectors. 2018;11(1):29.

26. Beckham JD, Tyler KL. Arbovirus Infections. Continuum (Minneapolis, Minn). 2015;21(6 Neuroinfectious Disease):1599-611.

27. McSherry JA. Some medical aspects of the Darien scheme: was it dengue? Scottish medical journal. 1982;27(2):183-4.

28. Hammon WM, Rudnick A, Sather GE. Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. Science. 1960;131(3407):1102-3.

29. Duong V, Lambrechts L, Paul RE, Ly S, Lay RS, Long KC, et al. Asymptomatic humans transmit dengue virus to mosquitoes. Proc Natl Acad Sci U S A. 2015;112(47):14688-93.

30. van Duijl-Richter MK, Hoornweg TE, Rodenhuis-Zybert IA, Smit JM. Early Events in Chikungunya Virus Infection-From Virus Cell Binding to Membrane Fusion. Viruses. 2015;7(7):3647-74.

31. Zhang R, Kim AS, Fox JM, Nair S, Basore K, Klimstra WB, et al. Mxra8 is a receptor for multiple arthritogenic alphaviruses. Nature. 2018;557(7706):570-4.

32. Flint J, Racaniello VR, Rall GF, Skalka AM. Principles of Virology, Volume I: Molecular Biology, Fourth Edition: American Society of Microbiology; 2015.

33. Schwartz O, Albert ML. Biology and pathogenesis of chikungunya virus. Nat Rev Microbiol. 2010;8(7):491-500.

34. Borgherini G, Poubeau P, Jossaume A, Gouix A, Cotte L, Michault A, et al. Persistent arthralgia associated with chikungunya virus: a study of 88 adult patients on reunion island. Clin Infect Dis. 2008;47(4):469-75.

35. Weaver SC, Osorio JE, Livengood JA, Chen R, Stinchcomb DT. Chikungunya virus and prospects for a vaccine. Expert review of vaccines. 2012;11(9):1087-101.

36. Volk SM, Chen R, Tsetsarkin KA, Adams AP, Garcia TI, Sall AA, et al. Genome-scale phylogenetic analyses of chikungunya virus reveal independent emergences of recent epidemics and various evolutionary rates. J Virol. 2010;84(13):6497-504.

37. Ross RW. The Newala epidemic. III. The virus: isolation, pathogenic properties and relationship to the epidemic. The Journal of hygiene. 1956;54(2):177-91.

38. Powers AM, Logue CH. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. J Gen Virol. 2007;88(Pt 9):2363-77.

39. Kuo SC, Chen YJ, Wang YM, Tsui PY, Kuo MD, Wu TY, et al. Cell-based analysis of Chikungunya virus E1 protein in membrane fusion. Journal of biomedical science. 2012;19:44.

40. Tsetsarkin KA, Weaver SC. Sequential adaptive mutations enhance efficient vector switching by Chikungunya virus and its epidemic emergence. PLoS Pathog. 2011;7(12):e1002412.

41. Ravi V. Re-emergence of chikungunya virus in India. Indian journal of medical microbiology. 2006;24(2):83-4.

42. Angelini R, Finarelli AC, Angelini P, Po C, Petropulacos K, Macini P, et al. An outbreak of chikungunya fever in the province of Ravenna, Italy. Euro Surveill. 2007;12(9):E070906.1.

43. Grandadam M, Caro V, Plumet S, Thiberge JM, Souares Y, Failloux AB, et al. Chikungunya virus, southeastern France. Emerg Infect Dis. 2011;17(5):910-3.

44. Centers for Disease Control and Prevention. Chikungunya virus in the United States 2016 [cited 2016 August 10]. Available from: https://www.cdc.gov/chikungunya/geo/united-states.html.

45. Brown JE, Evans BR, Zheng W, Obas V, Barrera-Martinez L, Egizi A, et al. Human impacts have shaped historical and recent evolution in Aedes aegypti, the dengue and yellow fever mosquito. Evolution; international journal of organic evolution. 2014;68(2):514-25.

46. Tabachnick WJ. The evolutionary relationships among arboviruses and the evolutionary relationships of their vectors provides a method for understanding vector-host interactions. J Med Entomol. 1991;28(3):297-8.

47. Weaver SC, Forrester NL. Chikungunya: Evolutionary history and recent epidemic spread. Antiviral research. 2015;120:32-9.

48. Shragai T, Tesla B, Murdock C, Harrington LC. Zika and chikungunya: mosquito-borne viruses in a changing world. Ann N Y Acad Sci. 2017;1399(1):61-77.

49. Kraemer MU, Sinka ME, Duda KA, Mylne AQ, Shearer FM, Barker CM, et al. The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus. eLife. 2015;4:e08347.

50. Weetman D, Kamgang B, Badolo A, Moyes CL, Shearer FM, Coulibaly M, et al. Aedes Mosquitoes and Aedes-Borne Arboviruses in Africa: Current and Future Threats. International journal of environmental research and public health. 2018;15(2).

51. Paixao ES, Teixeira MG, Rodrigues LC. Zika, chikungunya and dengue: the causes and threats of new and re-emerging arboviral diseases. BMJ global health. 2018;3(Suppl 1):e000530.

52. Franz AW, Kantor AM, Passarelli AL, Clem RJ. Tissue Barriers to Arbovirus Infection in Mosquitoes. Viruses. 2015;7(7):3741-67.

53. Liu T, Xu Y, Wang X, Gu J, Yan G, Chen XG. Antiviral systems in vector mosquitoes. Developmental and comparative immunology. 2018;83:34-43.

54. Cheng G, Liu Y, Wang P, Xiao X. Mosquito Defense Strategies against Viral Infection. Trends Parasitol. 2016;32(3):177-86.

55. Lee W-S, Webster JA, Madzokere ET, Stephenson EB, Herrero LJ. Mosquito antiviral defense mechanisms: a delicate balance between innate immunity and persistent viral infection. Parasit Vectors. 2019;12(1):165.

56. Sim S, Jupatanakul N, Dimopoulos G. Mosquito immunity against arboviruses. Viruses. 2014;6(11):4479-504.

57. Souza-Neto JA, Sim S, Dimopoulos G. An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. Proc Natl Acad Sci U S A. 2009;106(42):17841-6.

58. Sanchez-Vargas I, Scott JC, Poole-Smith BK, Franz AW, Barbosa-Solomieu V, Wilusz J, et al. Dengue virus type 2 infections of Aedes aegypti are modulated by the mosquito's RNA interference pathway. PLoS Pathog. 2009;5(2):e1000299.

59. Zhu Y, Zhang R, Zhang B, Zhao T, Wang P, Liang G, et al. Blood meal acquisition enhances arbovirus replication in mosquitoes through activation of the GABAergic system. Nat Commun. 2017;8(1):1262.

60. Oliveira JHM, Talyuli OAC, Goncalves RLS, Paiva-Silva GO, Sorgine MHF, Alvarenga PH, et al. Catalase protects Aedes aegypti from oxidative stress and increases midgut infection prevalence of Dengue but not Zika. PLoS Negl Trop Dis. 2017;11(4):e0005525.

61. Oliveira JH, Goncalves RL, Lara FA, Dias FA, Gandara AC, Menna-Barreto RF, et al. Blood meal-derived heme decreases ROS levels in the midgut of Aedes aegypti and allows proliferation of intestinal microbiota. PLoS Pathog. 2011;7(3):e1001320.

62. Hwang J, Jurado KA, Fikrig E. Genetics of War and Truce between Mosquitos and Emerging Viruses. Cell Host Microbe. 2016;19(5):583-7.

63. Sim S, Dimopoulos G. Dengue virus inhibits immune responses in Aedes aegypti cells. PLoS One. 2010;5(5):e10678.

64. Fragkoudis R, Chi Y, Siu RW, Barry G, Attarzadeh-Yazdi G, Merits A, et al. Semliki Forest virus strongly reduces mosquito host defence signaling. Insect molecular biology. 2008;17(6):647-56.

65. McFarlane M, Arias-Goeta C, Martin E, O'Hara Z, Lulla A, Mousson L, et al. Characterization of Aedes aegypti innate-immune pathways that limit Chikungunya virus replication. PLoS Negl Trop Dis. 2014;8(7):e2994.

66. Lin CC, Chou CM, Hsu YL, Lien JC, Wang YM, Chen ST, et al. Characterization of two mosquito STATs, AaSTAT and CtSTAT. Differential regulation of tyrosine phosphorylation and DNA binding activity by lipopolysaccharide treatment and by Japanese encephalitis virus infection. The Journal of biological chemistry. 2004;279(5):3308-17.

67. Kingsolver MB, Huang Z, Hardy RW. Insect antiviral innate immunity: pathways, effectors, and connections. J Mol Biol. 2013;425(24):4921-36.

68. Forrester NL, Coffey LL, Weaver SC. Arboviral bottlenecks and challenges to maintaining diversity and fitness during mosquito transmission. Viruses. 2014;6(10):3991-4004.

69. Parrish CR, Holmes EC, Morens DM, Park EC, Burke DS, Calisher CH, et al. Cross-species virus transmission and the emergence of new epidemic diseases. Microbiol Mol Biol Rev. 2008;72(3):457-70.

70. Ciota AT, Kramer LD. Insights into arbovirus evolution and adaptation from experimental studies. Viruses. 2010;2(12):2594-617.

71. Coffey LL, Vasilakis N, Brault AC, Powers AM, Tripet F, Weaver SC. Arbovirus evolution in vivo is constrained by host alternation. Proc Natl Acad Sci U S A. 2008;105(19):6970-5.

72. Souza-Neto JA, Powell JR, Bonizzoni M. Aedes aegypti vector competence studies: A review. Infection, Genetics and Evolution. 2019;67:191-209.

73. Waggoner JJ, Gresh L, Vargas MJ, Ballesteros G, Tellez Y, Soda KJ, et al. Viremia and clinical presentation in Nicaraguan patients infected with Zika virus, chikungunya virus, and dengue virus. Clin Infect Dis. 2016;63(12):1584-90.

74. Pakpour N, Akman-Anderson L, Vodovotz Y, Luckhart S. The effects of ingested mammalian blood factors on vector arthropod immunity and physiology. Microbes Infect. 2013;15(3):243-54.

75. Pakpour N, Riehle MA, Luckhart S. Effects of ingested vertebrate-derived factors on insect immune responses. Curr Opin Insect Sci. 2014;3:1-5.

76. Ruckert C, Ebel GD. How Do Virus-Mosquito Interactions Lead to Viral Emergence? Trends Parasitol. 2018;34(4):310-21.

77. Brault AC, Powers AM, Ortiz D, Estrada-Franco JG, Navarro-Lopez R, Weaver SC. Venezuelan equine encephalitis emergence: enhanced vector infection from a single amino acid substitution in the envelope glycoprotein. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(31):11344-9.

78. Moudy RM, Meola MA, Morin LL, Ebel GD, Kramer LD. A newly emergent genotype of West Nile virus is transmitted earlier and more efficiently by Culex mosquitoes. Am J Trop Med Hyg. 2007;77(2):365-70.

79. Severson DW, Behura SK. Genome Investigations of Vector Competence in Aedes aegypti to Inform Novel Arbovirus Disease Control Approaches. Insects. 2016;7(4).

80. Goncalves CM, Melo FF, Bezerra JM, Chaves BA, Silva BM, Silva LD, et al. Distinct variation in vector competence among nine field populations of Aedes aegypti from a Brazilian dengue-endemic risk city. Parasit Vectors. 2014;7:320.

81. Failloux AB, Vazeille M, Rodhain F. Geographic genetic variation in populations of the dengue virus vector Aedes aegypti. J Mol Evol. 2002;55(6):653-63.

82. Bennett KE, Olson KE, Munoz Mde L, Fernandez-Salas I, Farfan-Ale JA, Higgs S, et al. Variation in vector competence for dengue 2 virus among 24 collections of Aedes aegypti from Mexico and the United States. Am J Trop Med Hyg. 2002;67(1):85-92.

83. Beerntsen BT, James AA, Christensen BM. Genetics of mosquito vector competence. Microbiol Mol Biol Rev. 2000;64(1):115-37.

84. Lambrechts L, Chevillon C, Albright RG, Thaisomboonsuk B, Richardson JH, Jarman RG, et al. Genetic specificity and potential for local adaptation between dengue viruses and mosquito vectors. BMC Evol Biol. 2009;9:160.

85. Anderson JR, Rico-Hesse R. Aedes aegypti vectorial capacity is determined by the infecting genotype of dengue virus. Am J Trop Med Hyg. 2006;75(5):886-92.

86. Lambrechts L, Scott TW. Mode of transmission and the evolution of arbovirus virulence in mosquito vectors. Proc Biol Sci. 2009;276(1660):1369-78.

87. Fansiri T, Fontaine A, Diancourt L, Caro V, Thaisomboonsuk B, Richardson JH, et al. Genetic mapping of specific interactions between Aedes aegypti mosquitoes and dengue viruses. PLoS Genet. 2013;9(8):e1003621.

88. Obbard DJ, Dudas G. The genetics of host-virus coevolution in invertebrates. Curr Opin Virol. 2014;8:73-8.

89. Sackton TB, Lazzaro BP, Schlenke TA, Evans JD, Hultmark D, Clark AG. Dynamic evolution of the innate immune system in Drosophila. Nat Genet. 2007;39(12):1461-8.

90. Obbard DJ, Welch JJ, Kim KW, Jiggins FM. Quantifying adaptive evolution in the Drosophila immune system. PLoS Genet. 2009;5(10):e1000698.

91. Obbard DJ, Jiggins FM, Halligan DL, Little TJ. Natural selection drives extremely rapid evolution in antiviral RNAi genes. Curr Biol. 2006;16(6):580-5.

92. Lambrechts L, Quillery E, Noel V, Richardson JH, Jarman RG, Scott TW, et al. Specificity of resistance to dengue virus isolates is associated with genotypes of the mosquito antiviral gene Dicer-2. Proc Biol Sci. 2013;280(1751):20122437.

93. Zouache K, Raharimalala FN, Raquin V, Tran-Van V, Raveloson LH, Ravelonandro P, et al. Bacterial diversity of field-caught mosquitoes, Aedes albopictus and Aedes aegypti, from different geographic regions of Madagascar. FEMS Microbiol Ecol. 2011;75(3):377-89.

94. Wu P, Sun P, Nie K, Zhu Y, Shi M, Xiao C, et al. A Gut Commensal Bacterium Promotes Mosquito Permissiveness to Arboviruses. Cell Host Microbe. 2019;25(1):101-12.e5.

95. Ruckert C, Weger-Lucarelli J, Garcia-Luna SM, Young MC, Byas AD, Murrieta RA, et al. Impact of simultaneous exposure to arboviruses on infection and transmission by Aedes aegypti mosquitoes. Nat Commun. 2017;8:15412.

96. Goertz GP, Vogels CBF, Geertsema C, Koenraadt CJM, Pijlman GP. Mosquito co-infection with Zika and chikungunya virus allows simultaneous transmission without affecting vector competence of Aedes aegypti. PLoS Negl Trop Dis. 2017;11(6):e0005654.

97. Le Coupanec A, Tchankouo-Nguetcheu S, Roux P, Khun H, Huerre M, Morales-Vargas R, et al. Co-Infection of Mosquitoes with Chikungunya and Dengue Viruses Reveals Modulation of the Replication of Both Viruses in Midguts and Salivary Glands of Aedes aegypti Mosquitoes. International Journal of Molecular Sciences. 2017;18(8):1708.

98. Lim XN, Shan C, Marzinek JK, Dong H, Ng TS, Ooi JSG, et al. Molecular basis of dengue virus serotype 2 morphological switch from 29 degrees C to 37 degrees C. PLoS Pathog. 2019;15(9):e1007996.

99. Salas-Benito J, Reyes-Del Valle J, Salas-Benito M, Ceballos-Olvera I, Mosso C, del Angel RM. Evidence that the 45-kD glycoprotein, part of a putative dengue virus receptor complex in the mosquito cell line C6/36, is a heat-shock related protein. Am J Trop Med Hyg. 2007;77(2):283-90.

100. Reyes-Del Valle J, Chavez-Salinas S, Medina F, Del Angel RM. Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. J Virol. 2005;79(8):4557-67.

101. Chavez-Salinas S, Ceballos-Olvera I, Reyes-Del Valle J, Medina F, Del Angel RM. Heat shock effect upon dengue virus replication into U937 cells. Virus research. 2008;138(1-2):111-8.

102. Zhang M, Zheng X, Wu Y, Gan M, He A, Li Z, et al. Differential proteomics of Aedes albopictus salivary gland, midgut and C6/36 cell induced by dengue virus infection. Virology. 2013;444(1-2):109-18.

103. Lee RCH, Chu JJH. Proteomics Profiling of Chikungunya-Infected Aedes albopictus C6/36 Cells Reveal Important Mosquito Cell Factors in Virus Replication. PLoS Negl Trop Dis. 2015;9(3):e0003544.

104. Murdock CC, Paaijmans KP, Cox-Foster D, Read AF, Thomas MB. Rethinking vector immunology: the role of environmental temperature in shaping resistance. Nat Rev Microbiol. 2012;10(12):869-76.

105. Murdock CC, Moller-Jacobs LL, Thomas MB. Complex environmental drivers of immunity and resistance in malaria mosquitoes. Proc Biol Sci. 2013;280(1770):20132030.

106. Colinet H, Sinclair BJ, Vernon P, Renault D. Insects in fluctuating thermal environments. Annu Rev Entomol. 2015;60:123-40.

107. Turell MJ. Effect of environmental temperature on the vector competence of Aedes taeniorhynchus for Rift Valley fever and Venezuelan equine encephalitis viruses. Am J Trop Med Hyg. 1993;49(6):672-6.

108. Westbrook CJ, Reiskind MH, Pesko KN, Greene KE, Lounibos LP. Larval Environmental Temperature and the Susceptibility of Aedes albopictus Skuse (Diptera: Culicidae) to Chikungunya Virus. Vector Borne Zoonotic Dis. 2010;10(3):241-7.

109. Adelman ZN, Anderson MA, Wiley MR, Murreddu MG, Samuel GH, Morazzani EM, et al. Cooler temperatures destabilize RNA interference and increase susceptibility of disease vector mosquitoes to viral infection. PLoS Negl Trop Dis. 2013;7(5):e2239.

110. Catalan TP, Wozniak A, Niemeyer HM, Kalergis AM, Bozinovic F. Interplay between thermal and immune ecology: effect of environmental temperature on insect immune response and energetic costs after an immune challenge. J Insect Physiol. 2012;58(3):310-7.

111. Murdock CC, Paaijmans KP, Bell AS, King JG, Hillyer JF, Read AF, et al. Complex effects of temperature on mosquito immune function. Proc Biol Sci. 2012;279(1741):3357-66.

112. Neven LG. Physiological responses of insects to heat. Postharvest Biology and Technology. 2000;21:1003-111.

113. Reznik SY, Voinovich ND, Vaghina NP. Effect of temperature on the reproduction and development of Trichogramma buesi (Hymenoptera: Trichogrammatidae)2009. 535-44 p.

114. Liu Z, Zhang Z, Lai Z, Zhou T, Jia Z, Gu J, et al. Temperature Increase Enhances Aedes albopictus Competence to Transmit Dengue Virus. Front Microbiol. 2017;8:2337.

115. Cornel AJ, Jupp PG, Blackburn NK. Environmental temperature on the vector competence of Culex univittatus (Diptera: Culicidae) for West Nile virus. J Med Entomol. 1993;30(2):449-56.

116. Mbaika S, Lutomiah J, Chepkorir E, Mulwa F, Khayeka-Wandabwa C, Tigoi C, et al. Vector competence of Aedes aegypti in transmitting Chikungunya virus: effects and

implications of extrinsic incubation temperature on dissemination and infection rates. Virology journal. 2016;13:114.

117. Samuel GH, Adelman ZN, Myles KM. Temperature-dependent effects on the replication and transmission of arthropod-borne viruses in their insect hosts. Curr Opin Insect Sci. 2016;16:108-13.

118. Lambrechts L, Paaijmans KP, Fansiri T, Carrington LB, Kramer LD, Thomas MB, et al. Impact of daily temperature fluctuations on dengue virus transmission by Aedes aegypti. Proc Natl Acad Sci U S A. 2011;108(18):7460-5.

119. Carrington LB, Armijos MV, Lambrechts L, Scott TW. Fluctuations at a low mean temperature accelerate dengue virus transmission by Aedes aegypti. PLoS Negl Trop Dis. 2013;7(4):e2190.

120. Carrington LB, Seifert SN, Armijos MV, Lambrechts L, Scott TW. Reduction of Aedes aegypti vector competence for dengue virus under large temperature fluctuations. Am J Trop Med Hyg. 2013;88(4):689-97.

121. Zouache K, Fontaine A, Vega-Rua A, Mousson L, Thiberge JM, Lourenco-De-Oliveira R, et al. Three-way interactions between mosquito population, viral strain and temperature underlying chikungunya virus transmission potential. Proc Biol Sci. 2014;281(1792).

122. Gloria-Soria A, Armstrong PM, Powell JR, Turner PE. Infection rate of Aedes aegypti mosquitoes with dengue virus depends on the interaction between temperature and mosquito genotype. Proc Biol Sci. 2017;284(1864).

123. Hanley KA, Weaver SC. CHAPTER 16 - Arbovirus Evolution. In: Domingo E, Parrish CR, Holland JJ, editors. Origin and Evolution of Viruses (Second Edition). London: Academic Press; 2008. p. 351-91.

124. Besnard M, Lastere S, Teissier A, Cao-Lormeau V, Musso D. Evidence of perinatal transmission of Zika virus, French Polynesia, December 2013 and February 2014. Euro Surveill. 2014;19(13).

125. Hoonbok Y, R. DB, Jae-seung Y, Ki-cheol O, Jinhong K, Hyun-Jung K. Effects of global warming on mosquitoes & mosquito-borne diseases and the new strategies for mosquito control. Entomological Research. 2014;44(6):215-35.

126. Smith DL, Battle KE, Hay SI, Barker CM, Scott TW, McKenzie FE. Ross, macdonald, and a theory for the dynamics and control of mosquito-transmitted pathogens. PLoS Pathog. 2012;8(4):e1002588.

127. Halstead SB. Dengue virus-mosquito interactions. Annu Rev Entomol. 2008;53:273-91.

128. Mordecai E, Cohen J, Evans MV, Gudapati P, Johnson LR, Lippi CA, et al. Detecting the impact of temperature on transmission of Zika, dengue, and chikungunya using mechanistic models. PLoS Neglected Tropical Diseases. 2017;11(4):e0005568.

129. Morin CW, Comrie AC, Ernst K. Climate and Dengue Transmission: Evidence and Implications. Environ Health Perspect. 2013;121(11-12):1264-72.

130. Slon Campos JL, Mongkolsapaya J, Screaton GR. The immune response against flaviviruses. Nature Immunology. 2018;19(11):1189-98.

CHAPTER 3

ESTIMATING THE EFFECTS OF VARIATION IN VIREMIA ON MOSQUITO SUSCEPTIBILITY, INFECTIOUSNESS, AND R_0 OF ZIKA IN *AEDES AEGYPTI* [1]

[1] Tesla B., Demakovsky L.R., Packiam H.S., Mordecai E.A., Rodríguez A.D., Bonds M.H., Brindley M.A., Murdock C.C. 2018. *PLoS Negl Trop Dis* 12(8): e0006733. Accepted by PLoS Neglected Tropical Diseases. Reprinted here with permission of the publisher.

Abstract

Zika virus (ZIKV) is an arbovirus primarily transmitted by *Aedes* mosquitoes. Like most viral infections, ZIKV viremia varies over several orders of magnitude, with unknown consequences for transmission. To determine the effect of viral concentration on ZIKV transmission risk, we exposed field-derived *Ae. aegypti* mosquitoes to four doses (10^3, 10^4, 10^5, 10^6 PFU/mL) representative of potential variation in the field. We demonstrate that increasing ZIKV dose in the blood-meal significantly increases the probability of mosquitoes becoming infected and infectious, as well as the rate at which virus spreads to the saliva but found no effect on dissemination and transmission efficiencies or mosquito mortality. We also demonstrate that determining infection using RT-qPCR approaches rather than plaque assays potentially over-estimates key transmission parameters, including the time at which mosquitoes become infectious and viral burden. Finally, using these data to parameterize an R_0 model, we showed that increasing viremia from 10^4 to 10^6 PFU/mL increased relative R_0 3.8-fold, demonstrating that variation in viremia substantially affects transmission risk.

Author summary

The number of people at risk for contracting Zika virus (ZIKV) is difficult to estimate accurately because most infected hosts are asymptomatic and the relationship between variation in host viremia and transmission to local mosquitoes is unclear. Controlling ZIKV transmission remains a major challenge due to lack of basic information on transmission mechanisms and gaps in mechanistic models. Therefore, our study highlights the importance of variation in viral concentration that current modeling efforts ignore, which will enhance our ability to predict the number of people at risk for arbovirus infection, overall disease transmission, and the efficacy of current and future intervention strategies. We demonstrated that increased concentration of ZIKV in the blood significantly increases the probability and the rate at which mosquitoes become infectious, which increases the risk of ZIKV transmission.

Introduction

Although discovered in 1947 (1), Zika virus (ZIKV) has recently become a public health concern due to its rapid spread and newly identified teratogenic effects (2). Shortly after isolation from a rhesus macaque in Uganda, the virus caused several mild infections in humans (3, 4). ZIKV infections remained unapparent until the first major outbreak in 2007 on the island of Yap (5). The virus further spread across the Pacific, where it was first associated with Guillain-Barré syndrome during the 2014 French Polynesian outbreak (6). In 2015, transmission was confirmed in Brazil (7), after which the virus spread rapidly across the Americas (8). ZIKV was declared a "public health emergency of international concern" by WHO in 2016 due to rapid spread and increases in complications associated with congenital Zika virus syndrome (9).

The primary route of ZIKV transmission is through the bite of *Aedes* mosquitoes, but the virus can also be spread vertically (2), sexually (10), and through blood transfusion (11). The principal urban vector in the Americas is *Ae. aegypti*, while *Ae. albopictus* is believed to be a secondary vector (12). Although most cases of ZIKV infection are asymptomatic (5), 20% of individuals develop symptoms associated with Zika fever (13). Currently, human viremia is not well characterized. Studies suggest that ZIKV viremia in the blood is lower than other arboviruses and does not significantly differ between symptomatic and asymptomatic patients (14). In arboviral systems such as dengue, variation in viremia across infectious human hosts influences the number of mosquitoes that become infectious (15), yet this has only been minimally explored in the ZIKV system (16-18). Further, the impact of variation in host viremia on overall transmission has yet to be adequately addressed.

The number of people at risk for contracting ZIKV or other similarly transmitted arboviruses (e.g. dengue and chikungunya) is difficult to estimate accurately because most ZIKV infected hosts are asymptomatic, the distribution of hosts with varying viremia is unknown, and the relationship between variation in host viremia and transmission to local mosquito populations is unclear. R_0 (the basic reproductive number of a pathogen) represents the expected number of secondary cases that result from a single infection in a susceptible population and is comprised of a combination of human, mosquito, and pathogen traits (19, 20). R_0 models allow for the estimation of the epidemic spread of pathogens (19-21), are commonly used to assess the effectiveness of mosquito control strategies (22-25), and are routinely used to predict the coverage required for successful vaccination programs (26-28). Yet, our current ability to estimate the number of human hosts at risk or to control ZIKV transmission is limited by the lack of basic information on transmission mechanisms, leading to gaps in mechanistic models, the most fundamental of which is R_0. To address this limitation, we conducted experiments to assess the effect of variation in viral dose on vector competence, the extrinsic incubation rate (EIR), and mosquito survival. We used these results to parameterize a mechanistic R_0 model and to estimate the number of infectious bites contributed by mosquito populations feeding on hosts with varying viremia.

Methods

Mosquito rearing

We generated an outbred field-derived population of *Ae. aegypti* mosquitoes from ovitrap collections in Tapachula, Chiapas, Mexico, 2016. Larvae were reared in trays (200 larvae/1L ddH_2O) and fed with 4 fish food pellets (Hikari Cichlid Cod Fish pellets).

Larvae and adults were kept under standard insectary conditions at 27°C ± 0.5°C, 80% ± 10% relative humidity, and a 12:12 hours light:dark diurnal cycle. Mosquitoes were maintained on human blood (Interstate Blood Bank) and provided with 10% sucrose *ad libitum*. F2 - F4 generations of mosquitoes were used for all downstream experiments.

Virus culture

For all mosquito infections, we used the ZIKV MEX1-44 strain obtained from the University of Texas Medical Branch Arbovirus Reference Collection. The virus was isolated from *Ae. aegypti* in 2016 from Chiapas, Mexico and passaged in Vero cells nine times. Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) at 37°C and 5% CO_2. The virus was harvested four days after inoculation and stored at -80°C for at least seven days before titrating. Titers were determined by standard plaque assays on Vero cells as previously described (29), and expressed in plaque-forming units per milliliter (PFU/mL). Virus tested negative for *Mycoplasma* contamination using MycoSensor PCR Assay Kit (Agilent).

Experimental mosquito infections

All ZIKV infections were performed under ACL3 conditions at the University of Georgia, Athens, GA, USA. Two days prior to infectious feeding, we separated 1 to 3-day-old female mosquitoes and sorted them into eight 64 oz paper cups with 200 mosquitoes per cup. After separation, females were provided water and transported to the ACL3 facility. On the day of infection, we prepared infectious and control blood-meals by washing human blood three times in RPMI medium. We then mixed 50% red blood

cells with 33% DMEM, 20% FBS, 1% (wt/vol) sucrose, and ATP to a final concentration of 5 mmol/L. The blood-mixture was then mixed with virus at a 1:1 ratio. We fed three to five day-old mosquitoes on a water-jacketed membrane feeder containing uninfected (n = 80) or infectious blood-meals (n = 400 per treatment) with a final concentration of 10^3 (M = $4*10^3$, SD = $2.12*10^3$), 10^4 (M = $2.9*10^4$, SD = $2.65*10^3$), 10^5 (M = $2.27*10^5$, SD = $1.34*10^5$) or 10^6 (M = $2.13*10^6$, SD = $1.45*10^6$) PFU/mL for 30 min. We then randomly distributed 200 engorged mosquitoes from each dose treatment into 16 oz paper cups (n = 40 per cup) for destructive sampling every 4 days post-infection (dpi). An additional 40 mosquitoes engorged with uninfected blood were placed in a 16 oz paper cup to track mortality. At each sampling time point, 20 mosquitoes from each dose treatment group were removed from one cup for forced salivations (n = 100 total per dose treatment group, n = 400 per experiment). Every two days, we recorded the number of dead and alive mosquitoes across all the cups. The mosquitoes were housed at 27°C ± 0.5°C, 70% ± 5% relative humidity, and a 12:12 hours light:dark photoperiod with *ad libitum* access to 10% sucrose solution and water for up to 20 days. Three full biological replicates of this experiment were performed (n = 1200 total; Fig 3.1).

Quantifying mosquito infection via forced salivations

To determine the proportion of mosquitoes that were infected with ZIKV, had disseminated infections, and were infectious, we processed 20 mosquitoes per treatment group on days 4, 8, 12, 16 and 20 post-infection. Mosquitoes were cold anesthetized and kept on ice until their legs and wings were removed. After immobilization, we transferred the mosquitoes to a hot plate (35°C) and placed the proboscis of each mosquito into a pipet tip containing 35 µL FBS with 3 mmol/L ATP and red food dye, after which they

were allowed to salivate for 45 min. After salivation, mosquitoes were decapitated, and bodies, heads, and saliva were individually placed into tubes containing 600 μL of DMEM with 1x antibiotic/antimycotic. Bodies and heads were homogenized in a QIAGEN TissueLyzer at 30 cycles/second for 30 seconds, and centrifuged at 17,000xg for 5 minutes at 4°C. Samples were then assessed for the presence/absence of virus with plaque assays.

RT-qPCR analysis

To compare plaque assays with quantitative reverse transcription PCR (RT-qPCR), we performed RT-qPCR on saliva samples from mosquitoes exposed to 10^5 and 10^6 PFU/mL at days 4 and 20 post-infection. Viral RNA was extracted from saliva samples (QIAamp® Viral RNA Mini Kit, Qiagen) and reverse-transcribed to cDNA (High Capacity RNA-to-cDNA Kit, Applied Biosystems). ZIKV genome copies were measured with RT-qPCR reaction assay using TaqMan® Gene Expression Master Mix (Applied Biosystems), primers (F: ZIKV 1086, R: ZIKV 1162c; Invitrogen Custom Primers) and probes (ZIKV 1107-FAM; TaqMan MGB Probe) (30). Each sample was analyzed in duplicate, and each assay contained a standard curve (ZIKV molecular clone), no template, and no primer controls. We extrapolated ZIKV copy numbers from the generated standard curve using the Applied Biosystems protocol. The limit of detection was experimentally established to be 30 copies (10^{-16} g). Final copy numbers were adjusted by back-calculations to the total RNA and cDNA volume and expressed as copies per saliva sample.

Statistical analysis

From these data, we ran two general sets of analyses. The first set of analyses explored the effects of dose, day post-infection (dpi), and their interaction on the numbers of mosquitoes infected, with disseminated infections, and infectious out of the total number of mosquitoes exposed. These analyses were performed to estimate the effects of variation in viral dose on vector competence and the extrinsic incubation period (the rate of change in vector competence), both crucial parameters in estimating dose effects on transmission potential (R_0) and the force of infection. The second set of analyses investigated the effects of dose, dpi, and their interaction on the numbers of mosquitoes with disseminated infection and that are infectious out of total number of mosquitoes successfully infected, as well as on overall viral burden. These analyses are important for exploring the effects of variation in viral dose on different aspects of the virus infection in the mosquito (e.g. virus escape from the midgut and salivary gland tissue barriers) and for inferring how dose affects the mosquito-virus interaction.

We used mixed effects generalized linear models (IBM® SPSS® Statistics 1.0.0.407) to estimate the effects of ZIKV dose, dpi, and the interaction (fixed factors) on the number of mosquitoes, out of total mosquitoes exposed, that are infected (positive bodies: negative binomial distribution, log link function), have disseminated infection (positive heads: normal distribution, identify link function), and are infectious (positive saliva: normal distribution, identity link function). Similar models were also constructed to assess dose and dpi effects on dissemination efficiency (of those infected, the number of mosquitoes with disseminated infections; negative binomial, log link function), transmission efficiency (of those infected, the number of mosquitoes with positive saliva:

negative binomial distribution, log link function), and viral burdens in the saliva (normal distribution, log link function). Finally, we used a Cox mixed effects model (R version 3.3.3, package 'coxme' (31)) to estimate the effects of ZIKV infection (exposed / unexposed), dose, and their interaction on the daily probability of mosquito survival. Experimental replicate was included in all models as a random effect. Model fit and distributions were determined based on Akaike Information Criterion (AIC), the dispersion parameter, and by plotting residuals. Sequential Bonferroni tests were used to assess the significance of pairwise comparisons when relevant, and *p*-values greater than 0.05 were considered non-significant.

Mechanistic R_0 model

To estimate the effects of dose on transmission risk, we used two different approaches. First, we calculated relative R_0 as a function of dose (x) since the absolute magnitude of R_0 depends on other factors not considered here. We modified a function of R_0 used in previous work (20) (Equation 1):

$$R_0(x) = \sqrt{\frac{a^2\, bc(x)\, exp(-\mu(x)/EIR(x))\, EFD\, p_{EA}\, MDR}{N\, r\, \mu(x)^3}} \qquad (1)$$

with parameters for the daily biting rate (a), vector competence (bc), the daily adult mosquito mortality rate (μ), the extrinsic incubation rate (EIR), the probability of egg to adult survival (p_{EA}), the mosquito development rate (MDR), the density of humans (N), and the human recovery rate (r). For parameters we did not directly estimate (a, p_{EA}, MDR), we used estimates generated by Mordecai et al. (20) for *Ae. aegypti* at 27°C and assumed the human recovery rate to be the inverse of the average number of days ZIKV is detectable in the blood (6 days (32)). We used the experimental infection data to

estimate dose-dependent vector competence (*bc*), the *EIR*, and the daily mortality rate (μ) as follows. We fit logistic growth models to the proportion of infectious mosquitoes (*Y*) versus dpi (*t*) for each viral dose (*x*) (Equation 2),

$$Y(t) = \frac{bc(x)}{1+e^{-k(t-EIP(x))}} \quad (2)$$

using the "nls" package in R (33). Vector competence was defined as the maximum proportion of infectious mosquitoes out of total exposed achieved per dose (the asymptote, *bc*), *EIR* was estimated as the inverse of the extrinsic incubation period (*EIP*, the inflection point or the time it takes the mosquito population to achieve 50% of maximum vector competence), and *k* reflects the instantaneous rate of increase (slope at the inflection point). Then, to estimate the daily probability of mosquito mortality (μ) we fit a variety of non-linear curves (exponential, log-linear, Weibull, and Gompertz) to the daily survival probabilities of mosquitoes exposed to different doses with the "flexsurv" package in R (34). We used AIC to determine the best performing model and calculated the area under the curve to estimate the average lifespan (*lf*) of mosquitoes exposed to varying doses. The average daily probability of mortality was then estimated as the inverse of the dose-specific lifespan (1/*lf*).

Second, we performed an alternative calculation of transmission risk following previously described methods (35) to account for the substantial variation in infection outcomes observed across mosquitoes exposed to a given dose. Briefly, we multiplied the best fitting non-linear functions describing the daily relationship between survival and the proportion of infectious mosquitoes for each dose treatment, resulting in the number of infectious days/dose. We then estimated the area under the curve of the resulting function and multiplied by the daily biting rate (*a*) (20) to calculate the number of infectious

mosquito bites generated for each dose treatment for a mosquito population of a given size (n=100).

Results

The effect of viral dose on vector competence and EIR

To investigate how variation in ZIKV dose affects vector competence, transmission efficiency, and the extrinsic incubation period in *Ae. aegypti*, we orally infected mosquitoes with four different viral concentrations (10^3, 10^4, 10^5 and 10^6 PFU/mL) reflecting viremia in ZIKV-infected humans. We found that the mean proportion of infected mosquitoes, mosquitoes with disseminated infections, and infectious mosquitoes significantly increased with increasing viral dose (Table 3.1, Fig 3.2). The infectious dose required to infect 50% of the mosquito population (ID_{50}) was $10^{4.98}$ PFU/mL. We also observed a significant effect of days after infection on the probability that mosquitoes had disseminated infection or became infectious, but not on the probability that they became infected (Table 3.1). At the highest doses (10^5 and 10^6 PFU/mL), the virus was detectable in mosquito bodies at all tested time points (Fig 3.3A). On average, more than 4 days were required for ZIKV to disseminate into the head (Fig 3.3B) and more than 8 days to be present in the saliva (Fig 3.3C). Finally, the significant interaction between ZIKV dose and days post-infection indicates that increases in viral concentration significantly increased the rate at which mosquitoes disseminate infection and become infectious (Table 3.1, Fig 3.3). These results suggest that mosquitoes feeding on human hosts with varying levels of circulating virus could experience both different probabilities of infection and overall infection dynamics.

The effect of viral dose on ZIKV transmission efficiency

We measured the effect of viral dose on transmission efficiency; specifically, the proportion of infected mosquitoes that have disseminated infection (dissemination efficiency) and that became infectious (transmission efficiency). Despite the significant effects of dose and days post-infection on the number of mosquitoes that disseminate infection and that are infectious out of the total number of mosquitoes exposed, these main effects did not affect measures of dissemination and transmission efficiency. However, we did identify a significant interaction between ZIKV dose and days post-infection for both response variables (Fig 3.4, Table 3.1). This interaction demonstrates that variation in viral dose significantly affected the rate at which virus escapes the midgut and salivary gland barriers, with increases in viral dose resulting in more rapid dissemination and overall infectiousness.

The effect of ZIKV infection on mosquito survival

To determine if ZIKV infection and viral dose altered the probability of survival in *Ae. aegypti* mosquitoes, we included uninfected blood-fed controls in the study. We did not find any significant differences in the probability of survival between uninfected and ZIKV infected mosquitoes. Further, we observed no effects of increasing viral dose on mosquito survival among the infected mosquitoes (Table 3.2). On average, *Ae. aegypti* fed on viral doses of 10^3, 10^4, 10^5, and 10^6 PFU/mL experienced an average lifespan (*lf*) of 27, 24, 30, and 29 days, respectively.

Comparison between plaque assays and RT-qPCR

Most studies utilize qPCR to assess mosquito infection status. This method not only detects infectious particles, but also detects the viral genomic RNA in the infected cells, producing high RNA values that do not reflect the levels of infectious particles in the sample. When comparing the performance of plaque assays and RT-qPCR to assess infection status, we included the two highest doses (10^5 and 10^6 PFU/mL) because we had few to no positive saliva samples from the 10^3 and 10^4 treatment groups. Overall, the two methods gave similar numbers of positive samples (Table 3.3); however, we can detect the presence of ZIKV genome in mosquito saliva using RT-qPCR methods as early as 4 dpi, which was never the case with plaque assays. In fact, infectious particles were rarely detected at 8 dpi with plaque assays. The number of infectious particles ranged from 3 (the limit of detection) to 120 PFU per sample and RNA molecules ranged from 10^4 to 10^7 gRNA copies (Fig 3.5A and 3.5B). Both detection methods show that viral concentration does not have a significant effect on ZIKV levels in the saliva. However, we do see a significant effect of days after infection on viral gRNA copies detected by RT-qPCR (Table 3.4).

The effect of viral dose on overall transmission

The maximum proportion of the mosquito population that became infectious (vector competence; *bc*) increased with viral dose (Fig 3.6A). In contrast, the estimated *EIR* did not differ substantially among mosquitoes fed different viral doses (Fig 3.6B), further suggesting that variation in infection dynamics with viral dose is driven primarily by positive dose effects on viral infection and escape from the midgut. This in turn resulted in increases in the relative transmission risk (R_0) of mosquito populations feeding

on hosts of increasing viremias (Fig 3.6C) and the relative number of infectious bites a human population would experience from a mosquito population of a given size (Fig 3.7).

Discussion

Mosquito vectors are often exposed to hosts that individually vary in pathogen loads, which can result in variation in the proportion of the mosquito population that becomes infectious (15, 36). To date, only a few studies have explored how viral concentration impacts measures of vector competence for ZIKV (16-18), and no studies have explicitly linked this source of variation to metrics of transmission risk. In this study, we demonstrate that *Ae. aegypti* populations exposed to increasing ZIKV concentrations exhibit increases in vector competence and EIR, which in turn results in substantial increases in relative transmission risk, measured as either R_0 or the force of infection.

Consistent with previous studies (16-18), we show that increasing the blood-meal concentration of ZIKV increases the probability mosquitoes will become infected, disseminate infection, and become infectious. Vector competence of a mosquito is strongly affected by the ability of a particular arbovirus to infect and escape the midgut and salivary gland barriers (37). As in other studies, we demonstrate that increases in viral concentration facilitates ZIKV infection and midgut barrier escape (38). A dose of at least 10^4 PFU/mL was required for dissemination, and higher concentrations resulted in a higher proportion of mosquitoes with disseminated infections at earlier time points. Further, we show that increases in viral concentration increase the EIR of ZIKV, consistent with other studies (18, 39), in part due to the positive effects of dose on the

rate at which virus escapes the midgut and salivary gland tissue barriers. Finally, due to the lack of a main effect of dose on dissemination and transmission efficiency, we show that the effects of variation in viral dose on vector competence and *EIR* are largely driven by a carry-over effect of dose on initial midgut infection. This is not surprising considering the probability of dissemination and becoming infectious is first dependent on successful midgut infection, as well as subsequent midgut escape and salivary gland invasion, respectively.

Compared to previous studies of ZIKV infection in *Ae. aegypti*, we found higher infection rates and a lower infectious dose 50 (ID_{50}). Our estimated ID_{50} ($10^{4.98}$ PFU/mL) is much lower than previously reported ID_{50} $10^{7.4}$ PFU/mL (18). There is a substantial evidence for ZIKV, dengue, and chikungunya that vector competence can vary across mosquito populations due to genotype-by-genotype (G x G) interactions (17, 18, 39-41). Our higher infection rates could be due to the fact that we paired a Mexican ZIKV isolate with an *Ae. aegypti* population collected from the same region. Considering most ZIKV infected patients exhibit low viremia relative to other arboviruses, the mosquito-virus pairing may also explain why our infectious dose is more consistent with real-world viremias than previous estimates (17, 18, 42).

Mosquito longevity, along with EIR, are the strongest drivers of R_0. Together these two parameters determine the duration of time a mosquito is alive and infectious. We found no effect of ZIKV infection or viral concentration on mosquito survival. Although mosquito mortality was not checked daily, and the ability to detect small effects is limited, it is generally assumed that mosquitoes are fairly tolerant of viral pathogens, allowing the virus to persist in the host without incurring fitness costs (43). However,

most studies, including ours, have been performed in laboratory settings under relatively optimal conditions. Thus, if the costs of infection on mosquito survival and reproduction reflect underlying physiological trade-offs, fitness effects may only manifest in studies that incorporate relevant environmental stressors (e.g. variation in environmental temperature, food availability, competition, etc.) (44, 45). Finally, we assume that biting rates are equivalent between ZIKV-exposed and unexposed mosquitoes and with increasing ZIKV dose. However, there is evidence that exposure to malaria and dengue can alter mosquito feeding behavior and biting rates hosts experience (46). This is potentially an important avenue for future research, especially if ZIKV infection reduces or increases mosquito biting rates at specific points during the infection process and if dose modifies these relationships.

To understand how variation in viral dose affects potential transmission risk, we used our infection and mortality data to parameterize a relative R_0 model and determine the force of infection. Both R_0 and the force of infection are important measures of disease spread, representing the number of secondary cases in a susceptible population and the rate at which susceptible individuals acquire infectious disease, respectively. In our study, we show that mosquito populations feeding on increasing viral doses contribute more infectious bites and produce more secondary ZIKV cases due to increased vector competence and the rate at which virus escaped the midgut. For example, increasing viremia from 10^4 to 10^6 PFU/mL increased relative R_0 3.8-fold and the number of infectious bites 18-fold. Although populations of mosquitoes in the field are exposed to multiple doses, this was an important first step for understanding the implications of dose-dependent transmission. Knowing transmission risk will vary with

heterogeneity in host viremia, future studies should focus on characterizing the distribution of viremia in the host population and incorporating individual variation in infectiousness into mechanistic models of disease spread. Model predictions from some pathogen systems (e.g. SARS, measles, and smallpox) that account for individual variation in infectiousness differ greatly from predictions generated by average-based approaches (47).

We used plaque assays to determine infection status instead of RT-qPCR, a common technique used in other studies due to its rapidity and sensitivity (48). However, because this method will detect all viral RNA in infected tissues, it can overestimate the actual number of infectious particles present. While we found the results of RT-qPCR and plaque assays to be highly correlated, the number of genomes detected by RT-qPCR was much higher than the number of plaque-forming units. We detected ZIKV genome in mosquito saliva (4 dpi) well before our first ZIKV infectious saliva sample was detected by plaque assay (8 dpi). Other studies using RT-qPCR methods have reported ZIKV in mosquito saliva as early as 3 dpi (16, 49). Since virus can be transmitted only in the form of infectious particles, the use of RT-qPCR to determine transmission relevant phenotypes could lead to overestimates of transmission risk.

In general, ZIKV viremia does not differ between symptomatic and asymptomatic patients (14) and is on average lower than seen with other arbovirus systems (39, 50). Contrary to our study, in the dengue and malaria systems, asymptomatic and pre-symptomatic patients with lower pathogen loads can be more infectious to host-seeking mosquitoes than symptomatic hosts with high pathogen loads (15, 51, 52). This could be due to host factors that are absent in our study and related studies (16, 18, 39). Variation

in host blood quality (e.g. hematocrit) and mosquito attraction, or circulating host factors (e.g. differences in immune factors), could result in reduced infectivity of mosquitoes feeding on hosts with high pathogen burdens (15, 53). Even the current, most frequently used ZIKV mouse models use mice lacking a large component of the immune system and likely do not represent transmission in the field (17, 54). Thus, our study and others should be confirmed with mosquito feeding trials on human hosts of varying viremias.

In conclusion, we demonstrate that ingesting higher doses of ZIKV increases the proportion and the rate at which mosquito populations become infectious. This, in turn, results in an increase in the relative transmission risk and the force of infection experienced by susceptible human populations. Therefore, variation in viremia, as well as the frequency distribution of hosts of different viremias, should be accounted for when estimating R_0 and in assessing the efficacy of arbovirus prevention strategies.

Acknowledgements

We thank the University of Texas Medical Branch Arbovirus Reference Collection for providing the virus. We also thank the members of the Murdock and Brindley labs for thoughtful comments on the project and manuscript.

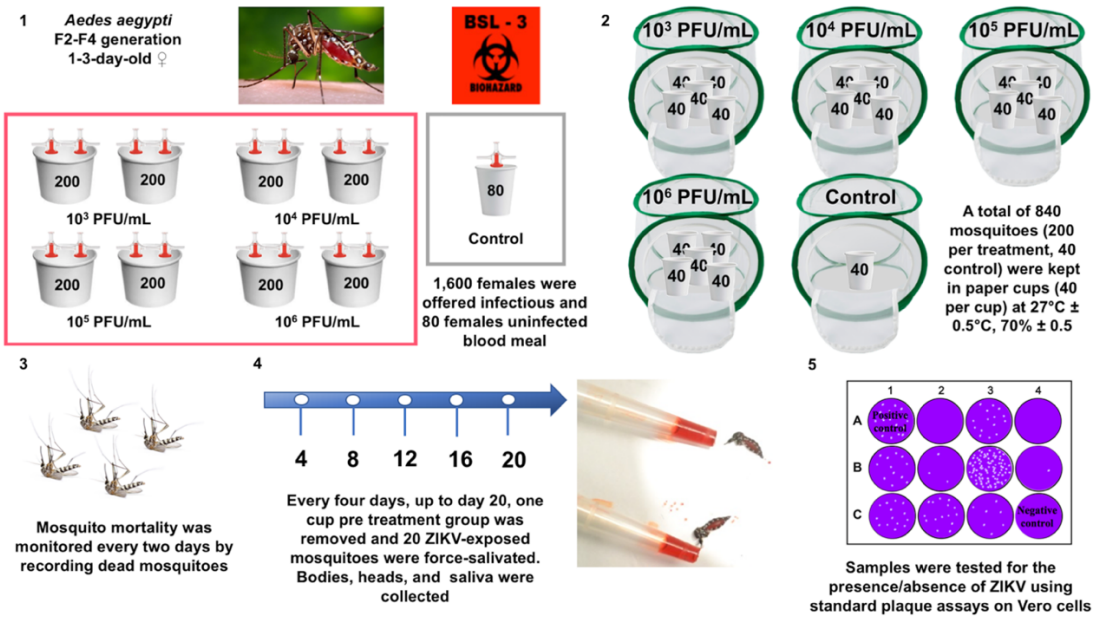


Fig 3.1. Experimental design. In each biological replicate, a total of 1,600 female *Aedes aegypti* mosquitoes were offered an infectious blood meal containing ZIKV at the final concentrations of 10^3 PFU/mL, 10^4 PFU/mL, 10^5 PFU/mL or 10^6 PFU/mL (400 females per treatment). Eighty females were offered uninfected, control blood meal. Two hundred ZIKV-exposed engorged mosquitoes for each treatment (800 total) and 40 engorged control mosquitoes were randomly distributed into mesh-covered paper cups (40 per cup) and housed at 27°C ± 0.5°C for 20 days. Mosquito mortality was checked every two-days. Every four days, twenty ZIKV-exposed mosquitoes per treatment group were force-salivated. After salivation, mosquito saliva, heads, and bodies were collected into separate tubes. Each tissue was tested for the presence/absence of the ZIKV using plaque assays on Vero cells. Three full biological replicates were performed.

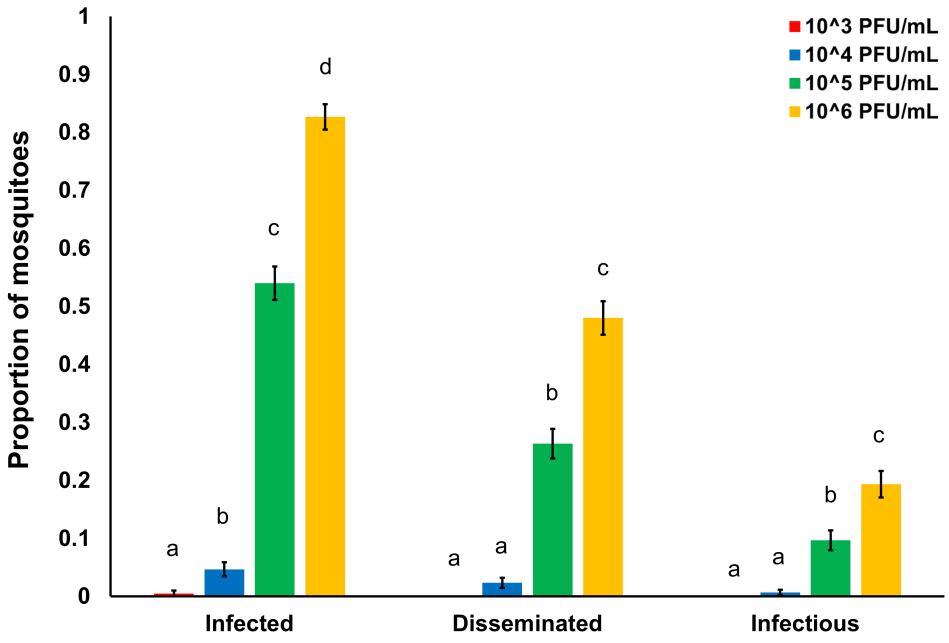


Fig 3.2. ZIKV dose and the proportion of mosquitoes infected, with disseminated infections, and infectious. Relationship between the ZIKV dose (10^3, 10^4, 10^5, and 10^6 PFU/mL) and the proportion of mosquitoes infected (ZIKV positive bodies compared to total number exposed), with disseminated infections (ZIKV positive heads compared to total number exposed), and infectious (ZIKV positive saliva compared to total number exposed). For each category, results with no common letters were significantly different ($p \leq 0.05$) and whiskers on each bar represent the standard error of the mean.

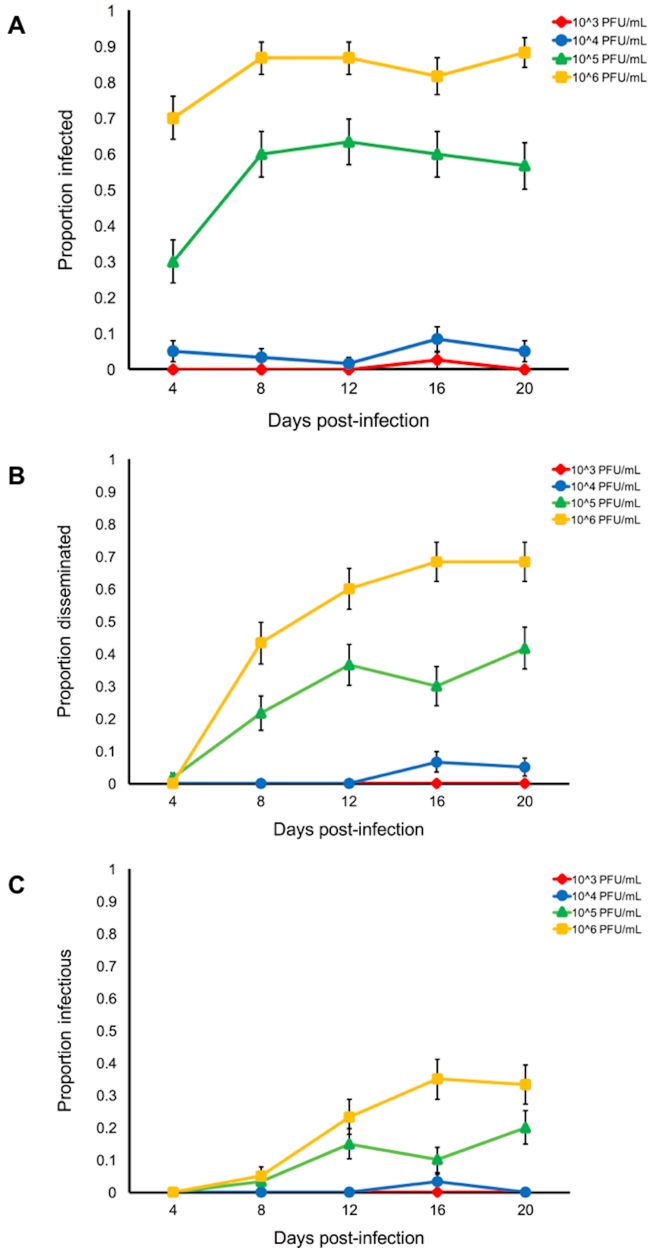


Fig 3.3. Days post-infection and the proportion of mosquitoes infected, with disseminated infections, and infectious. The relationship between days post-infection (4, 8, 12, 16, 20) and the proportion of mosquitoes infected (A), with disseminated infections (B), and infectious (C) after exposure to four different viral doses (10^3, 10^4, 10^5, and 10^6 PFU/mL). Whiskers on each bar represent the standard error of the mean.

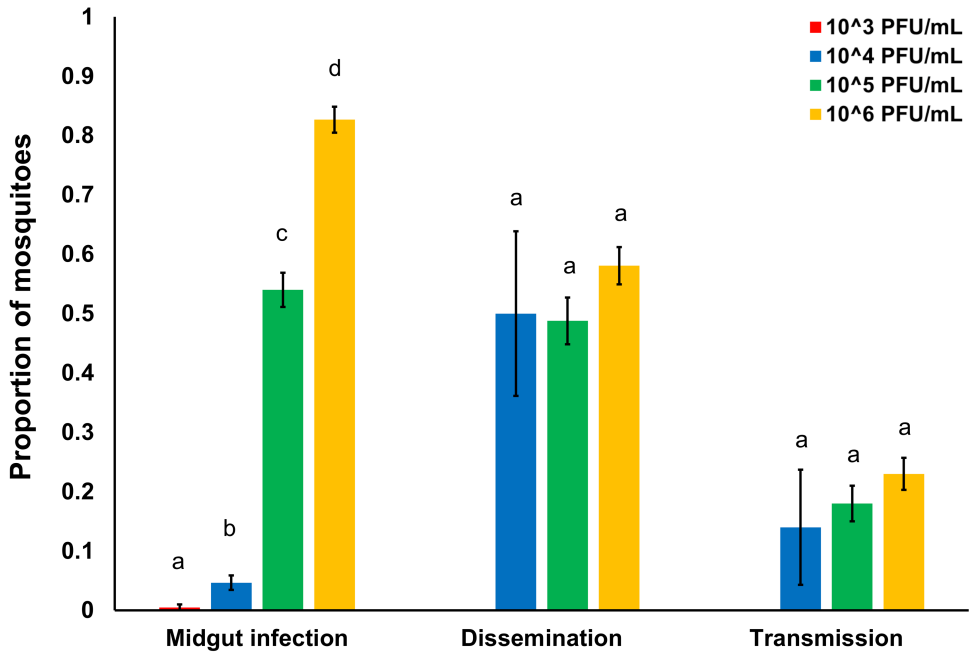


Fig 3.4. ZIKV dose and the efficiency of midgut infection, dissemination, and transmission. Relationship between the ZIKV dose (10^3, 10^4, 10^5, and 10^6 PFU/mL) and the efficiency of midgut infection (ZIKV positive bodies divided by total number exposed), of dissemination (ZIKV positive heads divided by positive bodies), and transmission (ZIKV positive saliva divided by positive bodies). For each category, results with no common letters were significantly different ($p \leq 0.05$) and whiskers on each bar represent the standard error of the mean.

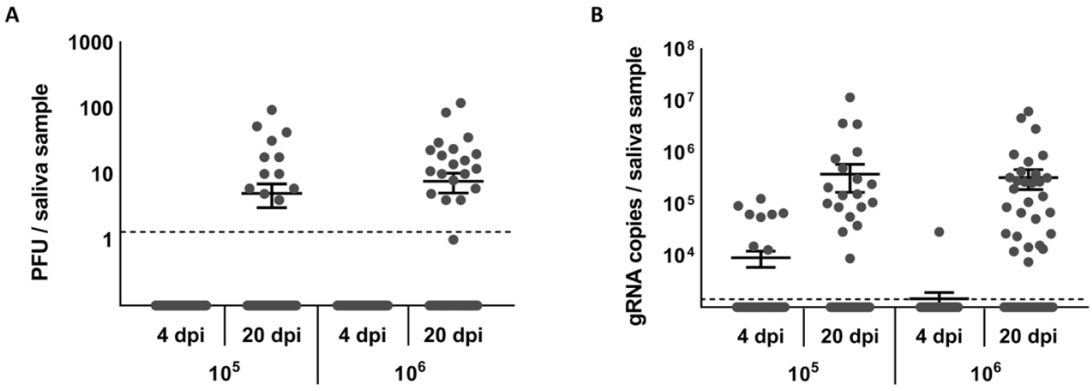


Fig 3.5. Viral loads in saliva determined by plaque assays and RT-qPCR. Viral load of ZIKV in saliva at 4 and 20 days post-infection (dpi) with 10^5 and 10^6 PFU/mL determined by standard plaque assays on Vero cells (A) and ZIKV-specific RT-qPCR (B). The limit of detection was experimentally established to be 3 plaque-forming units (PFU) for plaque assays and 30 gRNA copies for RT-qPCR.

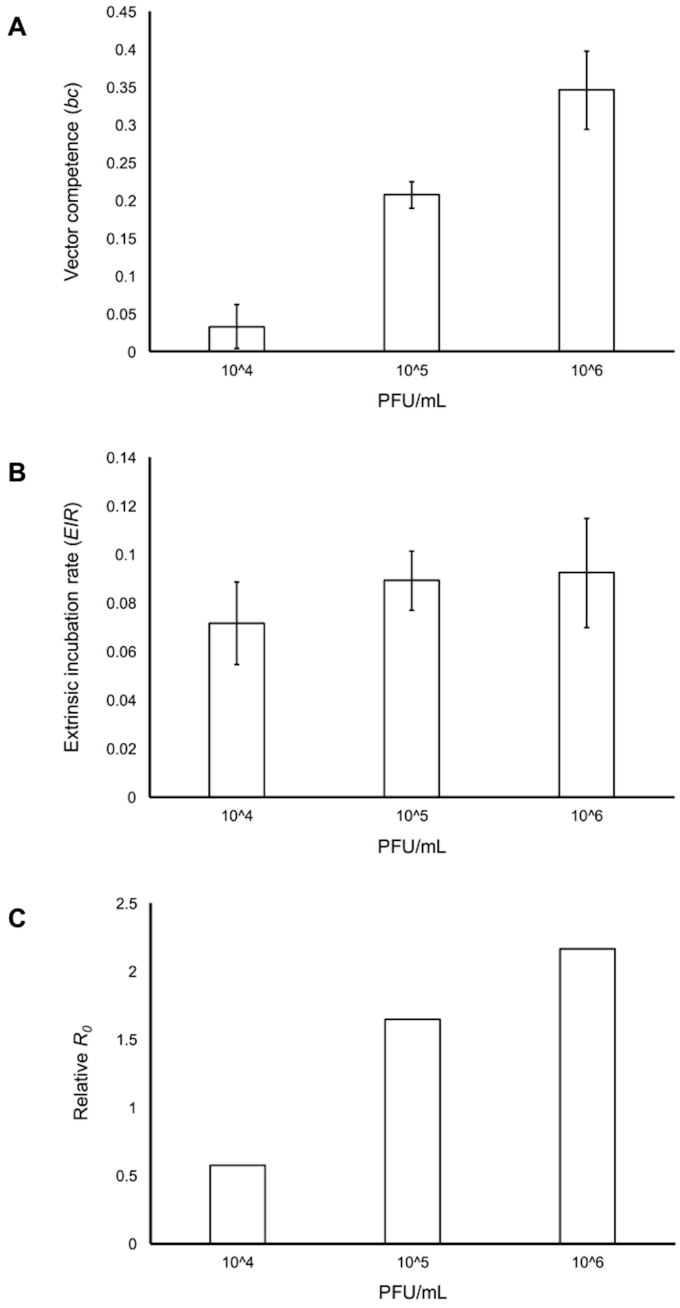


Fig 3.6. Viral dose and estimated vector competence, extrinsic incubation rate, and relative basic reproductive number R_0. Relationship between viral dose (10^3, 10^4, 10^5, and 10^6 PFU/mL) and estimated vector competence (A), the extrinsic incubation rate (B), and relative basic reproductive number R_0 (C).

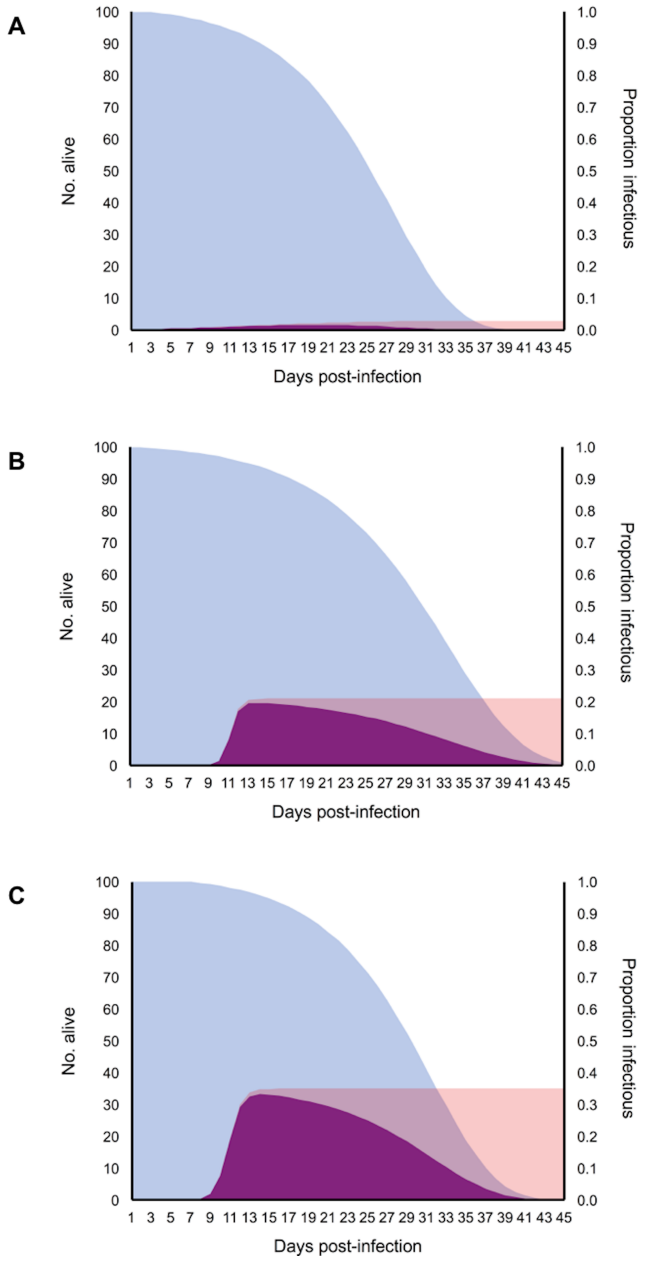


Fig 3.7. Daily proportion of mosquitoes alive, infectious, and both alive and infectious for mosquitoes exposed to different doses. Relationship between the daily proportion of mosquitoes alive (blue distributions), infectious (pink distributions), and those that are both alive and infectious (purple distributions) for mosquito populations exposed to 10^4 PFU/mL (A), 10^5 PFU/mL (B) and 10^6 PFU/mL (C).

Table 3.1. The effect of dose, day, and potential interaction on mosquito infection, dissemination, infectiousness, and dissemination and transmission efficiencies.

response variables	dose			day			dose x day		
	F	d.f.	*p*-value	F	d.f.	*p*-value	F	d.f.	*p*-value
probability of infection	33.898	3	**<0.0001**	0.004	4	1	0.501	12	0.9
probability of dissemination	52.61	3	**<0.0001**	11.929	4	**<0.0001**	4.295	12	**<0.0001**
probability of infectiousness	22.86	3	**<0.0001**	7.82	4	**<0.0001**	3.45	12	**0.002**
dissemination efficiency	0.852	3	0.466	1.102	4	0.355	2.328	8	**0.019**
transmission efficiency	0.011	3	0.998	0.027	4	0.999	3.862	8	**<0.0001**

Results from generalized linear mixed effects models examining the effects of dose, day, and the interaction on the numbers of mosquitoes infected (ZIKV positive bodies divided by total number exposed), with disseminated infections (ZIKV positive heads divided by total number exposed), infectiousness (ZIKV positive saliva divided by total number exposed), and measures of dissemination (ZIKV positive heads divided by positive bodies) and transmission (ZIKV positive saliva divided by positive bodies) efficiency.

Table 3.2. The effects of ZIKV dose on the daily probability of mosquito survival.

dose	z	*p*-value
uninfected	-1.44	0.15
10^3	0.64	0.52
10^4	1.05	0.29
10^5	-0.03	0.97
10^6	-0.55	0.58

Results from Cox mixed-effects model examining the effects of ZIKV dose (10^3, 10^4, 10^5, and 10^6 PFU/mL) on the daily probability of mosquito survival.

Table 3.3. Numbers of positive saliva samples determined by RT-qPCR and plaque assays.

replicate	dose (PFU/mL)*	day	gRNA	PFU
1	10^5	4	3	0
		20	7	4
	10^6	4	1	0
		20	12	9
2	10^5	4	2	0
		20	5	5
	10^6	4	0	0
		20	10	7
3	10^5	4	3	0
		20	7	3
	10^6	4	0	0
		20	7	4

Numbers of positive saliva samples determined by RT-qPCR (gRNA) and plaque assays (PFU) for 10^5 and 10^6 viral doses on days 4 and 20 post-infection for each experimental replicate.

Table 3.4. The effects of dose and day on the number of ZIKV gRNA copies and plaque-forming units.

	gRNA copies			plaque-forming units		
factors	F	d.f.	*p*-value	F	d.f.	*p*-value
dose	0.873	1	0.354	0.136	1	0.715
day	5.688	1	**0.021**	-	-	-

Results from generalized linear mixed effects models examining the effects of dose and day on the number of ZIKV gRNA copies vs. plaque-forming units

References

1. Dick GWA, Kitchen SF, Haddow AJ. Zika Virus (I). Isolations and serological specificity. Trans R Soc Trop Med Hyg. 1952;46(5):509-20.

2. Mlakar J, Korva M, Tul N, Popovic M, Poljsak-Prijatelj M, Mraz J, et al. Zika virus associated with microcephaly. N Engl J Med. 2016;374(10):951-8.

3. Smithburn KC. Neutralizing antibodies against certain recently isolated viruses in the sera of human beings residing in East Africa. J Immunol. 1952;69(2):223-34.

4. MacNamara FN. Zika virus : A report on three cases of human infection during an epidemic of jaundice in Nigeria. Trans R Soc Trop Med Hyg. 1954;48(2):139-45.

5. Duffy MR, Chen T-H, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med. 2009;360(24):2536-43.

6. Cao-Lormeau V-M, Blake A, Mons S, Lastère S, Roche C, Vanhomwegen J, et al. Guillain-Barre Syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. Lancet. 2016;387(10027):1531-9.

7. Zanluca C, Melo VC, Mosimann AL, Santos GI, Santos CN, Luz K. First report of autochthonous transmission of Zika virus in Brazil. Mem Inst Oswaldo Cruz. 2015;110(4):569-72.

8. Centers for Disease Control and Prevention. World map of areas with risk of Zika 2017 [cited 2017 July 19]. Available from: https://wwwnc.cdc.gov/travel/page/world-map-areas-with-zika.

9. World Health Organization. WHO statement on the first meeting of the International Health Regulations (2005) (IHR 2005) Emergency Committee on Zika virus

and observed increase in neurological disorders and neonatal malformations 2016 [Available from: http://www.who.int/mediacentre/news/statements/2016/1st-emergency-committee-zika/en/.

10. Musso D, Roche C, Robin E, Nhan T, Teissier A, Cao-Lormeau VM. Potential sexual transmission of Zika virus. Emerg Infect Dis. 2015;21(2):359-61.

11. Musso D, Nhan T, Robin E, Roche C, Bierlaire D, Zisou K, et al. Potential for Zika virus transmission through blood transfusion demonstrated during an outbreak in French Polynesia, November 2013 to February 2014. Euro Surveill. 2014;19(14).

12. Wong PS, Li MZ, Chong CS, Ng LC, Tan CH. Aedes (Stegomyia) albopictus (Skuse): a potential vector of Zika virus in Singapore. PLoS Negl Trop Dis. 2013;7(8):e2348.

13. Shragai T, Tesla B, Murdock C, Harrington LC. Zika and chikungunya: mosquito-borne viruses in a changing world. Ann N Y Acad Sci. 2017;1399(1):61-77.

14. Musso D, Rouault E, Teissier A, Lanteri MC, Zisou K, Broult J, et al. Molecular detection of Zika virus in blood and RNA load determination during the French Polynesian outbreak. J Med Virol. 2017;89(9):1505-10.

15. Duong V, Lambrechts L, Paul RE, Ly S, Lay RS, Long KC, et al. Asymptomatic humans transmit dengue virus to mosquitoes. Proc Natl Acad Sci U S A. 2015;112(47):14688-93.

16. Pompon J, Morales-Vargas R, Manuel M, Huat Tan C, Vial T, Hao Tan J, et al. A Zika virus from America is more efficiently transmitted than an Asian virus by Aedes aegypti mosquitoes from Asia. Sci Rep. 2017;7(1):1215.

17. Roundy CM, Azar SR, Rossi SL, Huang JH, Leal G, Yun R, et al. Variation in Aedes aegypti mosquito competence for Zika virus transmission. Emerg Infect Dis. 2017;23(4):625-32.

18. Ciota AT, Bialosuknia SM, Zink SD, Brecher M, Ehrbar DJ, Morrissette MN, et al. Effects of Zika virus strain and Aedes mosquito species on vector competence. Emerg Infect Dis. 2017;23(7):1110-7.

19. Liu-Helmersson J, Stenlund H, Wilder-Smith A, Rocklov J. Vectorial capacity of Aedes aegypti: effects of temperature and implications for global dengue epidemic potential. PLoS One. 2014;9(3):e89783.

20. Mordecai EA, Cohen JM, Evans MV, Gudapati P, Johnson LR, Lippi CA, et al. Detecting the impact of temperature on transmission of Zika, dengue, and chikungunya using mechanistic models. PLoS Negl Trop Dis. 2017;11(4):e0005568.

21. Ryan SJ, McNally A, Johnson LR, Mordecai EA, Ben-Horin T, Paaijmans K, et al. Mapping Physiological Suitability Limits for Malaria in Africa Under Climate Change. Vector Borne Zoonotic Dis. 2015;15(12):718-25.

22. Garrett-Jones C, Grab B. The assessment of insecticidal impact on the malaria mosquito's vectorial capacity, from data on the proportion of parous females. Bull World Health Organ. 1964;31:71-86.

23. Rivero A, Vézilier J, Weill M, Read AF, Gandon S. Insecticide control of vector-borne diseases: when is insecticide resistance a problem? PLoS Pathog. 2010;6(8).

24. Le Menach A, Takala S, McKenzie FE, Perisse A, Harris A, Flahault A, et al. An elaborated feeding cycle model for reductions in vectorial capacity of night-biting mosquitoes by insecticide-treated nets. Malar J. 2007;6:10.

25. Hancock PA, Sinkins SP, Godfray HCJ. Strategies for introducing Wolbachia to reduce transmission of mosquito-borne diseases. PLoS Negl Trop Dis. 2011;5(4).

26. Anderson RM, May RM. Vaccination and herd immunity to infectious diseases. Nature. 1985;318(6044):323-9.

27. Anderson RM, May RM. Immunisation and herd immunity. Lancet. 1990;335(8690):641-5.

28. Anderson RM. The concept of herd immunity and the design of community-based immunization programmes. Vaccine. 1992;10(13):928-35.

29. Goodfellow FT, Tesla B, Simchick G, Zhao Q, Hodge T, Brindley MA, et al. Zika virus induced mortality and microcephaly in chicken embryos. Stem Cells Dev. 2016;25(22):1691-7.

30. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis. 2008;14(8):1232-9.

31. Therneau TM. coxme: Mixed Effects Cox Models. R package version 2.2-5. 2015.

32. Centers for Disease Control and Prevention. Zika symptoms 2017 [Available from: https://www.cdc.gov/zika/symptoms/symptoms.html.

33. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2017.

34. Jackson C. flexsurv: A Platform for Parametric Survival Modeling in R. Journal of Statistical Software. 2016;70(8):1-33.

35. Shapiro LL, Murdock CC, Jacobs GR, Thomas RJ, Thomas MB. Larval food quantity affects the capacity of adult mosquitoes to transmit human malaria. Proc Biol Sci. 2016;283(1834).

36. Da DF, Churcher TS, Yerbanga RS, Yameogo B, Sangare I, Ouedraogo JB, et al. Experimental study of the relationship between Plasmodium gametocyte density and infection success in mosquitoes; implications for the evaluation of malaria transmission-reducing interventions. Exp Parasitol. 2015;149:74-83.

37. Franz AW, Kantor AM, Passarelli AL, Clem RJ. Tissue Barriers to Arbovirus Infection in Mosquitoes. Viruses. 2015;7(7):3741-67.

38. Kramer LD, Hardy JL, Presser SB, Houk EJ. Dissemination barriers for western equine encephalomyelitis virus in Culex tarsalis infected after ingestion of low viral doses. Am J Trop Med Hyg. 1981;30(1):190-7.

39. Chouin-Carneiro T, Vega-Rua A, Vazeille M, Yebakima A, Girod R, Goindin D, et al. Differential Susceptibilities of Aedes aegypti and Aedes albopictus from the Americas to Zika Virus. PLoS Negl Trop Dis. 2016;10(3):e0004543.

40. Lambrechts L, Chevillon C, Albright RG, Thaisomboonsuk B, Richardson JH, Jarman RG, et al. Genetic specificity and potential for local adaptation between dengue viruses and mosquito vectors. BMC Evol Biol. 2009;9:160.

41. Zouache K, Fontaine A, Vega-Rua A, Mousson L, Thiberge JM, Lourenco-De-Oliveira R, et al. Three-way interactions between mosquito population, viral strain and temperature underlying chikungunya virus transmission potential. Proc Biol Sci. 2014;281(1792).

42. Guerbois M, Fernandez-Salas I, Azar SR, Danis-Lozano R, Alpuche-Aranda CM, Leal G, et al. Outbreak of Zika virus infection, Chiapas State, Mexico, 2015, and first confirmed transmission by Aedes aegypti mosquitoes in the Americas. J Infect Dis. 2016;214(9):1349-56.

43. Goic B, Stapleford KA, Frangeul L, Doucet AJ, Gausson V, Blanc H, et al. Virus-derived DNA drives mosquito vector tolerance to arboviral infection. Nat Commun. 2016;7:12410.

44. Christofferson RC, Mores CN. Potential for extrinsic incubation temperature to alter interplay between transmission potential and mortality of dengue-infected Aedes aegypti. Environ Health Insights. 2016;10:119-23.

45. Vaidyanathan R, Fleisher AE, Minnick SL, Simmons KA, Scott TW. Nutritional stress affects mosquito survival and vector competence for West Nile virus. Vector Borne Zoonotic Dis. 2008;8(6):727-32.

46. Murdock CC, Luckhart S, Cator LJ. Immunity, host physiology, and behaviour in infected vectors. Curr Opin Insect Sci. 2017;20:28-33.

47. Lloyd-Smith JO, Schreiber SJ, Kopp PE, Getz WM. Superspreading and the effect of individual variation on disease emergence. Nature. 2005;438(7066):355-9.

48. Mackay IM, Arden KE, Nitsche A. Real-time PCR in virology. Nucleic Acids Res. 2002;30(6):1292-305.

49. Ruckert C, Weger-Lucarelli J, Garcia-Luna SM, Young MC, Byas AD, Murrieta RA, et al. Impact of simultaneous exposure to arboviruses on infection and transmission by Aedes aegypti mosquitoes. Nat Commun. 2017;8:15412.

50. Waggoner JJ, Gresh L, Vargas MJ, Ballesteros G, Tellez Y, Soda KJ, et al. Viremia and clinical presentation in Nicaraguan patients infected with Zika virus, chikungunya virus, and dengue virus. Clin Infect Dis. 2016;63(12):1584-90.

51. Nedelman J. Gametocytemia and infectiousness in falciparum malaria: observations and models. In: Harris KF, editor. Advances in Disease Vector Research. New York, NY: Springer New York; 1990. p. 59-89.

52. Boudin C, Olivier M, Molez JF, Chiron JP, Ambroise-Thomas P. High human malarial infectivity to laboratory-bred Anopheles gambiae in a village in Burkina Faso. Am J Trop Med Hyg. 1993;48(5):700-6.

53. Pakpour N, Akman-Anderson L, Vodovotz Y, Luckhart S. The effects of ingested mammalian blood factors on vector arthropod immunity and physiology. Microbes Infect. 2013;15(3):243-54.

54. Shan C, Xie X, Muruato AE, Rossi SL, Roundy CM, Azar SR, et al. An infectious cDNA clone of Zika virus to study viral virulence, mosquito transmission, and antiviral inhibitors. Cell Host Microbe. 2016;19(6):891-900.

CHAPTER 4

TEMPERATURE DRIVES ZIKA VIRUS TRANSMISSION: EVIDENCE FROM EMPIRICAL AND MATHEMATICAL MODELS [2]

[2] Tesla B., Demakovsky L.R., Mordecai E.A., Ryan S.J., Bonds M.H., Ngonghala C.N., Brindley M.A., Murdock C.C. 2018. *Proc. R. Soc. B* 285: 20180795. Accepted by Proceedings of the Royal Society B. Reprinted here with permission of the publisher.

Abstract

Temperature is a strong driver of vector-borne disease transmission. Yet, for emerging arboviruses we lack fundamental knowledge on the relationship between transmission and temperature. Current models rely on the untested assumption that Zika virus responds similarly to dengue virus, potentially limiting our ability to accurately predict the spread of Zika. We conducted experiments to estimate the thermal performance of Zika virus (ZIKV) in field-derived *Aedes aegypti* across eight constant temperatures. We observed strong, unimodal effects of temperature on vector competence, extrinsic incubation period, and mosquito survival. We used thermal responses of these traits to update an existing temperature-dependent model to infer temperature effects on ZIKV transmission. ZIKV transmission was optimized at 29°C, and had a thermal range of 22.7°C - 34.7°C. Thus, as temperatures move toward the predicted thermal optimum (29°C) due to climate change, urbanization, or seasonality, Zika could expand north and into longer seasons. In contrast, areas that are near the thermal optimum were predicted to experience a decrease in overall environmental suitability. We also demonstrate that the predicted thermal minimum for Zika transmission is 5°C warmer than that of dengue, and current global estimates on the environmental suitability for Zika are greatly over-predicting its possible range.

Introduction

Mosquito-borne viruses are an emerging threat impacting human health and well-being. Epidemics of dengue (DENV), chikungunya (CHIKV), and Zika (ZIKV) have spilled out of Africa to spread explosively throughout the world creating public health crises. Worldwide, an estimated 3.9 billion people living within 120 countries are at risk (1). In 2015-2016, ZIKV spread throughout the Americas including the continental U.S., resulting in over 360,000 suspected cases, with likely many more undetected (2). With the rise of neurological disorders and birth defects, such as Guillain-Barré and congenital Zika virus syndrome (3, 4), ZIKV became widely feared and was declared a "public health emergency of international concern" by the World Health Organization in 2016 (5). In spite of growing research efforts to develop new therapeutics, vaccines, and innovative mosquito control technologies, mitigating arbovirus disease spread still depends on conventional mosquito control methods and public education. Thus, substantial efforts have been made to predict how ZIKV will spread seasonally, geographically, and with the effects of climate change (6-9).

There are several key gaps that potentially affect our ability to predict, and ultimately, mitigate the factors that influence transmission risk and arbovirus emergence globally. First, current models predicting mosquito distributions or virus transmission are often limited by a relatively poor understanding of the relationships among mosquito vectors, pathogens, and the environment. There is substantial evidence that temperature variability is a key driver of disease transmission across diverse vector-borne pathogen systems (8, 10, 11). Mosquitoes are small ectothermic animals and their physiology (12, 13), life history (8, 14), and vectorial capacity (10, 15, 16) exhibit unimodal responses to

changes in temperature. Transmission depends in large part on the ability of mosquitoes to survive the extrinsic incubation period (EIP), become infectious, and bite new hosts, so differential (unimodal) impacts of temperature on survival, vector competence, and EIP have highly nonlinear effects on transmission. Warmer temperatures do not necessarily translate into more infectious mosquitoes (8, 17). Second, current models often ignore the low quality and quantity of existing data. Even in systems that are fairly well-studied (e.g. *Plasmodium falciparum* and DENV), key parameters are often estimated from only a few studies. Finally, current transmission models often assume, with little justification, that the relationship between temperature and EIP is monotonic (18), or that the relationships between temperature, EIP, and vector competence of less-studied arboviruses (e.g. CHIKV and ZIKV) are similar to DENV (8, 9, 19, 20).

To advance our fundamental scientific understanding of the relationship between temperature and ZIKV transmission, we conducted a series of laboratory experiments to estimate the thermal performance of ZIKV (vector competence, the extrinsic incubation rate, and the daily per capita mosquito mortality rate) in field-derived *Ae. aegypti* across eight different constant temperatures ranging from 16 – 38°C. We fit a series of nonlinear functions to estimate the thermal responses of the above traits. These thermal responses were incorporated into a temperature-dependent basic reproductive number (R_0) model developed for *Ae. aegypti* and DENV (8) to infer how temperature variation will impact ZIKV transmission.

Methods

Virus culture

We used the ZIKV isolate MEX1-44 obtained from the University of Texas Medical Branch (UTMB) Arbovirus Reference Collection. The virus was isolated in January 2016 from a field-caught *Ae. aegypti* mosquito from Tapachula, Chiapas, Mexico. For all mosquito infections, we used pass ten stock virus that was passaged four times at the UTMB and an additional six times at the University of Georgia. Four days after inoculation in Vero cells, we harvested the virus, centrifuged it at 2,500xg for 5 min, and stored it at -80°C. The virus tested negative for *Mycoplasma* contamination using MycoSensor PCR Assay Kit (Agilent) and was titrated using standard plaque assays on Vero cells (21). Briefly, we infected the cells with six 10-fold serial dilutions for 1-2 hours. After incubation, we removed the inoculum and replaced it with 1.5% agarose DMEM (UltraPure LMP Agarose, Fisher Scientific). The cells were kept at 37°C, 5% CO_2 for four days when they were fixed with 4% formalin and stained with crystal violet. The titers were expressed in plaque forming units per milliliter (PFU/mL).

Mosquito rearing

Outbred *Ae. aegypti* mosquito colonies were generated from ovitrap collections in Tapachula, Chiapas, Mexico, spring 2016. Mosquito eggs were hatched in ddH_2O under reduced pressure in a vacuum desiccator and dispersed larvae in rearing trays. Each tray contained 200 larvae in 1L ddH_2O and 4 fish food pellets (Hikari Cichlid Gold Large Pellets). Adult mosquitoes were kept in rearing cages and provided with 10% sucrose *ad libitum*. We maintained the colonies on whole human blood (Interstate Blood Bank) and

collected eggs on paper towels. Larvae and adults were maintained under standard, controlled insectary conditions at 27°C ± 0.5°C, 80% ± 10% relative humidity, and a 12:12 light: dark diurnal cycle in a dedicated environmental walk-in room (Percival Scientific).

Experimental mosquito infections and forced salivations

For each biological replicate, we separated 8,000 1 to 3-day-old females (field derived *Ae. aegypti,* F4 generation) and held them for 48 hours prior to ZIKV infection (Fig S1). Mosquitoes were kept in 64 oz. paper cups and provided with water, which was withdrawn 12 hours before feeding. We offered 3 – 5-day old mosquitoes either an infectious blood meal containing ZIKV at a final concentration of 10^6 PFU/mL or an uninfected, control blood meal. The blood meal was prepared by washing human blood three times in RPMI medium and the pelleted red blood cells (50%) were resuspended in 33% DMEM, 20% FBS, 1% sucrose, and 5 mmol/L ATP. For the infectious blood meal, we mixed the blood mixture with ZIKV diluted in DMEM ($2*10^6$ PFU/mL) at a 1:1 ratio. Mosquitoes were blood-fed through a water-jacketed membrane feeder for 30 min, after which we randomly distributed 2,000 ZIKV-exposed engorged mosquitoes and 2,000 unexposed blood-fed control mosquitoes into mesh-covered paper cups (250 mosquitoes per cup). We then placed one ZIKV-exposed and one control cup at each temperature treatment (Percival Scientific): 16°C, 20°C, 24°C, 28°C, 32°C, 34°C, 36°C, and 38°C ± 0.5°C. Chambers were set to 80% ± 5% relative humidity and a 12:12 light: dark cycle, and mosquitoes were maintained on 10% sucrose for the duration of the experiment. Mosquito mortality was monitored and recorded daily.

Every three days (up to day 21) we force-salivated 20 ZIKV-exposed mosquitoes per treatment group by immobilizing mosquitoes on ice, removing their legs and wings, and placing the proboscis of each mosquito into a pipet tip (containing 35 μL FBS with 3 mmol/L ATP) for 30 min on a 35°C warming plate. After salivation, we collected mosquito saliva, heads and legs, and bodies into 700 μL of DMEM with 1x antibiotic/antimycotic. Each tissue was homogenized in a QIAGEN TissueLyzer at 30 cycles/second for 30 seconds, and centrifuged at 17,000xg for 5 minutes at 4°C. To measure the proportion of mosquitoes that became infected, disseminated infection, and became infectious at each temperature, we tested for the presence/absence of ZIKV in mosquito bodies, legs and heads, and saliva, respectively, using plaque assays on Vero cells. Two full biological replicates were performed (Fig 4.1).

Statistical analysis

Generalized linear model (GLMM) analysis was used to estimate the effects of temperature (*T*; 16°C, 20°C, 24°C, 28°C, 32°C, 34°C, 36°C, 38°C) and days post infection (*dpi*; 3, 6, 9, 12, 15, 18, 21) on the probability of successful mosquito infection (proportion of mosquitoes with positive bodies), dissemination (proportion of mosquitoes with positive legs and heads), and becoming infectious (proportion of mosquitoes with positive saliva) after being exposed to a ZIKV infectious blood meal. We also used GLMM analysis to estimate the probability of becoming infectious after successful ZIKV infection (proportion of mosquitoes with positive bodies) as a measure of dissemination efficiency. As our response variables were presence or absence of virus in a particular tissue, we constructed our GLMM using a binomial distribution and logit link function. The covariates temperature and dpi were centered by subtracting the mean and scaled by

dividing by the standard deviation (SD). To account for differences in ZIKV infection metrics due to mosquito cohort, we used a random intercept for mosquito cohort in each analysis. Because a diversity of organismal traits exhibit non-linear, unimodal relationships with temperature (8, 22), and we observe non-monotonic effects of dpi on some of our response variables in specific temperature treatments, we incorporated a polynomial function into our statistical model to accommodate this non-linearity. We evaluated a series of eight candidate models which varied in fixed effects structure from a "base model" with only linear fixed effects of temperature and dpi to a "full" model, which included temperature and dpi polynomial terms that were squared (T^2 and dpi^2) and their interactions (R Core Team, 2018 (23), package lme4 (24)). We selected the most parsimonious model using the Akaike Information Criterion with a sample size correction (AICc). Finally, to estimate the effects of temperature, ZIKV exposure, and their interaction on the daily probability of mosquito survival, we used a Cox proportional hazards model (SAS® Studio, 3.6 Basic Edition) with temperature, infection status (ZIKV-exposed or control), and their interaction as fixed factors, with mosquito batch as a random factor.

Mechanistic R_0 model

In previous work, we assembled trait thermal response estimates from laboratory experiments that manipulated temperature and measured each of the following traits for *Ae. aegypti* and DENV: egg-to-adult development rate (*MDR*), survival probability (p_{EA}), fecundity (*EFD*; eggs per female per day), biting rate (a), adult mosquito mortality rate (μ), extrinsic incubation rate (*EIR*), and vector competence (bc; equal to the proportion of exposed mosquitoes that become infected times the proportion of infected mosquitoes

that become infectious, with virus in their saliva). We then synthesized them into an estimate for the thermal response of R_0, the expected number of new cases generated by a single infectious person or mosquito introduced into a fully susceptible population throughout the period within which the person or mosquito is infectious (8):

$$R_0(T) = \sqrt{\frac{a(T)^2\ bc(T)\ exp\left(-\mu(T)/EIR(T)\right)\ EFD(T)\ p_{EA}(T)\ MDR(T)}{N\ r\ \mu(T)^3}}$$

Where N is the density of humans, r is the human recovery rate and (T) indicates parameters that are dependent on environmental temperature, T. Here, we update three of these thermal response functions – average adult mosquito lifespan ($lf=1/\mu$), extrinsic incubation rate (EIR), and vector competence (bc) – using the new experimental data from *Ae. aegypti* mosquitoes exposed to ZIKV-infected blood meals across a range of constant temperatures.

Experimental data on lifespan, vector competence, and extrinsic incubation rates were used across temperatures to estimate trait thermal response functions for calculating $R_0(T)$. Because we destructively sampled mosquitoes to assess infection status and did not follow all mosquito cohorts to the end of their lifespan, we used Gompertz survival curves to estimate average lifespan. First, Kaplan-Meier daily probabilities of survival for each experimental replicate, infection status, and temperature were estimated. Then, we used the 'nls' function in R (23) to fit a Gompertz function to the daily survival probabilities for each infection status by trial and temperature combination. To estimate the average female lifespan of exposed and control mosquitoes for each temperature treatment and experimental replicate, we calculated the area under the curve by integrating the associated Gompertz function. Vector competence for each temperature

was estimated from the average proportion of mosquitoes observed to become infectious at each temperature. For estimating the ZIKV extrinsic incubation rate (*EIR*) at each temperature, we calculated the time required for half of the average proportion of the population to become infectious (and defined this as the average extrinsic incubation period, *EIP*), then inverted this time interval to estimate a daily rate of ZIKV development for each temperature (*1/EIP*).

Using these data, we fit thermal response functions for lifespan, EIR, and vector competence as either symmetric (Quadratic: $-c(T-T_0)(T-T_m)$) or asymmetric (Briere: $cT(T-T_0)(T_m-T)^{(1/2)}$) functions, where T is experimental temperature, T_0 is the minimum temperature, T_m is the maximum temperature, c is a rate constant, and both functions are truncated at zero for negative values (8, 25). As in previous work (8), we fit the thermal response functions using Bayesian inference with uninformative priors, which are restricted to biologically reasonable ranges: $T_0 \sim$ Uniform (0, 24), $T_m \sim$ Uniform (25, 45), $c \sim$ Gamma (1, 10) for Briere and $c \sim$ Gamma (1, 1) for Quadratic (8). In the model, we assume that the sampling process is a normal distribution centered on the thermal response function calculated at the experimental temperature, with precision τ (where $\tau=1/\sigma$) assigned the prior: Gamma ~ (0.0001, 0.0001). We fit the models using JAGS (26) and R (23) and the R package 'rjags' (27), by running five Markov Chain Monte Carlo simulations for a 5,000-step burn-in followed by 5,000 additional steps, then thinning the posterior samples by saving every fifth sample (8, 25).

The three new thermal response functions (*lf, EIR,* and *bc*) were combined with the remaining previously-fitted thermal response functions (8) to calculate $R_0(T)$ for

ZIKV. To do so, we propagated the posterior distribution of each parameter thermal response through the $R_0(T)$ function to calculate a posterior distribution on $R_0(T)$.

This expression for temperature-dependent R_0 assumes a constant temperature to calculate the per-generation rate of increase of a pathogen in a fully susceptible population. However, environmental temperatures in nature are variable, which affects the calculation and interpretation of R_0 (28-32). Here, as in previous work (8, 33), we use relative R_0 as a simple metric for the *relative* suitability of temperature for transmission, rather than as an absolute metric for secondary case distributions, invasion and extinction thresholds, or expected equilibrium prevalence (34-36). The relative R_0 approach allows us to estimate the thermal optimum and limits, at which R_0 is maximized or goes to zero, respectively, and compare them to a similar model previously parameterized for DENV (8). Because our estimate of $R_0(T)$ is relative (rescaled to range from zero to one), we cannot estimate the stable transmission threshold $R_0(T) > 1$, so we instead use the more conservative suitability threshold $R_0(T) > 0$. At temperatures outside of this suitable range transmission is impossible because one or more process necessary for transmission has gone to zero.

Mapping seasonal transmission range

To illustrate predicted temperature suitability for Zika transmission in the Americas, we mapped the number of months for which $R_0(T) > 0$ for the posterior median response, based on the temperature-dependent model derived here and previously (8). This conservative threshold of $R_0(T) > 0$ illustrates all pixels in which transmission is theoretically possible (given that the mosquito and pathogen are present), but not necessarily stable. We calculated $R_0(T)$ at 0.1°C increments and projected it onto the

landscape for monthly mean current temperatures from WorldClim data at a 5-minute resolution (approximately $10km^2$ at the equator). This calculation gives a snapshot of the relative temperature suitability for transmission in each pixel each month but does not account for the influence of short- or long-term variation in temperature. Climate data layers were extracted for the geographic area and defined using the Global Administrative Boundaries Databases (37). All map calculations and manipulations were run in R using packages 'raster' (38), 'maptools', and 'Rgdal' (39). Resulting GeoTiffs were rendered in ArcGIS 10.3.1 (40), and mapped as figures. We then used the area represented by 6 months and 12 months of transmission suitability to calculate and display the difference between a previous model parameterized on the *Ae. aegypti* – DENV system (8) and our current predictions. This model was then validated using spatially explicit ZIKV case records from Columbia reported at the municipality level (41, 42).

Spatial validation of temperature dependent R_0 maps

We performed a spatial join to mapped administrative boundaries for municipalities in Colombia and summed reported Zika cases at the level of municipality for the entirety of the dataset (total cases=94,975). We overlaid this municipal level data onto the model predictions for months suitable for ZIKV transmission as a function of temperature. In absence of finer scale data than municipality level reporting, we simply extracted the model pixel value at the geographic centroid of each municipality represented and report the number and proportion of 'hits' and 'misses' of cases.

Results

Our GLMM analysis found that our data and response variables (probability of infection, dissemination, infectiousness, and dissemination efficiency) were best explained by temperature, dpi, and their interaction. Further, the best model for all of our response variables was the full model containing both linear and squared terms for temperature and dpi, as well as their interactions. This model captured the observed delayed ZIKV infection dynamics at the cool temperatures and the observed declines in ZIKV infection over time in the warmer temperature treatments due to increased mosquito mortality. Our best model suggests that the effects of temperature and dpi combine to shape relative R_0 (i.e. predicted risk of transmission for ZIKV), which differs from previous estimates generated from DENV-specific models.

The effect of temperature on ZIKV infection and infection dynamics

We observed strong, unimodal effects of temperature on the number of mosquitoes infected, with disseminated infections, and that became infectious (Table 4.1, Fig 4.2). While all three response variables dropped at both cool and warm temperatures, this decrease was more pronounced as the infection progressed (Fig 4.2). The likelihood of becoming infected was the most permissive to temperature variation, with the number of infected mosquitoes minimized at 16°C (6%), maximized from 24°C – 34°C (75% – 89%), and again minimized at 38°C (7%). The likelihood of viral dissemination was more constrained, with the probability of mosquitoes disseminating infections minimized at 16 – 20°C (4% – 3%), maximized at 28 – 34°C (65% – 77%), and again minimized at 38°C (5%). Finally, the likelihood of mosquitoes becoming infectious was the most sensitive to temperature, with the probability of mosquitoes becoming infectious

minimized from 16 – 24°C (0% – 4%), maximized between 28 – 34°C (23% – 19%), and again minimized from 36 – 38°C (5% – 0.4%).

Temperature also affected the rate of ZIKV infection, dissemination, and detection in saliva (Table 4.1, Fig 4.3). In general (with the exception of 36°C and 38°C), we observed an increase in the probability of mosquitoes with ZIKV in the bodies, legs and heads, and saliva with time (Fig 4.3) suggesting that rate of ZIKV detection in these samples decreased with increasing temperature. However, at 36°C and 38°C, we see declines in these response variables with dpi due to high mosquito mortality.

The effects of temperature on ZIKV dissemination efficiency

We observed effects of temperature, dpi, and their interaction on the dissemination efficiency of ZIKV (the probability of becoming infectious after successful ZIKV infection – positive bodies; Table 4.2). ZIKV dissemination efficiency was maximized from 28 – 34°C, suggesting that ZIKV infection process (e.g. escape from the midgut and salivary gland invasion) was most efficient at these temperatures (Fig 4.4). In contrast, dissemination efficiency was minimized at both cooler (16 – 20°C) and warmer temperatures (38°C). Cooler temperatures had a more dramatic effect on dissemination efficiency than warmer temperatures. Although 60% of exposed mosquitoes became successfully infected at 20°C, we had very low salivary gland invasion, with only one mosquito across both trials becoming infectious. In contrast, at warm temperatures infection and dissemination efficiencies were very robust (Fig 4.5), but the mortality associated with the warm temperatures resulted in low numbers of mosquitoes that were capable of being infectious. Finally, of those successfully infected, we observed

successful salivary gland invasion to occur earlier in the infection process as temperatures warmed (Fig 4.4).

The effect of temperature on mosquito survival

We observed effects of temperature and an interaction between temperature and ZIKV exposure on the daily probability of mosquito survival (Fig 4.6, Table 4.3). Overall, the daily probability of mosquito survival was highest for mosquitoes housed at 24°C and 28°C relative to cooler (16 – 20°C) and warmer (32 – 38°C) temperatures. Mosquito survival was lowest at the warmest temperature of 38°C, with no mosquitoes surviving past 3 dpi. ZIKV-exposed mosquitoes experienced a higher daily probability of survival at 24°C and 28°C relative to unexposed, control mosquitoes with greater than 90% daily survival at the optimal temperatures.

The effect of temperature on ZIKV transmission risk

Trait thermal responses for lifespan, vector competence, and extrinsic incubation rate were all unimodal (Fig 4.7, Table 4.4, Fig 4.8). Mosquito lifespan and vector competence thermal responses were symmetrical, peaking at 24.2°C (95% credible interval (CI): 21.9 – 25.9°C) and 30.6°C (95% CI: 29.6 – 31.4°C), respectively, while the extrinsic incubation rate thermal response was asymmetrical with a peak at 36.4°C (95% CI: 33.6 – 39.1°C). Applying these new trait thermal responses to the $R_0(T)$ model (8), we found that $R_0(T)$ peaked at 28.9°C (95% CI: 28.1 – 29.5°C), with lower and upper limits of 22.7°C (95% CI: 21.0 – 23.9°C) and 34.7°C (95% CI: 34.1 – 35.8°C), respectively (Fig 4.9). The seasonal transmission of ZIKV was predicted to be more constricted in latitudinal range from this temperature – transmission relationship than what has been

predicted previously (8), primarily because the predicted thermal minimum for ZIKV was 5^{o}C warmer than for DENV (Fig 4.9). This represents a 4.3 million km^2 estimated change in endemic (12-month, year-round suitability) land area, and a 6.03 million km^2 change in overall predicted range (1 – 12 months suitability) in the Americas (Fig 4.10).

The spatial validation for Columbia showed that 71.5% (67,934) of all ZIKV cases fell within 1-12 months of predicted suitability, with 68% (64,286) ZIKV cases overlaid areas predicted to have 12 months of suitability. In contrast, our spatial validation predicted 28.5% (27,041) ZIKV cases to occur in areas predicted to be unsuitable for transmission (0 months of suitability, Fig 4.11). Upon visual inspection, large clusters of cases occurred in valleys where the R_0 model predicted transmission suitability.

While there is some evidence that mosquito longevity varies for virus-exposed versus control mosquitoes, where unexposed mosquitoes had shorter lifespans at near-optimal temperatures (24°C and 28°C; Fig 4.6 and 4.7), we did not include this difference in the R_0 model for two reasons. First, with limited data to parameterize the low temperature range for survival, we are unable to characterize the differences in the lower end of the thermal response functions in detail. Second, the standard R_0 model does not incorporate differences in survival for infected versus uninfected mosquitoes because it assumes that the pathogen is rare and that all mosquitoes are uninfected. For this reason, we fit a single thermal response function for lifespan to the full dataset and used it in the R_0 model.

Discussion

The dynamics and distribution of vector-borne diseases depend on the interplay between the pathogen, the mosquito, and the environment (43). Temperature is a strong driver of vector-borne disease transmission and characterizing the thermal range and optimum for transmission is essential for accurately predicting how arbovirus emergence and transmission will be affected by seasonality, geography, climate and land use change. Yet current models of recently emerging arboviruses like ZIKV are constrained by a lack of data on the thermal sensitivity of key pathogen traits (e.g. 6, 7, 9). In this study, we experimentally estimated the relationship between temperature and measures of ZIKV vector competence, extrinsic incubation rate, and mosquito mortality. By incorporating these temperature-trait relationships into an existing mechanistic model, we demonstrate that, like malaria (15, 25) and dengue virus (8), ZIKV transmission has a strong unimodal relationship with temperature.

As studies have demonstrated in other arbovirus systems, temperature significantly affects vector competence (16, 44, 45). We show that temperature has a unimodal relationship with vector competence, with an estimated optimum at 30.6°C and an estimated thermal minimum and maximum of 22.9°C and 38.4°C, respectively (based on posterior median estimates for T_0 and T_m). ZIKV infectiousness was limited by different mechanisms at the thermal minimum and maximum. Cool temperatures limited midgut escape and dissemination, resulting in a lower proportion of the mosquito population that was infectious. This could be due to temperature effects on mosquito physiology (46), immunity (12, 47), and viral binding to specific receptors in the midgut, secondary tissues, and salivary glands (48). Warmer temperatures, on the other hand,

were very permissive for ZIKV infection, resulting in 95% and 100% infection among surviving mosquitoes at 36°C and 38°C, respectively (Fig 4.5). However, high mosquito mortality at these temperatures constrained the proportion of the mosquito population that became infectious (Fig 4.3, Fig 4.5) A similar nonlinear effect of cool and warm temperatures on vector competence was observed with *Ae. albopictus* infected with DENV-2 (49). In contrast, Adelman et al. (13) demonstrated that cooler temperatures in the larval stage resulted in increased susceptibility to CHIKV and yellow fever virus by impairing the RNAi pathway. However, mosquitoes in our study were exposed to different constant temperatures in the adult stage. Temperature variation experienced in both the larval and adult stage will likely be important in shaping mosquito and pathogen traits comprising arbovirus transmission.

We also observed an asymmetrical unimodal relationship between temperature and the extrinsic incubation rate of ZIKV, with the extrinsic incubation rate optimized at 36.4°C and minimized at 19.7°C and 42.5°C (based on posterior median estimates for T_0 and T_m). Consistent with previous studies (e.g. 45, 49, 50), we show that the extrinsic incubation rate of ZIKV increased with warming temperatures, with no infectious mosquitoes observed at 16°C after 21 days post infection and the first infectious mosquito detected at day 3 post infection at 38°C. The extrinsic incubation rate was ultimately constrained at the warmer temperatures due to high mosquito mortality. This is not surprising as metabolic reaction rates tend to increase exponentially to an optimal temperature, then decline rapidly due to protein degradation and other processes (22, 51).

The optimal temperature for mosquito fitness and viral dissemination need not be equivalent, and the impacts of temperature on mosquito mortality relative to the extrinsic

incubation rate of arboviruses strongly affect the total proportion of the mosquito population that is alive and infectious (52, 53). In our study, mosquito lifespan was optimized at 24.2°C and minimized at 11.7°C and 37.2°C, respectively (based on posterior median estimates for T_0 and T_m). The non-linear relationship between metrics of mosquito mortality or lifespan and temperature has also been demonstrated for *Ae. aegypti* (8), *Ae. albopictus* (8, 14) and various *Anopheles* spp. (15, 54). Despite the fact that the extrinsic incubation rate was optimized at a warm temperature (36.4°C), the optimal temperature for overall ZIKV transmission (R_0) was predicted to be cooler (28.9°C) because mosquitoes experience a shortened lifespan above 32°C. In contrast, even though mosquitoes are predicted to have relatively longer lifespans at cooler temperatures, the time required for mosquitoes to become infectious (>21 days at 16°C and 18 days at 20°C) may be longer than most mosquitoes experience in the field. As a result, large vector populations may not be sufficient for transmitting the virus if viral replication is inhibited or if the lifespan of the mosquito is shorter than the extrinsic incubation period (55). One surprising result was that mosquitoes exposed to ZIKV were predicted to live significantly longer than unexposed mosquitoes at temperatures already optimal for mosquito survival (37 vs. 87 days at 24°C; 45 vs. 54 days at 28°C). A similar phenomenon has been noted in the *Ae. aegypti* – DENV-2 system (56). Additionally, the temperature that optimizes mosquito lifespan might also vary between ZIKV exposed mosquitoes (24°C) and their uninfected counterparts (28°C). However, more data characterizing mosquito survival of uninfected and ZIKV exposed mosquitoes at the cool range of transmission are needed to better understand the consequences of survival differences between ZIKV infected and uninfected mosquitoes. If temperature

consistently has different effects on the mortality rates of uninfected and infected mosquitoes in other arbovirus systems, current modeling efforts may be underestimating virus transmission potential under certain environmental scenarios and estimating mosquito mortality in the field for mosquitoes of different infection statuses are important areas for future research.

After incorporating the relationships between temperature and vector competence, the extrinsic incubation rate, and mosquito lifespan into a mechanistic model, we demonstrated that ZIKV transmission is optimized at a mean temperature of approximately 29°C, and has a thermal range of 22.7°C to 34.7°C. Because this relationship is nonlinear and unimodal, we can expect as temperatures move toward the thermal optimum due to future climate change or increasing urbanization (57), environmental suitability for ZIKV transmission should increase, potentially resulting in expansion of ZIKV further north and into longer seasons. There is evidence that this is already occurring with warming at high elevations in the Ethiopian and Columbian highlands leading to increased incidence of malaria (11). In contrast, in areas that are already permissive and near the thermal optimum for ZIKV transmission, future warming and urbanization may lead to decreases in overall environmental suitability (17). Accurately estimating the optimal temperature for transmission is thus paramount for predicting where climate warming will expand, contract, or shift transmission potential.

By using a mechanistic model originally parameterized for DENV (data from serotypes 1 and 2), we also explored a common assumption made by multiple models that DENV transmission has a similar relationship with temperature as ZIKV (6-9, 20). While the temperature optimum and maximum for R_0 changed very little from our previous

DENV R_0 model, the temperature minimum for transmission increased by nearly five degrees in the ZIKV-specific model (Fig 4.9). This is mainly due to a higher thermal minimum for both vector competence and the extrinsic incubation rate for ZIKV as compared to DENV (Fig 4.8). Differences in the thermal niche of ZIKV relative to DENV or our field derived *Ae. aegypti* relative to those populations synthesized in Mordecai et al. (8) could explain this difference. There is evidence that the effects of environmental variation on disease transmission are often modified by the genetic background of the mosquito and infecting pathogen (44, 58, 59). Thus, more work is required to validate the generalizability of these models.

Our mapped seasonal ranges underscore the impact of a more refined empirical derivation of a pathogen-specific temperature dependent R_0, contrasted with the *Ae. aegypti* – DENV prediction of previous studies (6-8). The higher predicted thermal minimum for ZIKV resulted in a contraction in the areas of the Americas where year-round, endemic transmission suitability (12 months only) are predicted to occur. This area corresponds to a change of approximately 4.3 million km^2 in land area (Fig 4.10). Additionally, this higher thermal minimum contributes to a reduction in the overall estimated suitability for ZIKV transmission (all 1-12 months of transmission) resulting in an estimated difference of 6.03 million km^2. In particular, in the Florida peninsula where the primary focus of ZIKV cases within the U.S. occurred, our updated model (the median model – 50^{th} percentile posterior) now predicts only around six months of temperature suitability during the year (Fig 4.10) vs. almost year-round as predicted by a previous temperature-dependent R_0 model parameterized on the *Ae. aegypti* – DENV system (8). This contrast in seasonal suitability where ZIKV established in the USA is

striking and emphasizes the value of increasing empirical data and reexamining these types of model, as the capacity to do so becomes possible, in the face of an emerging epidemic. This result also largely concurs with a previous study that generated an R_0 ZIKV map of the Americas using a spatially explicit individual based SEI-SEIR compartmental model. This model incorporates unimodal temperature-trait relationships for mosquito lifespan, probability of transmission parameterized from the *Ae. aegypti* – DENV system, and mosquito abundance with pre-existing data layers for *Ae. aegypti* and *Ae. albopictus* distributions, spatially explicit human population and economic data, and ZIKV case data (20). While our models generally agree in the geographical extent to which ZIKV transmission is predicted to occur in the Americas, Zhang et al. 2017 show more heterogeneity in R_0 across space than our model would predict, which simply describes the temperature boundaries for potential ZIKV transmission on the landscape. However, our model, which uses a broader life history explicit parameterization including ZIKV-specific thermal responses for relevant transmission, provides important validation of the predictions in Zhang et al. 2017 (20).

Our spatial validation of this model revealed fairly robust predictive qualities (Fig 4.11), despite limitations of spatial resolution and scale at which ZIKV cases are reported. For example, aggregating at the municipality scale created a much larger minimum areal unit than the model pixels. Further, this region is subject to high variation in local conditions due to the Andes climate, which are likely not accurately captured by global temperature model outputs. Thus, we might expect the R_0 model to over or under predict fine-scale variation in local microclimate (60). Regardless, this highlights a need

for more spatially detailed health datasets to be available to this type of model validation exercise, as well as regionalized modeling efforts in climate-health initiatives.

Finally, although we estimated the effects of mean constant temperatures on ZIKV transmission, mosquitoes and their pathogens live in a variable world where temperatures fluctuate daily and seasonally. Temperature-trait relationships have been shown to differ in fluctuating environments relative to constant temperature environment (17, 61, 62). While characterizing trait responses to mean constant temperatures and incorporating these relationships into models of disease transmission is tractable, more effort is needed in validating computational approaches to infer transmission in a fluctuating environment (i.e. rate summation (8)).

Accurately predicting arbovirus transmission will be influenced by variation in other sources of abiotic (e.g. relative humidity, rainfall), biotic (e.g. availability and quality of oviposition and resting habitats), and socioeconomic factors that influence human exposure to biting mosquitoes (20). However, this is a fundamental first step for empirically defining and validating current models on the environmental suitability for ZIKV transmission, in which temperature will be a strong driver. R_0 models have been used as a tool to guide vector-borne disease interventions and represent a comprehensive metric of pathogen fitness. We anticipate, as with other vector-borne diseases, that environmental suitability for ZIKV transmission could expand northwards with future warming but will be more constrained than DENV at low temperatures. We also predict areas that are already at or near the thermal optimum of 29°C to experience a decrease in environmental suitability for ZIKV transmission (15, 17). Further, land use change that modifies the microclimates mosquitoes experience and human density and exposure

could have immediate impacts on ZIKV transmission, which might explain the explosive spread of ZIKV in urban centers throughout the Americas.

Acknowledgements

We thank the University of Texas Medical Branch Arbovirus Reference Collection for providing the virus. We thank Dr. Américo Rodríguez from the Instituto Nacional de Salud Pública for providing mosquito eggs. We gratefully acknowledge the members of the Murdock and Brindley labs for thoughtful comments on the project and manuscript, and Gregory R. Jacobs for feedback and coding help associated with the statistical analysis. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the Centers for Disease Control and Prevention. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

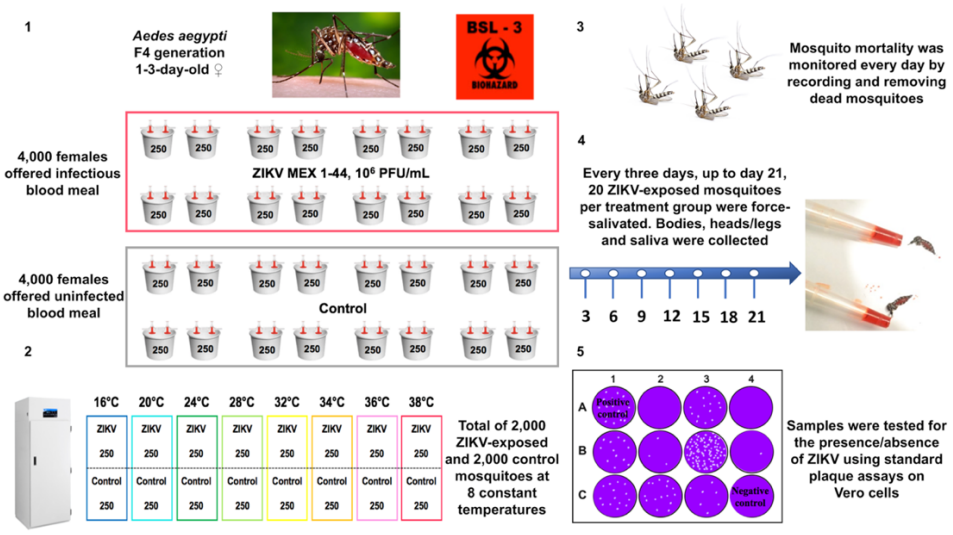


Fig 4.1. Experimental design. In each biological replicate, eight thousand female *Ae. aegypti* mosquitoes were offered either an infectious blood meal containing ZIKV at the final concentration of 10^6 PFU/mL or an uninfected, control blood meal (1). Two thousand ZIKV-exposed and two thousand control engorged mosquitoes were randomly distributed into mesh-covered paper cups (250 per cup) and put at one of eight temperature treatments (16°C, 20°C, 24°C, 28°C, 32°C, 34°C, 36°C, and 38°C; 2). Across all treatment groups, dead mosquitoes were removed and counted daily to monitor mortality (3). Every three days, up to day twenty-one, twenty ZIKV-exposed mosquitoes per treatment group were force-salivated. After salivation, mosquito saliva, heads, legs, and bodies were collected into separate tubes (4). Each tissue was tested for the presence/absence of the ZIKV using plaque assays on Vero cells (5). Two full biological replicates were performed.

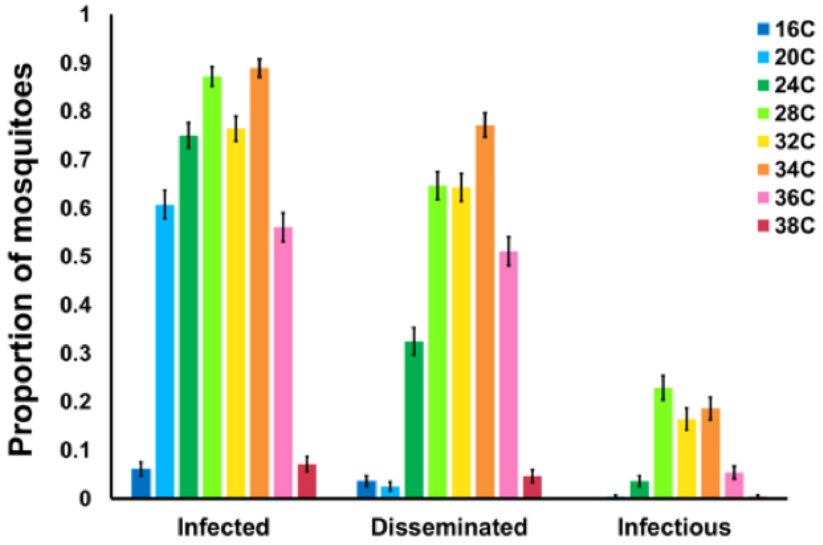


Fig 4.2. Temperature effect on the proportion of mosquitoes infected, with disseminated infections, and infectious. The effect of eight different constant temperatures (16°C, 20°C, 24°C, 28°C, 32°C, 34°C, 36°C, 38°C) on the proportion of mosquitoes infected (ZIKV positive bodies compared to total number of exposed), with disseminated infections (ZIKV positive heads compared to total number exposed), and infectious (ZIKV positive saliva compared to total number exposed).

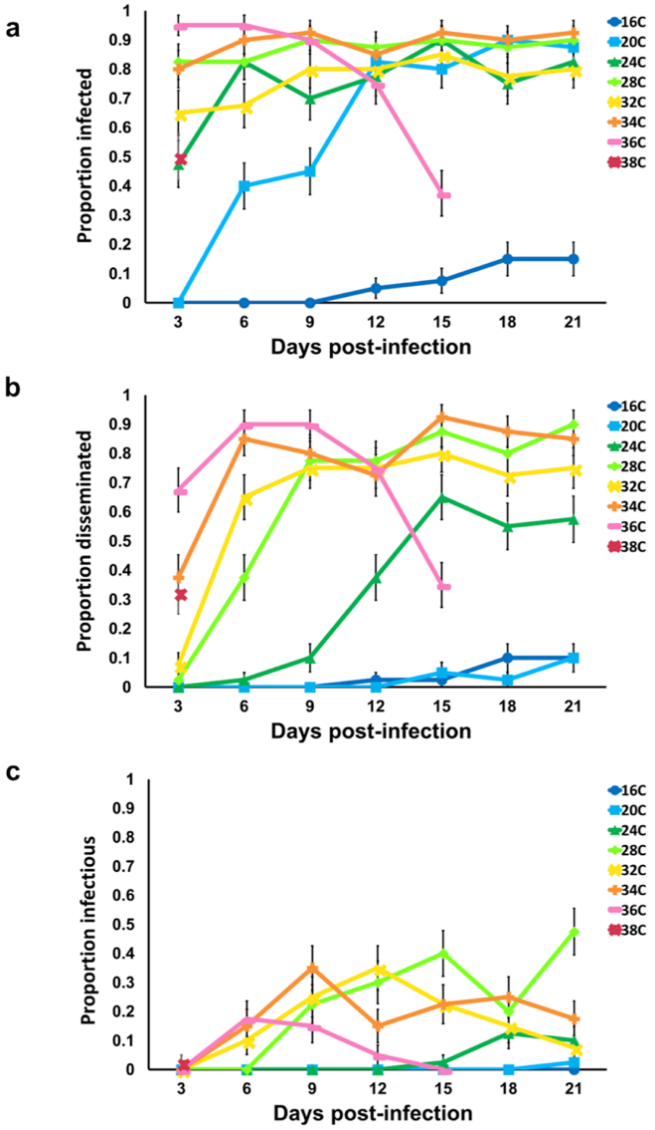


Fig 4.3. Days post-infection and the proportion of mosquitoes infected, with disseminated infections, and infectious. The relationship between days post-infection (3, 6, 9, 12, 15, 18, 21) and the proportion of mosquitoes infected (a, ZIKV positive bodies), with disseminated infections (b, ZIKV positive legs and heads), and infectious (c, ZIKV positive saliva) out of the total mosquitoes exposed to ZIKV at eight different constant temperatures (16°C, 20°C, 24°C, 28°C, 32°C, 34°C, 36°C, 38°C).

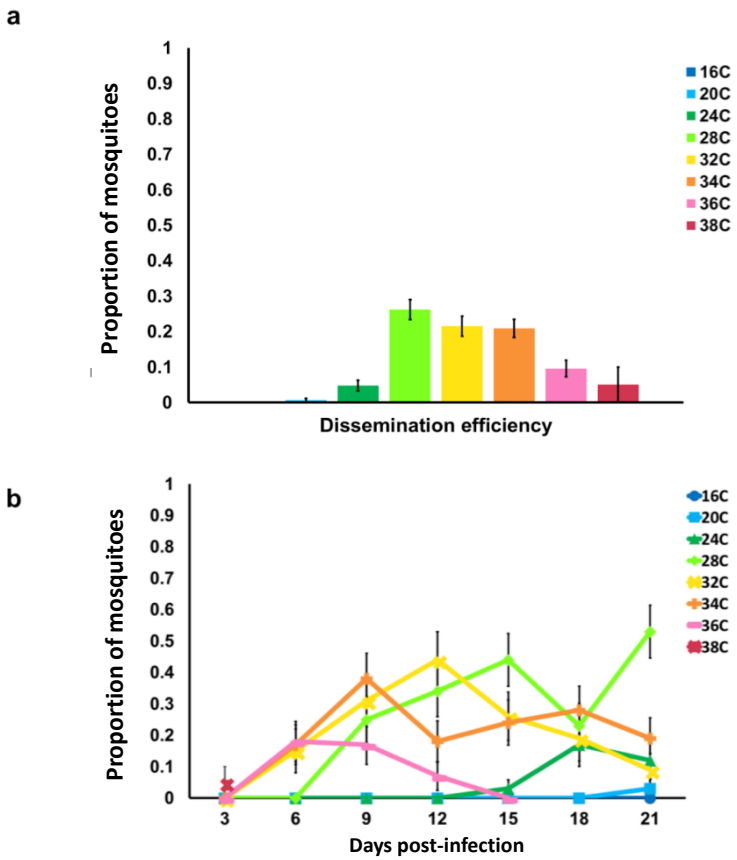
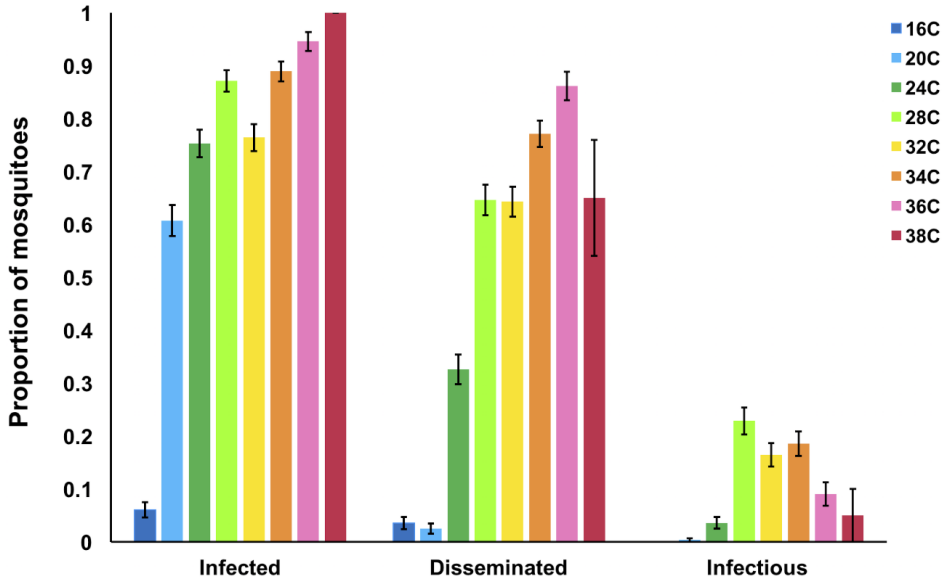


Fig 4.4. Temperature effect on the dissemination efficiency (a) The effect of eight different constant temperatures (16°C, 20°C, 24°C, 28°C, 32°C, 34°C, 36°C, 38°C) and (b) days post-infection (3, 6, 9, 12, 15, 18, 21) on the dissemination efficiency (proportion of mosquitoes with ZIKV positive saliva relative to those with positive bodies).



4.5. Temperature effect on the proportion of total mosquitoes infected, with disseminated infections, and infectious. The effect of eight different constant temperatures (16°C, 20°C, 24°C, 28°C, 32°C, 34°C, 36°C, 38°C) on the proportion of mosquitoes infected (proportion of ZIKV positive bodies out of total number of processed mosquitoes), with disseminated infections (proportion of ZIKV positive heads out of total number of processed mosquitoes), and infectious (proportion of ZIKV positive saliva out of total number of processed mosquitoes).

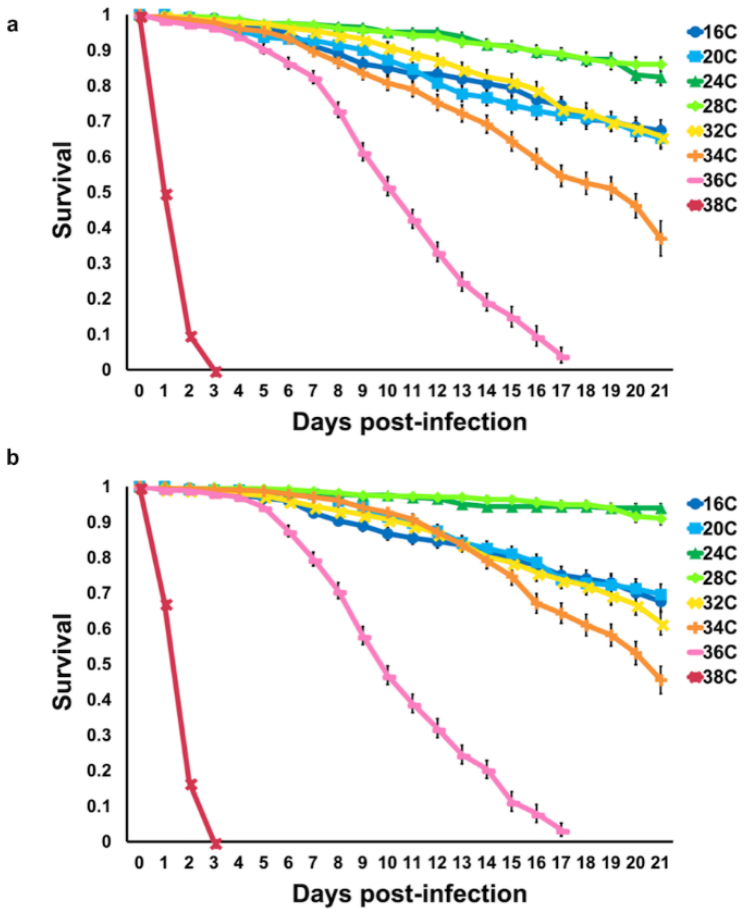


Fig 4.6. Daily probability of mosquito survival. Kaplan-Meier estimates of daily probability of mosquito survival for unexposed (a) and ZIKV exposed (b) field-derived *Ae. aegypti* mosquitoes across eight different constant temperatures (16°C, 20°C, 24°C, 28°C, 32°C, 34°C, 36°C, 38°C).

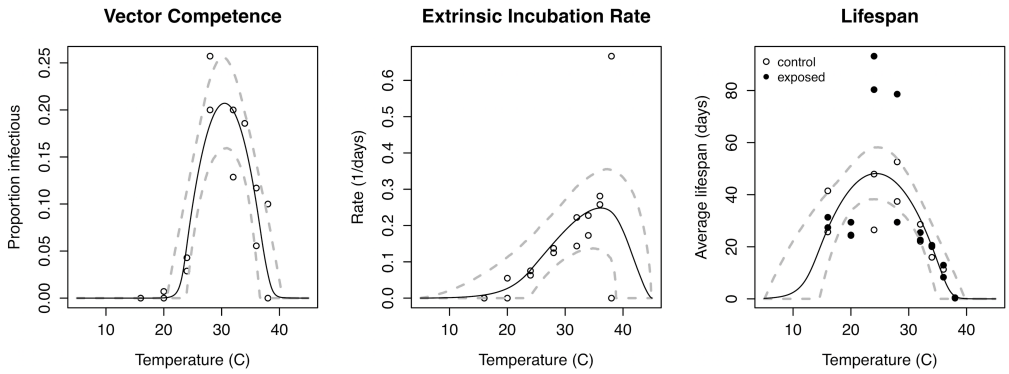


Fig 4.7. Effect of temperature and estimated vector competence, extrinsic incubation rate and mosquito lifespan. Trait thermal responses with fit from laboratory experimental data. Vector competence (left), is the maximum proportion of virus-exposed mosquitoes with virus in their saliva, across temperatures and replicates. Extrinsic incubation rate (middle) is the inverse of the time required to reach half of the maximum proportion infectious ($days^{-1}$) for each temperature and replicate. Lifespan (right) is the average lifespan of mosquitoes in each temperature and replicate (days), shown in filled (virus-exposed) and open (sham-inoculated) points. Solid lines represent posterior means; dashed lines represent 95% credible intervals.

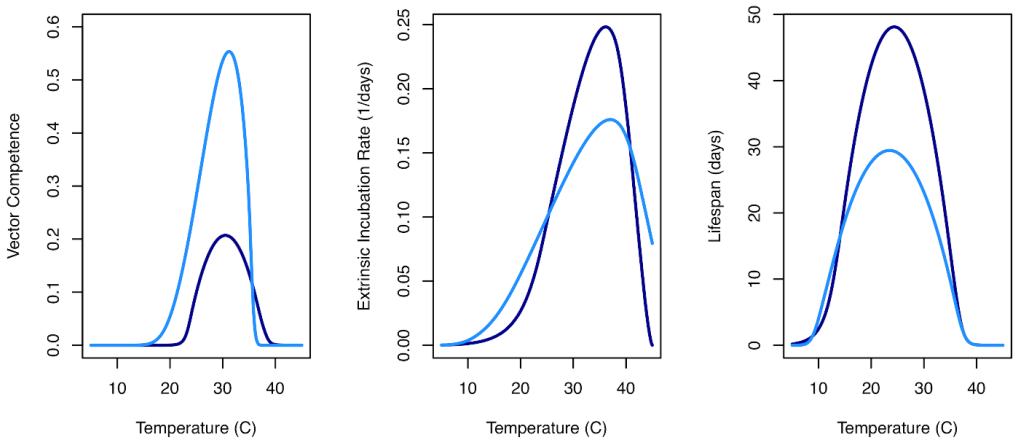


Fig 4.8. Trait thermal response means. Trait thermal response means for vector competence (left), extrinsic incubation rate (center), and lifespan (right) for the new experimental data presented here (ZIKV; dark blue) and the previously published data (DENV, light blue) (8)

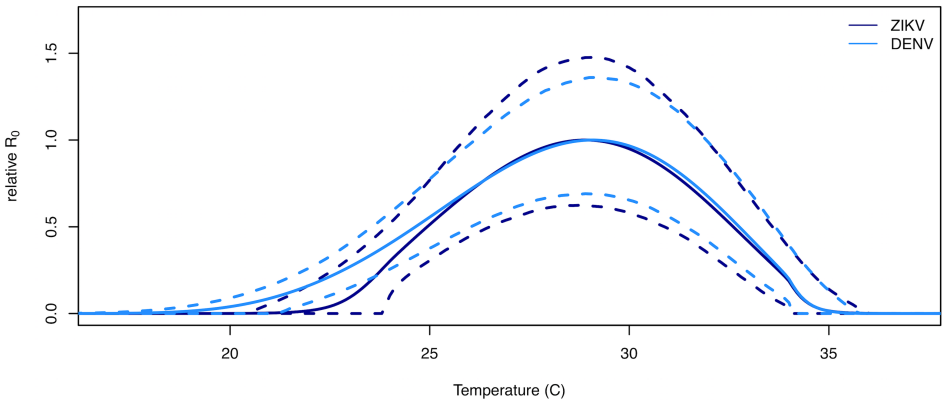


Fig 4.9. Effect of temperature on R_0. Effect of temperature on relative R_0 for DENV (light blue) and ZIKV (dark blue). Solid lines represent the mean and dashed lines are the 95% credible intervals.

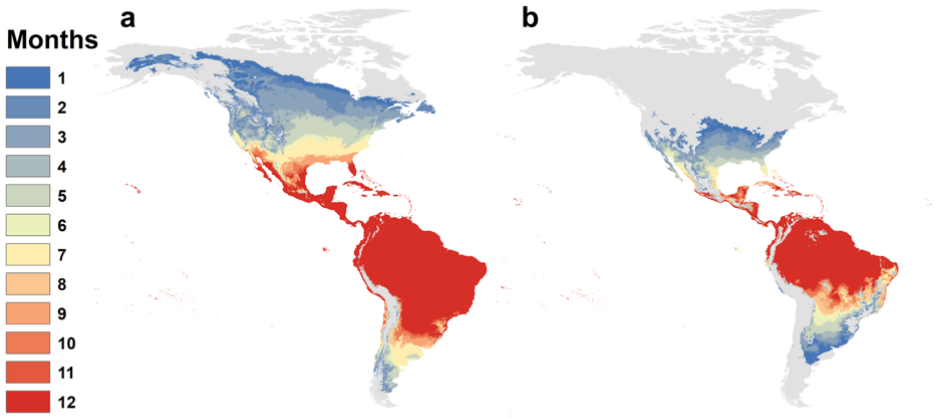
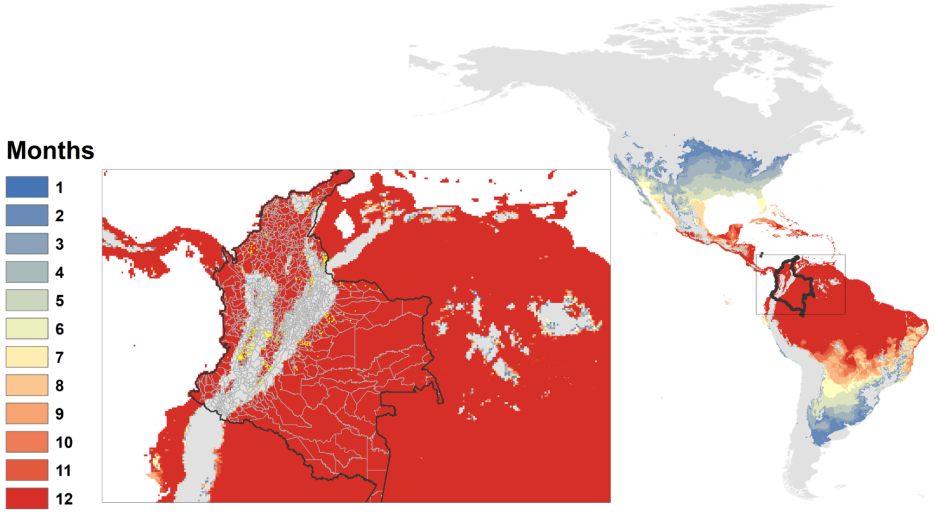


Fig 4.10. Months of transmission suitability in the Americas. The number of months of transmission suitability (R_0>0) for DENV derived in Mordecai et al 2017 (a) and ZIKV derived in this study (b), for median (posterior 50th percentile) models.



4.11. Spatial validation of the ZIKV suitability model. Spatial validation of the ZIKV suitability model, using case data from Colombia. Colombia is shown (black) in the Americas, on a backdrop of the ZIKV model mapped as months of suitability, described in Figure 4.10. Inset displays the case data at municipality level, dithered as dot densities (yellow, 300 cases per dot). Note contrasting suitability at the rapidly rising edge of the Andes, the most densely populated part of Colombia.

Table 4.1. The effects of temperature, day, and the interaction on the probability of mosquitoes becoming infected, disseminating infection, and becoming infectious after being exposed to ZIKV.

Fixed Effects	df	AICc	*Intercept*	*T*	*dpi*	*T: dpi*	T^2	D^2	T^2: D^2
Infected ~ $T + dpi$	4	2418.30	0.228	1.268	0.026				
Infected ~ $T + T^2 + dpi$	5	2132.60	2.184	-0.228	0.031		-1.995		
Infected ~ $T + dpi + dpi^2$	5	2412.40	0.391	1.273	0.026			-0.162	
Infected ~ $T + T^2 + dpi + dpi^2$	6	2125.6	2.381	-0.229	0.030		-2.004	-0.187	
Infected ~ $T + dpi + T: dpi$	5	2238.60	0.176	1.406	0.000	-0.721			
Infected ~ $T + dpi + T: dpi + T^2$	6	1870.80	2.612	-0.407	0.244	-1.091	-2.552		
Infected ~ $T + dpi + T:dpi + dpi^2$	6	2225.50	0.408	1.432	-0.006	-0.756		-0.242	
Infected ~ $T + dpi + T: dpi + T^2 + dpi^2 + T^2: dpi^2$	**8**	**1861.60**	**2.804**	**-0.373**	**0.232**	**-1.115**	**-2.494**	**-0.226**	**-0.027**
Disseminated ~ $T + dpi$	4	2330.70	-0.791	1.724	0.332				
Disseminated ~ $T + T^2 + dpi$	5	2133.70	0.813	1.018	0.354		-2.087		
Disseminated ~ $T + dpi + dpi^2$	5	2288.30	-0.407	1.764	0.358			-0.406	
Disseminated ~ $T + T^2 + dpi + dpi^2$	6	2090.00	1.244	1.040	0.370		-2.122	-0.424	
Disseminated ~ $T + dpi + T: dpi$	5	2217.00	-1.008	2.079	0.562	-0.717			
Disseminated ~ $T + dpi + T: dpi + T^2$	6	1895.40	0.969	1.662	1.055	-1.473	-3.027		
Disseminated ~ $T + dpi + T:dpi + dpi^2$	6	2134.90	-0.527	2.292	0.726	-0.976		-0.618	
Disseminated ~ $T + dpi + T: dpi + T^2 + dpi^2 + T^2: dpi^2$	**8**	**1782.70**	**2.021**	**1.922**	**1.244**	**-1.749**	**-3.837**	**-1.103**	**0.618**
Infectious ~ $T + dpi$	4	1125.70	-3.046	1.356	0.387				
Infectious ~ $T + T^2 + dpi$	5	1048.30	-1.331	1.357	0.392		-2.949		
Infectious ~ $T + dpi + dpi^2$	5	1101.80	-2.626	1.371	0.530			-0.527	
Infectious ~ $T + T^2 + dpi + dpi^2$	6	1024.30	-0.890	1.367	0.534		-2.975	-0.531	
Infectious ~ $T + dpi + T: dpi$	5	1111.90	-3.242	1.679	0.592	-0.542			
Infectious ~ $T + dpi + T: dpi + T^2$	6	1013.10	-1.514	2.126	0.883	-1.205	-3.550		
Infectious ~ $T + dpi + T:dpi + dpi^2$	6	1079.00	-2.859	1.877	0.900	-0.848		-0.648	
Infectious ~ $T + dpi + T: dpi + T^2 + dpi^2 + T^2: dpi^2$	**8**	**971.80**	**-0.953**	**2.667**	**1.461**	**-1.950**	**-4.259**	**-0.946**	**0.425**

Results from candidate generalized linear mixed effects models (binomial distribution, log link function, random effect of mosquito cohort) examining the fixed effects of temperature, day, and the interaction on the probability of mosquitoes becoming infected, disseminating infection, and becoming infectious after being exposed to ZIKV. Bolded model outputs represent the most parsimonious model as determined by Akaike Information criterion scores corrected for small sample sizes (AICc).

Table 4.2. The effects of temperature, day, and the interaction on the probability of mosquitoes becoming infectious after being successfully infected with ZIKV.

Fixed Effects	df	AICc	*Intercept*	*T*	*dpi*	*T: dpi*	T^2	D^2	T^2: D^2
Infectious ~ $T + dpi$	4	987.40	-2.120	0.848	0.472				
Infectious ~ $T + T^2 + dpi$	5	945.70	-1.149	0.713	0.448		-1.193		
Infectious ~ $T + dpi + dpi^2$	5	966.50	-1.719	0.868	0.598			-0.485	
Infectious ~ $T + T^2 + dpi + dpi^2$	6	926.00	-0.742	0.716	0.571		-1.210	-0.478	
Infectious ~ $T + dpi + T: dpi$	5	984.80	-2.310	1.034	0.567	-0.277			
Infectious ~ $T + dpi + T: dpi + T^2$	6	927.70	-1.180	1.074	0.727	-0.707	-1.630		
Infectious ~ $T + dpi + T:dpi + dpi^2$	6	952.80	-1.988	1.290	0.891	-0.642		-0.648	
Infectious ~ **$T + dpi + T: dpi + T^2 + dpi^2 + T^2: dpi^2$**	**8**	**891.20**	**-0.594**	**1.422**	**1.192**	**-1.224**	**-2.076**	**-0.867**	**0.250**

Results from candidate generalized linear mixed effects models (binomial distribution, log link function, random effect of mosquito cohort) examining the fixed effects of temperature, day, and the interaction on the probability of mosquitoes becoming infectious after being successfully infected with ZIKV (positive body infection). Bolded model outputs represent the most parsimonious model as determined by Akaike Information criterion scores corrected for small sample sizes (AICc).

Table 4.3. The effects of temperature, infection status and the interaction on the daily probability of mosquito survival.

effect	Chi-Square	d.f.	*p*-value
temperature	1138.226	7	**<0.0001**
infection	0.227	1	0.6338
temperature x infection	25.871	7	**0.0005**

Results from Cox mixed-effects model examining the effects of temperature (16°C, 20°C, 24°C, 28°C, 32°C, 34°C, 36°C, 38°C), infection status (exposed or not exposed) and the interaction on the daily probability of mosquito survival.

Table 4.4. Data used on the *Ae. aegypti* Zika virus R_0 model.

Trait		Refs	Function	c			To			Tmax		
				mean	95% CI		mean	95% CI		mean	95% CI	
MDR	mosquito egg-to-adult development rate (1/days)	1	Briere	6.38E-05	4.67E-05	8.23E-05	8.60	4.43	12.29	39.66	37.78	41.70
pEA	mosquito egg-to-adult survival probability	1	Quad	-3.61E-03	-4.74E-03	-2.59E-03	9.04	6.37	11.67	39.33	37.17	41.62
EFD	eggs laid per female per gonotrophic cycle no. / female)	1	Briere	4.88E-02	3.21E-02	6.72E-02	8.02	3.18	13.08	35.65	35.00	36.51
a	biting rate, reciprocal of oviposition cycle length (1/days)	1	Briere	1.93E-04	1.27E-04	2.61E-04	10.25	5.84	14.82	38.32	36.60	40.51
lf	mosquito adult lifespan (days)	2	Quad	-3.02E-01	-4.68E-01	-1.34E-01	11.25	6.30	15.06	37.22	34.79	39.57
bc	vector competence: average proportion of the mosquito population that is infectious	2	Quad	-3.54E-03	-5.56E-03	-1.82E-03	22.72	21.09	24.00	38.38	36.46	40.25
EIR	ZIKV extrinsic incubation rate (reciprocal of the extrinsic incubation period: the time required for half of the mosquito population to reach average vector competence	2	Briere	1.74E-04	5.38E-05	3.04E-04	18.27	7.68	24.00	42.31	39.26	45.00

[1]Mordecai E., Cohen J., Evans M.V., Gudapati P., Johnson L.R., Lippi C.A., Miazgowicz K., Murdock C.C., Rohr J.R., Ryan S.J., et al. 2017 Detecting the impact of temperature on transmission of Zika, dengue, and chikungunya using mechanistic models. *PLoS Negl Trop Dis* **11**(4), e0005568.
[2]Estimated from data generated in the current study

Data used on the *Ae. aegypti* Zika virus R_0 model. Each trait parameter symbol, definition, data sources, and thermal response function (Quad = quadratic) are shown on the left. Mean and 95% credible interval (95% HPD interval) for the critical thermal minimum (T_0), maximum, (T_m), and a rate constant (c) are given for each trait in the three right sections.

References

1. Brady OJ, Gething PW, Bhatt S, Messina JP, Brownstein JS, Hoen AG, et al. Refining the global spatial limits of dengue virus transmission by evidence-based consensus. PLoS Negl Trop Dis. 2012;6(8):e1760.

2. PAHO. Pan American Health Organisation 2018 [Available from: http://www.paho.org/.

3. Cao-Lormeau V-M, Blake A, Mons S, Lastère S, Roche C, Vanhomwegen J, et al. Guillain-Barre Syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. Lancet. 2016;387(10027):1531-9.

4. Mlakar J, Korva M, Tul N, Popovic M, Poljsak-Prijatelj M, Mraz J, et al. Zika virus associated with microcephaly. N Engl J Med. 2016;374(10):951-8.

5. World Health Organization. WHO statement on the first meeting of the International Health Regulations (2005) (IHR 2005) Emergency Committee on Zika virus and observed increase in neurological disorders and neonatal malformations 2016 [Available from: http://www.who.int/mediacentre/news/statements/2016/1st-emergency-committee-zika/en/.

6. Messina JP, Kraemer MU, Brady OJ, Pigott DM, Shearer FM, Weiss DJ, et al. Mapping global environmental suitability for Zika virus. eLife. 2016;5.

7. Samy AM, Thomas SM, Wahed AA, Cohoon KP, Peterson AT. Mapping the global geographic potential of Zika virus spread. Mem Inst Oswaldo Cruz. 2016;111(9):559-60.

8. Mordecai EA, Cohen JM, Evans MV, Gudapati P, Johnson LR, Lippi CA, et al. Detecting the impact of temperature on transmission of Zika, dengue, and chikungunya using mechanistic models. PLoS Negl Trop Dis. 2017;11(4):e0005568.

9. Caminade C, Turner J, Metelmann S, Hesson JC, Blagrove MS, Solomon T, et al. Global risk model for vector-borne transmission of Zika virus reveals the role of El Nino 2015. Proc Natl Acad Sci U S A. 2017;114(1):119-24.

10. Liu-Helmersson J, Stenlund H, Wilder-Smith A, Rocklov J. Vectorial capacity of Aedes aegypti: effects of temperature and implications for global dengue epidemic potential. PLoS One. 2014;9(3):e89783.

11. Siraj AS, Santos-Vega M, Bouma MJ, Yadeta D, Ruiz Carrascal D, Pascual M. Altitudinal changes in malaria incidence in highlands of Ethiopia and Colombia. Science. 2014;343(6175):1154-8.

12. Murdock CC, Paaijmans KP, Bell AS, King JG, Hillyer JF, Read AF, et al. Complex effects of temperature on mosquito immune function. Proc Biol Sci. 2012;279(1741):3357-66.

13. Adelman ZN, Anderson MA, Wiley MR, Murreddu MG, Samuel GH, Morazzani EM, et al. Cooler temperatures destabilize RNA interference and increase susceptibility of disease vector mosquitoes to viral infection. PLoS Negl Trop Dis. 2013;7(5):e2239.

14. Delatte H, Gimonneau G, Triboire A, Fontenille D. Influence of temperature on immature development, survival, longevity, fecundity, and gonotrophic cycles of Aedes albopictus, vector of chikungunya and dengue in the Indian Ocean. J Med Entomol. 2009;46(1):33-41.

15. Mordecai EA, Paaijmans KP, Johnson LR, Balzer C, Ben-Horin T, de Moor E, et al. Optimal temperature for malaria transmission is dramatically lower than previously predicted. Ecol Lett. 2013;16(1):22-30.

16. Kilpatrick AM, Meola MA, Moudy RM, Kramer LD. Temperature, viral genetics, and the transmission of West Nile virus by Culex pipiens mosquitoes. PLoS Pathog. 2008;4(6):e1000092.

17. Murdock CC, Sternberg ED, Thomas MB. Malaria transmission potential could be reduced with current and future climate change. Sci Rep. 2016;6:27771.

18. Ruiz-Moreno D, Vargas IS, Olson KE, Harrington LC. Modeling dynamic introduction of Chikungunya virus in the United States. PLoS Negl Trop Dis. 2012;6(11):e1918.

19. Johansson MA, Powers AM, Pesik N, Cohen NJ, Staples JE. Nowcasting the spread of chikungunya virus in the Americas. PLoS One. 2014;9(8):e104915.

20. Zhang Q, Sun K, Chinazzi M, Pastore YPA, Dean NE, Rojas DP, et al. Spread of Zika virus in the Americas. Proc Natl Acad Sci U S A. 2017;114(22):E4334-e43.

21. Willard KA, Demakovsky L, Tesla B, Goodfellow FT, Stice SL, Murdock CC, et al. Zika Virus Exhibits Lineage-Specific Phenotypes in Cell Culture, in Aedes aegypti Mosquitoes, and in an Embryo Model. Viruses. 2017;9(12).

22. Dell AI, Pawar S, Savage VM. The thermal dependence of biological traits. Ecology. 2013;94(5):1205-6.

23. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2017.

24. Bates D, Maechler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. J Stat Softw. 2015;67:1-48.

25. Johnson LR, Ben-Horin T, Lafferty KD, McNally A, Mordecai E, Paaijmans KP, et al. Understanding uncertainty in temperature effects on vector-borne disease: a Bayesian approach. Ecology. 2015;96(1):203-13.

26. Plummer M, editor JAGS: A program for analysis of Bayesian graphical models using Gibbs sampling. Proceedings of the 3rd International Workshop on Distributed Statistical Computing; 2003; Vienna, Austria.

27. Plummer M. rjags: Bayesian Graphical Models using MCMC. R package version 4-6. 2016.

28. Bacaër N, Guernaoui S. The epidemic threshold of vector-borne diseases with seasonality. Journal of Mathematical Biology. 2006;53(3):421-36.

29. Bacaër N. Approximation of the basic reproduction number Ro for vector-borne diseases with a periodic vector population. Bulletin of Mathematical Biology. 2007;69(3):1067-91.

30. Bacaër N, Ait Dads EH. On the biological interpretation of a definition for the parameter Ro in periodic population models. Journal of Mathematical Biology. 2012;65(4):601-21.

31. Diekmann O, Heesterbeek JAP, Roberts MG. The construction of next-generation matrices for compartmental epidemic models. Journal of the Royal Society Interface. 2010;7(47):873-85.

32. Parham PE, Michael E. Modeling the effects of weather and climate change on malaria transmission. Environmental Health Perspectives. 2010;118(5):620-6.

33. Diekmann O, Heesterbeek JAP, Metz JAJ. On the definition and the computation of the basic reproduction ratio Ro in models for infectious diseases in heterogeneous populations. Journal of Mathematical Biology. 1990;28(4):365-82.

34. Heesterbeek JAP. A brief history of Ro and a recipe for its calculation. Acta Biotheoretica. 2002;50(3):189-204.

35. Heffernan JM, Smith RJ, Wahl LM. Perspectives on the basic reproductive ratio. Journal of the Royal Society Interface. 2005;2(4):281-93.

36. GADM. GADM database of Global Administrative Boundaries. 2012.

37. Hijmans RJ. raster: Geographic Data Analysis and Modeling2016.

38. Bivand R, Lewin-Koh N. maptools: Tools for Reading and Handling Spatial Objects2017.

39. Ryan SJ, McNally A, Johnson LR, Mordecai EA, Ben-Horin T, Paaijmans K, et al. Mapping Physiological Suitability Limits for Malaria in Africa Under Climate Change. Vector Borne Zoonotic Dis. 2015;15(12):718-25.

40. ESRI. ArcGIS Desktop. In: Institute ESR, editor. Redlands, CA2015.

41. Siraj AS, Rodriguez-Barraquer I, Barker CM, Tejedor-Garavito N, Harding D, Lorton C, et al. Spatiotemporal incidence of Zika and associated environmental drivers for the 2015-2016 epidemic in Colombia. Scientific data. 2018;5:180073.

42. Siraj AS, Rodriguez-Barraquer I, Barker CM, Tejedor-Garavito N, Harding D, Lorton C, et al. Data from: Spatiotemporal incidence of Zika and associated environmental drivers for the 2015-2016 epidemic in Colombia. Dryad Data Repository. 2018.

43. Parham PE, Waldock J, Christophides GK, Hemming D, Agusto F, Evans KJ, et al. Climate, environmental and socio-economic change: weighing up the balance in vector-borne disease transmission. Philos Trans R Soc Lond B Biol Sci. 2015;370(1665):20130551.

44. Zouache K, Fontaine A, Vega-Rua A, Mousson L, Thiberge JM, Lourenco-De-Oliveira R, et al. Three-way interactions between mosquito population, viral strain and temperature underlying chikungunya virus transmission potential. Proc Biol Sci. 2014;281(1792).

45. Johansson MA, Arana-Vizcarrondo N, Biggerstaff BJ, Staples JE. Incubation periods of Yellow fever virus. Am J Trop Med Hyg. 2010;83(1):183-8.

46. Houk EJ, Obie F, Hardy JL. Peritrophic membrane formation and the midgut barrier to arboviral infection in the mosquito, Culex tarsalis Coquillett (Insecta, Diptera). Acta Trop. 1979;36(1):39-45.

47. Murdock CC, Paaijmans KP, Cox-Foster D, Read AF, Thomas MB. Rethinking vector immunology: the role of environmental temperature in shaping resistance. Nat Rev Microbiol. 2012;10(12):869-76.

48. Franz AW, Kantor AM, Passarelli AL, Clem RJ. Tissue Barriers to Arbovirus Infection in Mosquitoes. Viruses. 2015;7(7):3741-67.

49. Xiao FZ, Zhang Y, Deng YQ, He S, Xie HG, Zhou XN, et al. The effect of temperature on the extrinsic incubation period and infection rate of dengue virus serotype 2 infection in *Aedes albopictus*. Arch Virol. 2014;159(11):3053-7.

50. Christofferson RC, Chisenhall DM, Wearing HJ, Mores CN. Chikungunya viral fitness measures within the vector and subsequent transmission potential. PLoS One. 2014;9(10):e110538.

51. Johnson FH, Eyring H, Stover BJ. The Theory of Rate Processes in Biology and Medicine. Eyring H, Stover BJ, editors. New York: Wiley; 1974.

52. Shapiro LLM, Whitehead SA, Thomas MB. Quantifying the effects of temperature on mosquito and parasite traits that determine the transmission potential of human malaria. PLoS Biol. 2017;15(10):e2003489.

53. Brady OJ, Godfray HC, Tatem AJ, Gething PW, Cohen JM, McKenzie FE, et al. Adult vector control, mosquito ecology and malaria transmission. Int Health. 2015;7(2):121-9.

54. Paaijmans KP, Blanford S, Chan BH, Thomas MB. Warmer temperatures reduce the vectorial capacity of malaria mosquitoes. Biol Lett. 2012;8(3):465-8.

55. Morin CW, Comrie AC, Ernst K. Climate and Dengue Transmission: Evidence and Implications. Environ Health Perspect. 2013;121(11-12):1264-72.

56. Christofferson RC, Mores CN. Potential for extrinsic incubation temperature to alter interplay between transmission potential and mortality of dengue-infected *Aedes aegypti*. Environ Health Insights. 2016;10:119-23.

57. Murdock CC, Evans MV, McClanahan TD, Miazgowicz KL, Tesla B. Fine-scale variation in microclimate across an urban landscape shapes variation in mosquito population dynamics and the potential of Aedes albopictus to transmit arboviral disease. PLoS Negl Trop Dis. 2017;11(5):e0005640.

58. Gloria-Soria A, Armstrong PM, Powell JR, Turner PE. Infection rate of Aedes aegypti mosquitoes with dengue virus depends on the interaction between temperature and mosquito genotype. Proc Biol Sci. 2017;284(1864).

59. Chouin-Carneiro T, Vega-Rua A, Vazeille M, Yebakima A, Girod R, Goindin D, et al. Differential Susceptibilities of Aedes aegypti and Aedes albopictus from the Americas to Zika Virus. PLoS Negl Trop Dis. 2016;10(3):e0004543.

60. Carrington LB, Seifert SN, Willits NH, Lambrechts L, Scott TW. Large diurnal temperature fluctuations negatively influence Aedes aegypti (Diptera: Culicidae) life-history traits. J Med Entomol. 2013;50(1):43-51.

61. Lambrechts L, Paaijmans KP, Fansiri T, Carrington LB, Kramer LD, Thomas MB, et al. Impact of daily temperature fluctuations on dengue virus transmission by Aedes aegypti. Proc Natl Acad Sci U S A. 2011;108(18):7460-5.

62. Worner SP. Performance of Phenological Models Under Variable Temperature Regimes: Consequences of the Kaufmann or Rate Summation Effect. Environ Entomol. 1992;21(4):689-99.

CHAPTER 5

COOL TEMPERATURES DIMINISH ZIKA VIRUS REPLICATION IN MOSQUITO CELLS [3]

[3] Tesla B., Scott J.E., Murdock C.C., Brindley M.A. To be submitted to Journal of Virology.

Abstract

With half of the human population at risk, arboviral diseases represent a substantial global health burden. Zika virus is a mosquito-borne flavivirus known to cause severe birth defects and neuroimmunological disorders. Temperature is one of the strongest drivers of vector-borne disease transmission. We have previously demonstrated that Zika virus transmission is optimized at 29°C with a thermal range of 22.7°C to 34.7°C. Although there is substantial evidence of temperature effects on arbovirus replication and dissemination inside mosquitoes, little is known about whether temperature affects virus replication directly or indirectly through mosquito physiology. In order to determine the mechanisms behind temperature-induced changes in Zika transmission potential, we investigated Zika virus replication rates in mosquito (C6/36) cells across six temperatures (16°C, 20°C, 24°C, 28°C, 32°C, and 36°C). Similar to our *in vivo* study, Zika virus replication was diminished at cool temperatures. By investigating the effects of temperature on different steps of the virus replication cycle, we found that cool temperatures limit ZIKV replication after viral entry but before genome replication. We also demonstrated that this phenotype is specific to the Asian-lineage of ZIKV and is not observed among African-lineage ZIKV strains at 20°C.

Introduction

Diseases such as Zika, dengue, and chikungunya, once considered tropical and sub-tropical diseases, have spread explosively throughout the world due to climate change, globalization, and increased urbanization. With half of the human population at risk, arboviral diseases represent a substantial global health burden (1). Zika virus (ZIKV) is a mosquito-borne flavivirus known to cause sporadic and mild infections in humans. In 2015, ZIKV emerged in the Americas and within a year quickly spread to approximately 65 countries worldwide, resulting in over 360,000 suspected cases (2). Shortly after it was linked to birth defects (3) and neuroimmunological disorders (Guillain–Barré syndrome) (4), ZIKV was declared a "public health emergency of international concern" (5). With no therapeutics or vaccines to mitigate disease, ZIKV was quickly put in the forefront of research interest to fulfill the urgent need to better understand disease transmission, pathogenesis, and prevention.

Temperature is one of the strongest drivers of vector-borne disease transmission due to its profound impact on ectothermic mosquito vectors, pathogens, and their interactions (6). Numerous studies have investigated the effects of temperature on mosquito infection, the extrinsic incubation period, and overall transmission potential in a diversity of vector-borne disease systems (7-10). Although the exact mechanisms of how temperature shapes arboviral transmission are rarely elucidated, temperature can either affect virus replication directly, or indirectly by altering mosquito physiology, immunity, and cellular processes. Ambient temperature influences every aspect of mosquito life, from development and size, to its physiology, immunity, and metabolism, as well as behavior, distribution, and abundance, all of which can affect virus transmission. Studies

have shown that mosquitoes reared at low temperatures are more susceptible to some viruses (11, 12), potentially due to destabilized RNAi pathways (13), while warm temperatures can positively impact mosquito immune performance (14). Temperature can alter cellular processes such as metabolism, protein synthesis, enzymatic activity, and membrane fluidity, and induce heat- shock responses, which can further facilitate or hinder virus replication. Even though arboviruses evolved to replicate across a wide range of temperatures, from within invertebrate vectors to febrile mammalian and avian hosts, temperature can also modify virus replication. Studies have shown that temperature can alter virus structure and induce the fluctuation of viral surface proteins required for entry in the host cell. Dengue virus (DENV), for example, has a distinct structure at temperatures characteristic for mammalian host in comparison to those in the invertebrate host (15, 16). Although few studies have investigated ZIKV structure and thermal stability (17-19), no studies have investigated how temperature affects ZIKV replication.

In our previous work, we demonstrated that ZIKV transmission in *Aedes aegypti* mosquitoes was optimized at 29°C and had a thermal range of 22.7°C to 34.7°C. Although warm temperatures facilitated rapid virus replication, increased mosquito mortality decreased transmission potential. Cool temperatures, however, prevented the mosquitoes from becoming infectious due to poor midgut infection and escape (20). In order to understand the mechanisms of reduced ZIKV transmission potential at sub-optimal temperatures, we investigated ZIKV replication *in vitro* using mosquito cells incubated at cool temperatures. We formulated two hypotheses to address our observations that are not mutually exclusive: 1) The stress from sub-optimal temperatures alters the cellular environment, either limiting host factors necessary for viral replication

or overproducing an inhibitory factor not produced at permissive temperatures. 2) The sub-optimal temperatures prevent a viral function required to complete the viral replication cycle and produce progeny virus. By identifying the step in the virus replication cycle that is inhibited in cool conditions, we will provide the field with a deeper understanding of the host-pathogen interactions. Understanding the link between pathogen replication and environmental conditions can potentially be exploited to develop new vector control strategies in the future. These results will also have implications in modeling the temperature suitability for arbovirus transmission as global environmental patterns change.

Methods

Cell lines and viruses

C6/36 mosquito cells (ATCC CRL-1660) from *Ae. albopictus* were maintained in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (FBS) at 28°C. Some C6/36 cells were adapted to grow at 20°C. The cells were initially maintained in L-15 medium with the higher FBS content (20%) and the amount of FBS was reduced to 10% over several months. Vero (African green monkey kidney) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 5% FBS at 37°C, 5% CO_2.

In all experiments, we used the tenth passage of a ZIKV Mex 1-44 strain that was isolated from a field-caught *Ae. aegypti* mosquito in Chiapas, Mexico in 2016, and obtained from the University of Texas Medical Branch. Another Asian-lineage ZIKV strain used in this study was SPH from a Brazilian clinical sample in 2015, obtained from Oswaldo Cruz Foundation. Two African-lineage isolates, MR766 and IbH, were

purchased from American Type Culture Collection (catalog number VR-1838™ and VR-1829™, respectively). The DENV-2 isolate (PRS 225488) used in the study originated from human serum in Thailand in 1974. All ZIKV and DENV stocks were generated and titrated in Vero cells. Attenuated CHIKV strain (181/25) was generated in 293T cells and titrated in Vero cells. All ZIKV isolates tested negative for *Mycoplasma* contamination using a MycoSensor PCR Assay Kit (Agilent).

Determination of viral titers

All viral titers were determined using the 50% tissue culture infectious dose ($TCID_{50}$) method. Briefly, Vero cells were seeded in 96-well tissue culture plates and inoculated with virus diluted in DMEM. Viruses were serially diluted (10-fold) six to eight times before they were transferred onto Vero cells. Cells infected with ZIKV were observed and scored for cytopathic effect on day six after inoculation, and cells infected with DENV and CHIKV were scored on days seven and four, respectively. The number of wells showing cytopathic effect were quantified and converted into $TCID_{50}$/mL units using the Spearman-Karber formula.

Virus replication curves

C6/36 cells were seeded in 12-well plates at a density of 4×10^5 cells/mL several hours prior to infection. Cells were inoculated with ZIKV using a multiplicity of infection (MOI) of 0.1, DENV with an MOI of 0.1, or CHIKV with an MOI of 0.005. Plates were incubated for 2 hours at 28°C, then the infectious media was removed and replaced with fresh media. The cells and media were kept at the following constant temperatures: 16°C, 20°C, 24°C, 28°C, 32°C, and 36°C. Every 24 hours, half of the supernatant was collected

and replaced with fresh media equilibrated to each temperature, for a period of ten days. Similarly, Vero cells were plated at a density of 2.5×10^5 cells/mL and inoculated with ZIKV (MOI 0.1) for one hour at 37°C. The cells were incubated at one of six constant temperatures: 28°C, 32°C, 37°C, 39°C, 40°C, and 41°C, and half of the cell supernatant was collected every 24 hours for seven days. ZIKV, DENV, and CHIKV replication rates were also assessed in C6/36 cells adapted to grow at 20°C. The cells were infected in the same manner as previously described. 20°C-adapted cells were infected and kept at 20°C throughout the experiment, while unadapted cells were infected at 28°C and kept at 20°C or 28°C. Every two days for ten days, half of the media was collected and replaced with fresh media equilibrated to 20°C or 28°C. Replication rates of ZIKV Mex 1-44 strain were compared to SPH, MR766, and IbH. The cells were infected at an MOI of 0.1 for two hours at 28°C and incubated at either 20°C or 28°C for ten days. Cell supernatant was collected every two days. All replication curves were replicated three times and virus titers were determined using $TCID_{50}$ method on Vero cells as described above.

Cell proliferation assay

C6/36 cells were stained using the CellTrace™ Violet Cell Proliferation Kit (Invitrogen) as per manufacturer's instructions and plated at a density of 4×10^5 cells/mL in 24-well plates. Once the cells adhered to the plates, they were infected with ZIKV, DENV, or CHIKV as previously described and incubated at each of the six temperatures (16°C-36°C) for four days. One well contained uninfected cells in order to assess how temperature affects cell proliferation. Intracellular fluorescence, which decreases with every generation, was determined using flow cytometry and normalized to uninfected

cells that were stained and processed on day 0. This assay was also used to compare the cell proliferation of 20°C-adapted cells and unadapted cells when maintained at 20°C for 8 days. Fluorescence was measured every two days and normalized to cells maintained at 28°C.

Cell viability assay

C6/36 cells were plated at a density of 4×10^5 cells/mL and infected with ZIKV, DENV, or CHIKV as was described above. After six days of incubation at different temperatures (16°C-36°C), cell viability was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) and a GloMax plate reader (Promega) as per the manufacturer's instructions. Cell viability was normalized to uninfected cells maintained at 28°C.

ZIKV spread

C6/36 cells with established ZIKV infection were maintained in a T-75 flask at 28°C for several weeks. 1 mL of cell supernatant was collected on two consecutive days, after which it was removed, and the cells were seeded in two 6-well plates at a density of 6×10^5 cells/mL. Each plate was incubated at either 20°C or 28°C for five days. Cell supernatant was collected and replaced with 2 mL of fresh media every 24-hours. Similarly, C6/36 cells were seeded in a T-25 flask at a density of 6×10^5 cells/mL. The following day, the cells were infected with ZIKV (MOI 0.1) for two hours at 28°C. After incubation, the infectious media was removed, and the cells were washed once before fresh media was added. 1 mL of cell supernatant was collected immediately and after 24 and 48 hours. After the third collection, cell supernatant was removed, and the cells were

washed and seeded in two 6-well plates. As with persistently infected cells, one plate was incubated at 20°C while the other was incubated at 28°C. Every 24-hours for five days, cell supernatant was collected and replaced with 2 mL of fresh media. The same experimental setup was repeated, except that cells were distributed to each temperature after 36 hours instead of 48 hours. All three experimental settings were done in three replicates and virus titers were determined using the $TCID_{50}$ method on Vero cells.

Time of addition assay

C6/36 cells were plated at a density of 6×10^5 cells/mL and infected with ZIKV (MOI 0.1). Chlorpromazine (10 μM) or ammonium chloride (25 mM) was added to the cells for four hours at the following time points: 2 hours prior to infection, during infection, or 2, 4, 6, 8, 12, or 16 hours post-infection. After 48 hours, cell supernatant was collected, and virus titers were determined using $TCID_{50}$.

ZIKV entry

We assessed ZIKV entry at a sub-optimal temperature using two distinct approaches. In the first experiment, 6×10^5 C6/36 cells/mL were infected with ZIKV (MOI 0.1) for two hours. ZIKV-infected C6/36 cells were transferred from 28°C to 20°C at 2 hours prior to infection, during infection, or 2, 4, 6, 8, 12, or 16 hours post-infection, and cell supernatants were collected after 96 hours. In the second experiment, the same number of C6/36 cells were incubated at 20°C two hours prior to infection. Cells were inoculated with ZIKV (MOI 1) and moved to 28°C at 2, 4, or 6 hours post-infection. As a control, one set of cells was maintained at 20°C while another was maintained at 28°C. Cell supernatants were collected after 48 hours and titers were determined using $TCID_{50}$.

Results

ZIKV, DENV, and CHIKV replication curves at different temperatures

To characterize the effect of temperature on ZIKV replication in mosquito cells, we infected C6/36 cells with ZIKV and incubated them at six temperatures ranging from 16°C to 36°C (Fig 5.1A). Initial ZIKV replication and peak titers occurred more quickly at higher temperatures, while virus replication was limited at cool temperatures. Incubation at 20°C resulted in delayed particle release and a more than 5-log reduction in peak titers, and incubation at 16°C resulted in almost complete inhibition of virus production in C6/36 cells over the 10-day period. ZIKV replication rates increased at 36°C within the first few days, however the virus titers subsequently diminished. Although virus replication was reduced at 36°C in mosquito cells over time, this phenotype resulted from temperature-related cellular stress, and was not observed in ZIKV-infected mammalian cells that optimally produce virus at 37°C. To demonstrate that the decrease in viral titers at 36°C in mosquito cells are due to cellular heat-shock, we incubated ZIKV-infected Vero cells at even higher temperatures (Fig 5.1B). ZIKV peak titers were similar at 37°C, 39°C and 40°C in Vero cells; however, virus production was inhibited at 41°C, due to heat-induced cell death. While virus replication peaked at 2.67×10^9 $TCID_{50}$/mL at 28°C in mosquito cells, the peak titer was 3.5-log lower at 28°C in Vero cells. In comparison, virus titers were significantly reduced at 36°C in C6/36 cells, yet virus replication peaked at 37°C in Vero cells (5.05×10^7 $TCID_{50}$/mL), suggesting that virus replication is determined by optimal intracellular environment at these temperatures.

Next, we compared ZIKV replication across six temperatures to another flavivirus (DENV) and to an alphavirus (CHIKV). DENV had similar replication dynamics to ZIKV (Fig 5.1C), yet DENV replicated significantly better at cool temperatures, reaching 1.45×10^3 $TCID_{50}$/mL at 16°C and 3.74×10^6 $TCID_{50}$/mL at 20°C. CHIKV replication kinetics and its response to temperature greatly differed relative to both flaviviruses (Fig 5.1D). CHIKV replication proceeded at a faster rate, and temperature had unimodal and not linear effect on virus yields within first few days of infection. Interestingly, CHIKV peak $TCID_{50}$ values were similar at 28°C and 20°C, suggesting C6/36 cells are capable of producing virus particles at 20°C; however, cellular responses to low temperatures might affect ZIKV and CHIKV replication differently.

Temperature and virus effects on cell proliferation and viability

Temperature profoundly affects cell physiology and metabolism, and it can therefore alter cell proliferation and viability. To investigate the effect of temperature and virus infection on cells, we examined cell proliferation on day 4 and cell viability on day 6 after exposure. The number of generations increased proportionally with temperature with the exception of cells incubated at 36°C, where cell proliferation decreased (Fig 5.2A). While DENV infection did not affect cell proliferation at any temperature, ZIKV infection reduced the number of generations at 32°C and CHIKV decreased cell proliferation at all temperatures. Sub-optimal and high temperatures also affected cell viability, represented by lower ATP levels, after cells were exposed for six days (Fig 5.2B). CHIKV was more cytopathic than ZIKV and DENV across all temperatures, while ZIKV affected cell viability at warm temperatures. DENV-infected cells showed little-to-no reduction in ATP levels in comparison to uninfected cells.

ZIKV, DENV, and CHIKV replication in 20°C-adapted C6/36 cells

When performing the replication curves across the temperature range, cells normally maintained at 28°C were infected and transferred to the different temperature treatments. Therefore, cells were subjected to the temperature change during the initial infection time and may have triggered acute cellular responses that would not be present if cells were maintained at the various temperature. To determine if virus replication was inhibited at 20°C due to acute cellular stress responses, we adapted C6/36 cells to grow at 20°C. After maintaining C6/36 cells at 20°C for several months, we compared the adapted cell morphology to normal cells and observed no changes. We also determined that 20°C-adapted cells proliferated faster at 20°C than the cells that were shifted from 28°C to 20°C (Fig 5.3A). We then investigated ZIKV, DENV, and CHIKV replication in 20°C-adapted cells and found there was no difference in virus yields at 20°C between the cells undergoing cool temperature stress and the cells that were adapted to these environmental conditions (Fig 5.3B-D). This suggests that the reduced ZIKV replication was not a result of acute cellular stress brought on by the temperature shift.

Effects of sub-optimal temperatures on ZIKV spread

We next sought out to determine which part of the virus replication cycle is affected by cool temperatures. We first examined if cells with established ZIKV infection are capable of producing infectious virus particles at 20°C (Fig 5.4A). Persistently infected C6/36 were distributed into two dishes, and one was transferred to 20°C while the other was kept at 28°C. Interestingly, the amount of infectious virus produced every 24 hours for five days was similar at 20°C and 28°C. We observed a slight decrease in

virus yields, which was likely due to decreased cellular metabolism at 20°C. Overall, this suggests that later steps in virus replication, such as transcription, translation, budding and maturation are not inhibited at cool temperatures.

Next, we infected C6/36 cells at an MOI of 0.1 and incubated them at 28°C for 48 or 36 hours, during which ZIKV infection was established in a small proportion of cells (Fig 5.4B-C). The cells were then split and incubated at 20°C or 28°C for five more days. While virus titers increased 2 to 3 logs at 28°C, virus titers plateaued in C6/36 cells maintained at 20°C. Taken together, these data suggest that the cells with an established infection are capable of producing infectious particles, however the virus produced might not be able to efficiently establish infection in uninfected cells at 20°C.

Effects of sub-optimal temperatures on ZIKV entry

In order to elucidate the kinetics of ZIKV internalization and fusion in C6/36 cells, infected cells were treated with chlorpromazine or ammonium chloride before, during or after infection. Chlorpromazine is known to inhibit clathrin-mediated endocytosis, an endocytic pathway for ZIKV entry. Ammonium chloride is a weak base that increases the endosomal pH and inhibits pH-dependent fusion. Since neither chlorpromazine nor ammonium chloride were previously characterized in C6/36 cells during ZIKV infection, we first identified the concentrations at which both drugs reduced virus entry while remaining non-cytotoxic. We then performed a time-of-addition assay to determine the dynamics of early replication steps in C6/36 cells (Fig 5.5A). We treated cells with chlorpromazine every two hours, ranging from 2 hours prior to infection to 16 hours post-infection, to determine when ZIKV internalization occurs. Chlorpromazine

decreased virus titers when added prior-to or during infection. However, adding the compound 2 hours post-infection or later did not have an effect on virus yields. In order to determine the timing of ZIKV fusion in C6/36 cells, we treated cells with ammonium chloride at the same times as indicated above. Ammonium chloride inhibited virus fusion and decreased viral titers only when added before or during infection, suggesting that ZIKV enters C6/36 cells within first two hours of infection at the optimal temperature.

Next, we wanted to evaluate the effect of transferring infected cells to 20°C during entry on virus yields (Fig 5.5B). If the cells were kept at 20°C prior to, during, or up to 4 hours post-infection, no virus was detected in the supernatant after 96 hours. Infected cells needed to be incubated at 28°C for at least the first 8 hours in order to detect virus particles in the supernatant, and the amount of time cells were maintained at the optimal temperature was proportional to virus yields. Alternatively, if we kept cells at 20°C for 2, 4, or 6 hours post-infection, there was no difference in virus titers in comparison to the cells maintained at 28°C (Fig 5.5C). In the cells that were infected and maintained at 20°C for the duration of the experiment, the virus yields were more than 3 logs lower than cells transferred to 28°C. Taken together, these data suggest that temperature impairs the virus replication cycle after fusion yet before genome replication.

Replication curves of different ZIKV strains at cool temperatures

Lastly, we wanted to explore if limited virus replication in C6/36 cells at 20°C was common among different ZIKV strains. We compared replication curves of ZIKV Mex 1-44 to another Asian-lineage strain, SPH, and to two African-lineage strains, MR766 and IbH (Fig 5.6A-B). Interestingly, the observed phenotype was specific for

Asian-lineage strains. While Mex 1-44 and SPH yields decreased more than 2.5 and 3.5 logs at 20°C, respectively, there were no differences in virus yields between 20°C and 28°C among African-lineage strains.

Discussion

Transmission of arboviruses is shaped by environmental temperature as it affects mosquito vectors, viruses, and their interaction. Many studies have investigated how temperature influences arboviral transmission (9, 11, 21-24), and we were first to specifically characterize the relationship between environmental temperature and ZIKV transmission in *Ae. aegypti* mosquitoes (20). We demonstrated that ZIKV transmission is optimized at 29°C with a thermal range of 22.7°C-34.7°C. By comparing thermal suitability models between DENV-2 and Mexican isolate of ZIKV, we found that predicted thermal minimum for ZIKV is 5°C warmer than that of DENV. We hypothesized that sub-optimal temperatures alter intracellular environment which consequently inhibits virus replication and/or that temperature directly inhibits virus replication. We demonstrated that ZIKV replication is inhibited at sub-optimal temperatures even when cells are not undergoing acute temperature stress, and we further clarified the steps of the virus replication cycle that are most affected by cool temperatures. By exposing ZIKV-infected cells to 20°C at different times during infection and monitoring the virus yields, we concluded that virus replication is inhibited after virus enters the cells but before genome replication begins.

After exposing ZIKV-infected C6/36 cells to the same temperatures we previously exposed ZIKV-infected *Ae. aegypti* mosquitoes (20), we observed similar trends in our *in vitro* and *in vivo* experiments. Despite faster initial replication at high

temperatures, virus yields decreased due to poor cell viability, while virus replication at cool temperatures was slow and limited. Although temperature causes systemic changes in mosquitoes that alter virus replication, such as altered physiology, immunity or blood meal digestion (e.g. peritrophic membrane formation and managing oxidative stress) (14, 25, 26), the effect of temperature on ZIKV replication was still visible at the cellular level. Temperature also alters intracellular processes, pathways, enzymatic activity, cell metabolism, membrane fluctuations, cell viability, and induces temperature-shock responses, all of which can indirectly affect virus replication.

The initial increases in replication rates of ZIKV and DENV we observed at high temperatures could be due to increased cellular metabolism, enzymatic activity and fluidity of cellular membranes. Moreover, there is evidence that heat-shock response can facilitate virus entry and replication. Heat-shock proteins (HSP) that play an important role in protein folding and protection during stress are part of a receptor complex for multiple viruses, including ZIKV and DENV (27, 28). Hsp70 was shown to mediate ZIKV entry, replication, and egress in mammalian cells (27), while antibodies against 70-kD, 80-kD, and 90-kD proteins in C6/36 cells blocked entry of DENV, West Nile, and Japanese encephalitis virus (29). However, prolonged heat-shock response affects cell viability (Fig 5.2B), which likely contributed to the decrease in virus yields we observed after initial infection. The higher temperatures that reduced ZIKV titers in mosquito cells did not affect virus titers in mammalian cells, which are accustomed to exposure to these higher temperatures. Cold temperatures, on the other hand, diminished virus replication regardless of cellular adaptation to these temperatures. Although cold temperatures decrease cellular metabolism, enzymatic activity, membrane fluidity (30, 31), none of

these potential alterations in the intracellular environment seemed to inhibit CHIKV or DENV replication in C6/36 cells at 20°C to the same extent that ZIKV replication was inhibited. Taking into consideration that CHIKV can reach same peak titers at 20°C and 28°C, and that ZIKV replication is significantly less efficient at 20°C even in 20°C-adapted cells, these data suggest that reduced ZIKV replication at sub-optimal temperature is not caused by the stress-induced altered cellular environment, but could be due to a direct effect of temperature on virus replication.

Temperature can also affect the stability of virus particles and alter viral protein conformational changes necessary for virus entry and maturation. Both the *in vitro* and *in vivo* studies demonstrated that DENV replication is more efficient at cool temperatures in comparison to ZIKV. ZIKV is known to have tighter packing of E-proteins, resulting in higher thermal stability compared to DENV (32). Since the most characterized ZIKV entry mechanism is utilizing a phosphatidylserine (PS) receptor (33), exposure of the viral envelope underneath the tightly packed E and M proteins is crucial for viral entry. Temperature can alter the conformational fluctuation of viral glycoproteins on the surface of the particle (also known as "breathing") exposing PS on the membrane (34). Temperature can also influence virus structure. Cryo-electronic microscopy studies have shown that DENV's structure differs depending on its incubation in human host temperatures versus mosquito host temperatures (15, 35). DENV displays a "bumpy" surface at 37°C and a "smooth" surface at 28°C. The virus' ability to switch from "smooth" to "bumpy" is caused by the destabilization in the multiple regions of the E protein that can cause large scale changes on the surface of the particle (16).

By adding the chlorpromazine and ammonium chloride at different times after ZIKV-infection, we established that ZIKV enters C6/36 cells within two hours at optimal temperature. However, incubating the cells at 20°C during the first 6 hours of infection did not affect virus yields. On the other hand, we have demonstrated that incubating the cells at 28°C for the first eight hours of the infection is necessary to detect infectious virus particles in the supernatant. Since cells with an established infection can produce a similar number of infectious particles at 20°C and 28°C, suggests the later steps in the virus replication cycle, such as transcription, translation, budding and maturation are not inhibited at cool temperatures. Before genome replication, ZIKV must induce extensive ultrastructural changes and rearrangement of the intracellular membranes to provide the platforms required for formation of replication complexes (36, 37). Replication inside membrane compartments concentrates the molecules necessary for replication and particle assembly, but also helps evade host antiviral defense mechanisms (38).

Studies have shown that DENV utilizes fatty acid biosynthesis and that DENV-infected cells are enriched with lipids that have the capacity to destabilize and change the curvature and permeabilization of the membranes (39). DENV NS3 protein redistributes fatty acid synthase (FAS) to the site of viral replication and increases fatty acid synthesis. Inhibition of FAS causes intracellular lipid redistribution and significantly reduces DENV replication in both mosquito and mammalian cells especially when induced 8 hours post-infection (40). Since we observed that cool temperatures limited ZIKV replication post-entry, but before establishing replication, we propose that sub-optimal temperatures affect the ability of ZIKV NS3 protein to increase fatty acid synthesis and

membrane rearrangement, which leads to reduced virus replication and ultimately reduced transmission. However, future experiments will need to confirm this hypothesis.

Surprisingly, cool temperatures in this study affected only Asian-lineage ZIKV strains. While Mex 1-44 and SPH replicated at a slower rate and had lower virus titers at 20°C, MR766 and IbH were not affected by sub-optimal temperatures. African and Asian lineages were previously shown to have different phenotypes in cell culture, *Ae. aegypti* mosquitoes and chicken embryos (41). While African isolates (MR766 and IbH) have faster replication rates in C6/36 cells and induce higher mortality in embryos than Asian-lineage isolates (Mex 1-44 and SPH), they seem to be less infectious to *Ae. aegypti* mosquitoes (41). Moreover, ZIKV isolates from Asian and African lineages displayed different replication kinetics, cytopathic effects, and impacts on human neural progenitor cell function (42, 43), as well as the ability to infect human placental trophoblast (44), and cause severe brain damage (45), *in utero* infection in pigs (46), and disease progression in mice (47).These results suggest that genetic variation between ZIKV isolates can significantly alter experimental outcomes and . There are several amino acid changes in the NS3 protein between African- and Asian-lineage strains (Fig 5.6C) and future experiments should characterize the phenotypes at sub-optimal temperatures. It would also be interesting to see if serial passaging of Asian-lineage strains at cool temperatures could select for the mutants that are adapted to replicate at low temperatures and whether their sequence would be more similar to African-lineage strains.

Understanding transmission dynamics and epidemiology of the infectious diseases is crucial for successfully controlling current and preventing future outbreaks. Characterizing the link between pathogen replication and environmental conditions is

important to better understand how environmental factors shape transmission. This knowledge can be used for modeling temperature suitability and predicting arboviral spread which are necessary for successful implementation of vector-control strategies.

Acknowledgements

We thank the University of Texas Medical Branch Arbovirus Reference Collection for providing the virus and Dr. Ted Ross for providing C6/36 cells. We gratefully thank the members of the Brindley and Murdock labs for thoughtful comments on the project and manuscript.

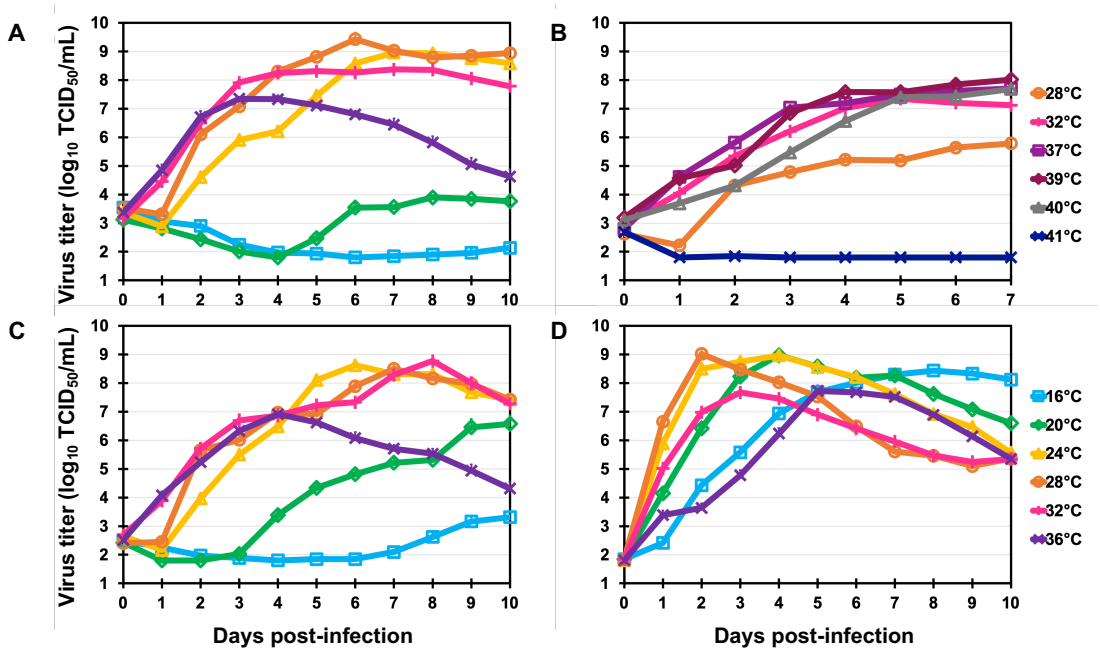


Fig 5.1. Temperature effects on ZIKV, DENV, and CHIKV replication. ZIKV replication rates in C6/36 cells at 16°C, 20°C, 24°C, 28°C, 32°C, and 36°C (A), and in Vero cells at 28°C, 32°C, 37°C, 39°C, 40°C, and 41°C (B). DENV (C) and CHIKV (D) replication rates in C6/36 cells at 16°C, 20°C, 24°C, 28°C, 32°C, and 36°C. Cell supernatants were collected every 24 hours for 10 days in C6/36 cells and every 24 hours for 7 days in Vero cells. Virus titers were determined using $TCID_{50}$ method.

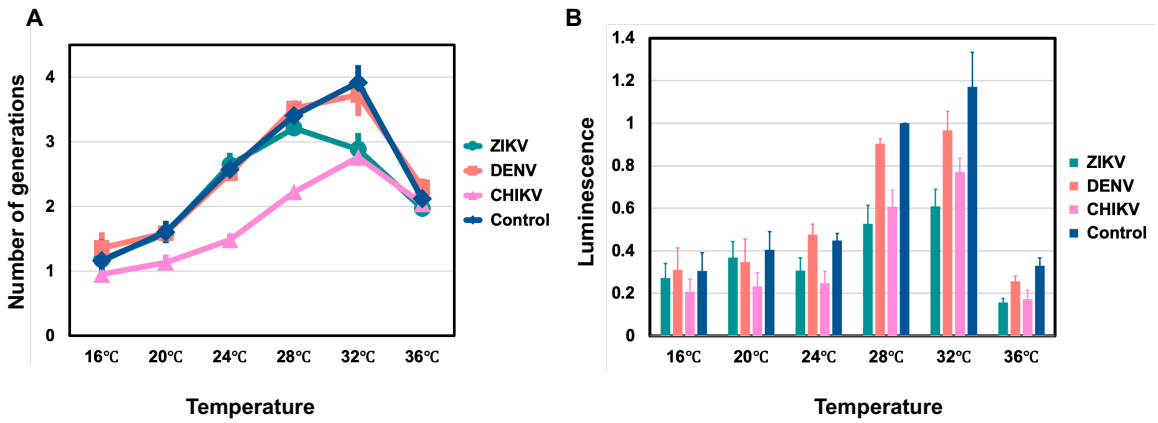


Fig 5.2. Temperature and virus infection effects on cell proliferation and viability. Uninfected or infected C6/36 cells with ZIKV, DENV, or CHIKV were incubated at 16°C, 20°C, 24°C, 28°C, 32°C, and 36°C. Cell proliferation was determined using CellTrace™ dye after four days (A) and cell viability was determined using CellTiter-Glo® after six days (B).

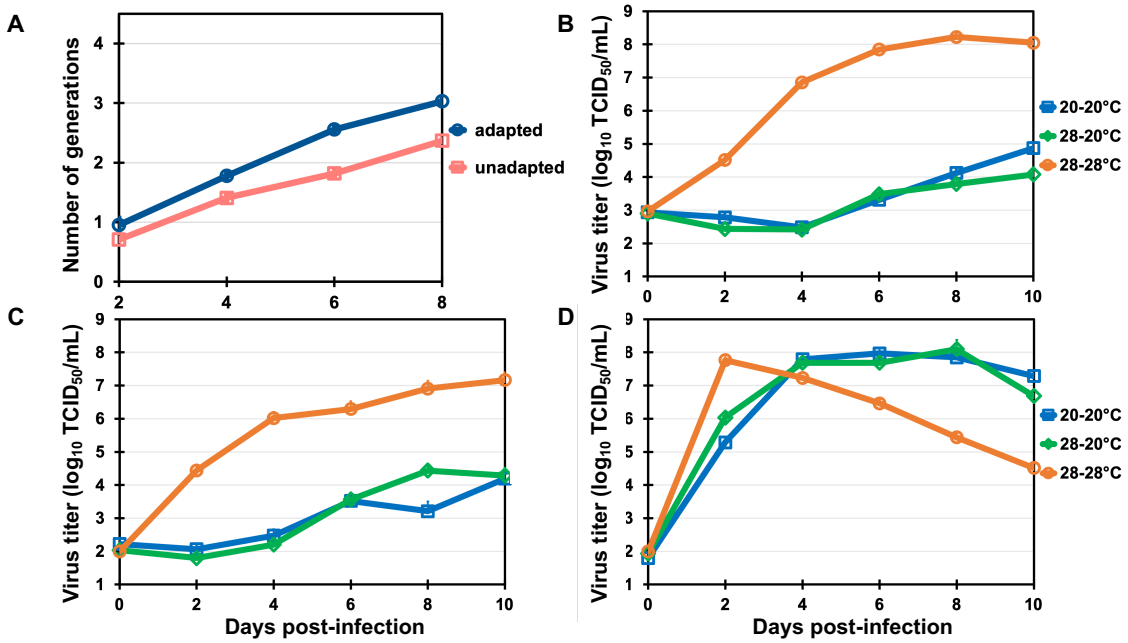


Fig 5.3. Replication curves in 20°C -adapted C6/36 cells. Cell proliferation of 20°C-adapted C6/36 cells in comparison to unadapted cells at 20°C (A). ZIKV (B), DENV (C), and CHIKV (D) replication kinetics in 20°C-adapted cells in comparison to unadapted cells at 20°C and 28°C. Cell supernatants were collected every 48 hours and virus titers were determined using $TCID_{50}$ method.

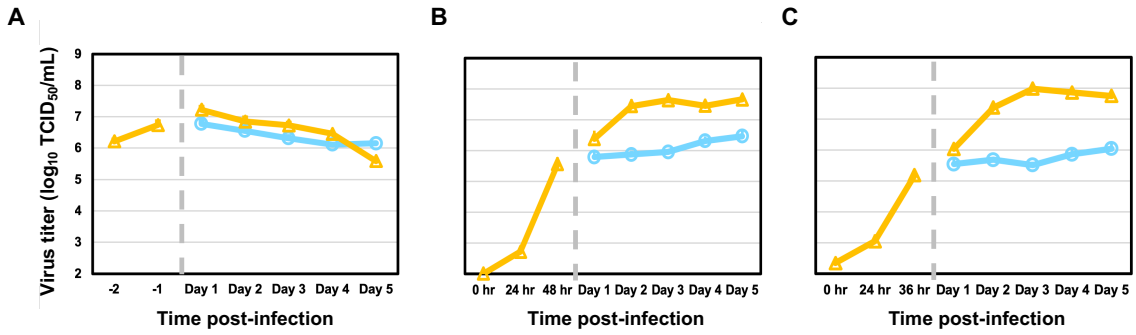


Fig 5.4. ZIKV spread at sub-optimal temperature. C6/36 cells with persistent ZIKV infection were split and incubated at 20°C and 28°C for five days (A). C6/36 cell infected at the MOI of 0.1 were split at 48 (B) or 36 (C) hours post-infection and incubated at 20°C and 28°C for five days. Virus titers were determined using $TCID_{50}$ method.

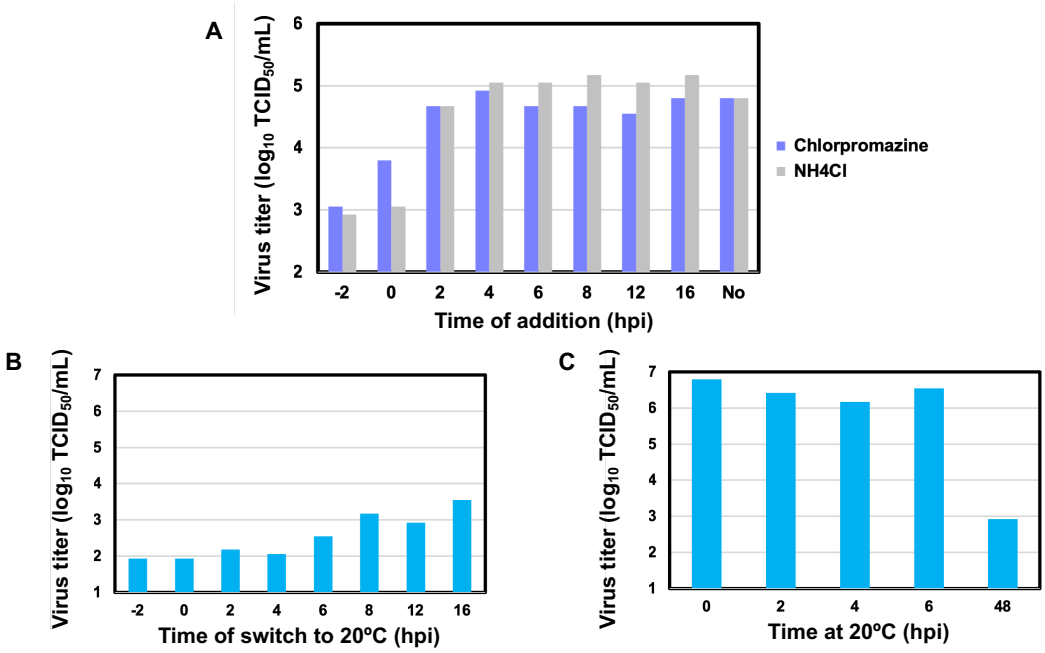


Fig 5.5. ZIKV entry dynamics in C6/36 cells. Chlorpromazine or ammonium chloride were added to C6/36 two hours prior to ZIKV infection, during infection, or 2, 4, 6, 8, 12, and 16 hours post-infection and cell supernatants were collected 48 hours post-infection (A). At the same time points, ZIKV-infected C6/36 cells were transferred from 28°C to 20°C (B) and cell supernatants were collected 96 hours post-infection. C6/36 cell were put to 20°C two hours prior to infection and transferred back to 28°C at 2, 4, or 6 hours post-infection (C). One control group was kept at 28°C and the other was kept at 20°C throughout the experiment. Cell supernatants were collected 48 hours post-infection. Virus titers were determined using $TCID_{50}$ method.

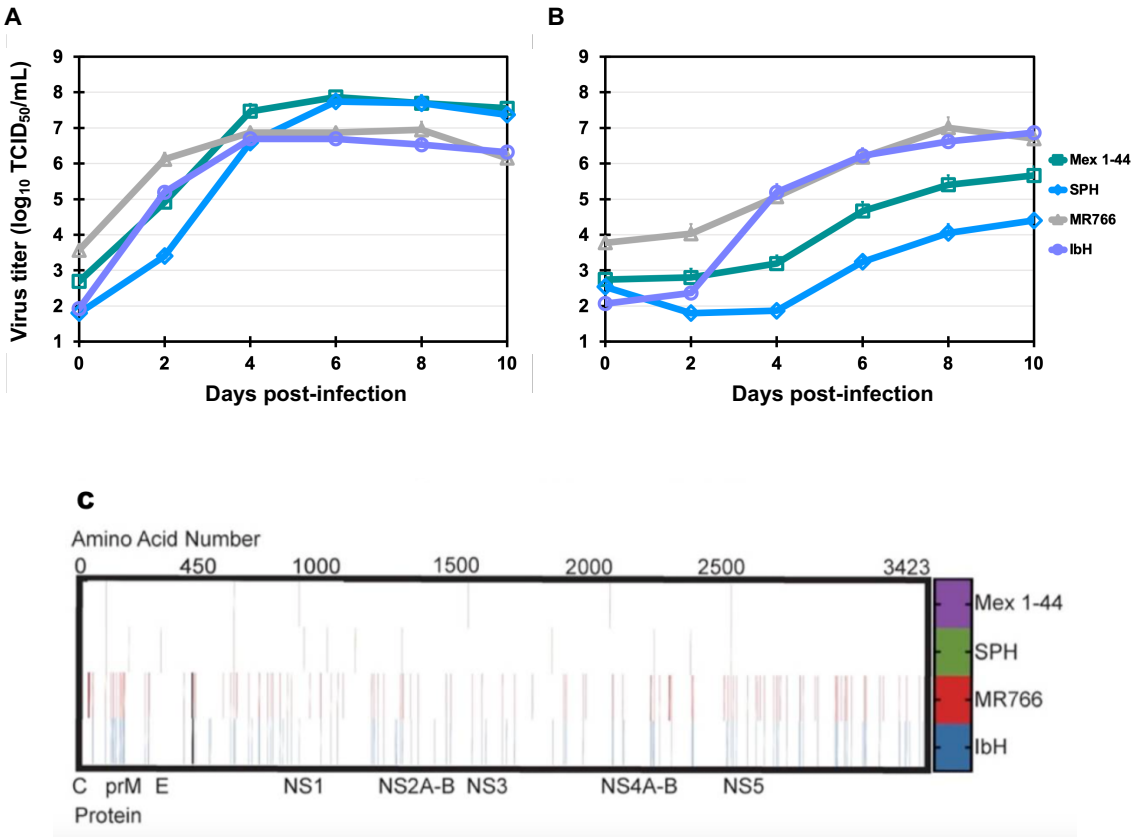


Fig 5.6. Replication of different ZIKV strains at sub-optimal temperatures. C6/36 cells were infected with different Asian-lineage (Mex 1-44 and SPH) or African-lineage (MR766 and IbH) ZIKV strains and incubated at 28°C (A) or 20°C (B) for ten days. Cell supernatants were collected every 48 hours and virus titers were determined using $TCID_{50}$ method. (C) Changes from the ZIKV consensus sequence across ZIKV genome. Each line represents an amino acid change from the standard consensus sequence of ZIKV (PRVABC59- Genbank accession number KX087101.3)

References

1. Brady OJ, Gething PW, Bhatt S, Messina JP, Brownstein JS, Hoen AG, et al. Refining the global spatial limits of dengue virus transmission by evidence-based consensus. PLoS Negl Trop Dis. 2012;6(8):e1760.

2. PAHO. Pan American Health Organisation 2018 [Available from: http://www.paho.org/.

3. Mlakar J, Korva M, Tul N, Popovic M, Poljsak-Prijatelj M, Mraz J, et al. Zika virus associated with microcephaly. N Engl J Med. 2016;374(10):951-8.

4. Cao-Lormeau V-M, Blake A, Mons S, Lastère S, Roche C, Vanhomwegen J, et al. Guillain-Barre Syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. Lancet. 2016;387(10027):1531-9.

5. World Health Organization. WHO statement on the first meeting of the International Health Regulations (2005) (IHR 2005) Emergency Committee on Zika virus and observed increase in neurological disorders and neonatal malformations 2016 [Available from: http://www.who.int/mediacentre/news/statements/2016/1st-emergency-committee-zika/en/.

6. Mordecai EA, Caldwell JM, Grossman MK, Lippi CA, Johnson LR, Neira M, et al. Thermal biology of mosquito-borne disease. Ecol Lett. 2019;22(10):1690-708.

7. Mordecai EA, Cohen JM, Evans MV, Gudapati P, Johnson LR, Lippi CA, et al. Detecting the impact of temperature on transmission of Zika, dengue, and chikungunya using mechanistic models. PLoS Negl Trop Dis. 2017;11(4):e0005568.

8. Lambrechts L, Paaijmans KP, Fansiri T, Carrington LB, Kramer LD, Thomas MB, et al. Impact of daily temperature fluctuations on dengue virus transmission by Aedes aegypti. Proc Natl Acad Sci U S A. 2011;108(18):7460-5.

9. Xiao FZ, Zhang Y, Deng YQ, He S, Xie HG, Zhou XN, et al. The effect of temperature on the extrinsic incubation period and infection rate of dengue virus serotype 2 infection in Aedes albopictus. Arch Virol. 2014;159(11):3053-7.

10. Mbaika S, Lutomiah J, Chepkorir E, Mulwa F, Khayeka-Wandabwa C, Tigoi C, et al. Vector competence of Aedes aegypti in transmitting Chikungunya virus: effects and implications of extrinsic incubation temperature on dissemination and infection rates. Virology journal. 2016;13:114.

11. Turell MJ. Effect of environmental temperature on the vector competence of Aedes taeniorhynchus for Rift Valley fever and Venezuelan equine encephalitis viruses. Am J Trop Med Hyg. 1993;49(6):672-6.

12. Westbrook CJ, Reiskind MH, Pesko KN, Greene KE, Lounibos LP. Larval Environmental Temperature and the Susceptibility of Aedes albopictus Skuse (Diptera: Culicidae) to Chikungunya Virus. Vector Borne Zoonotic Dis. 2010;10(3):241-7.

13. Adelman ZN, Anderson MA, Wiley MR, Murreddu MG, Samuel GH, Morazzani EM, et al. Cooler temperatures destabilize RNA interference and increase susceptibility of disease vector mosquitoes to viral infection. PLoS Negl Trop Dis. 2013;7(5):e2239.

14. Murdock CC, Paaijmans KP, Bell AS, King JG, Hillyer JF, Read AF, et al. Complex effects of temperature on mosquito immune function. Proc Biol Sci. 2012;279(1741):3357-66.

15. Zhang X, Sheng J, Plevka P, Kuhn RJ, Diamond MS, Rossmann MG. Dengue structure differs at the temperatures of its human and mosquito hosts. Proc Natl Acad Sci U S A. 2013;110(17):6795-9.

16. Lim XN, Shan C, Marzinek JK, Dong H, Ng TS, Ooi JSG, et al. Molecular basis of dengue virus serotype 2 morphological switch from 29 degrees C to 37 degrees C. PLoS Pathog. 2019;15(9):e1007996.

17. Kostyuchenko VA, Lim EX, Zhang S, Fibriansah G, Ng TS, Ooi JS, et al. Structure of the thermally stable Zika virus. Nature. 2016;533(7603):425-8.

18. Xie X, Yang Y, Muruato AE, Zou J, Shan C, Nunes BT, et al. Understanding Zika Virus Stability and Developing a Chimeric Vaccine through Functional Analysis. MBio. 2017;8(1).

19. Slon Campos JL, Marchese S, Rana J, Mossenta M, Poggianella M, Bestagno M, et al. Temperature-dependent folding allows stable dimerization of secretory and virus-associated E proteins of Dengue and Zika viruses in mammalian cells. Sci Rep. 2017;7(1):966.

20. Tesla B, Demakovsky LR, Mordecai EA, Ryan SJ, Bonds MH, Ngonghala CN, et al. Temperature drives Zika virus transmission: evidence from empirical and mathematical models. Proc Biol Sci. 2018;285(1884).

21. Watts DM, Burke DS, Harrison BA, Whitmire RE, Nisalak A. Effect of temperature on the vector efficiency of Aedes aegypti for dengue 2 virus. Am J Trop Med Hyg. 1987;36(1):143-52.

22. Rohani A, Wong YC, Zamre I, Lee HL, Zurainee MN. The effect of extrinsic incubation temperature on development of dengue serotype 2 and 4 viruses in Aedes aegypti (L.). Southeast Asian J Trop Med Public Health. 2009;40(5):942-50.

23. Zouache K, Fontaine A, Vega-Rua A, Mousson L, Thiberge JM, Lourenco-De-Oliveira R, et al. Three-way interactions between mosquito population, viral strain and temperature underlying chikungunya virus transmission potential. Proc Biol Sci. 2014;281(1792).

24. Reisen WK, Fang Y, Martinez VM. Effects of temperature on the transmission of west nile virus by Culex tarsalis (Diptera: Culicidae). J Med Entomol. 2006;43(2):309-17.

25. Neven LG. Physiological responses of insects to heat. Postharvest Biology and Technology. 2000;21:1003-111.

26. Reinhold JM, Lazzari CR, Lahondère C. Effects of the Environmental Temperature on Aedes aegypti and Aedes albopictus Mosquitoes: A Review. Insects. 2018;9(4):158.

27. Pujhari S, Brustolin M, Macias VM, Nissly RH, Nomura M, Kuchipudi SV, et al. Heat shock protein 70 (Hsp70) mediates Zika virus entry, replication, and egress from host cells. Emerg Microbes Infect. 2019;8(1):8-16.

28. Chavez-Salinas S, Ceballos-Olvera I, Reyes-Del Valle J, Medina F, Del Angel RM. Heat shock effect upon dengue virus replication into U937 cells. Virus research. 2008;138(1-2):111-8.

29. Chu JJ, Leong PW, Ng ML. Characterization of plasma membrane-associated proteins from Aedes albopictus mosquito (C6/36) cells that mediate West Nile virus binding and infection. Virology. 2005;339(2):249-60.

30. Zachariassen KE. Hypothermia and cellular physiology. Arctic Med Res. 1991;50 Suppl 6:13-7.

31. Quinn PJ. Effects of temperature on cell membranes. Symp Soc Exp Biol. 1988;42:237-58.

32. Sirohi D, Kuhn RJ. Zika Virus Structure, Maturation, and Receptors. J Infect Dis. 2017;216(suppl_10):S935-s44.

33. Persaud M, Martinez-Lopez A, Buffone C, Porcelli SA, Diaz-Griffero F. Infection by Zika viruses requires the transmembrane protein AXL, endocytosis and low pH. Virology. 2018;518:301-12.

34. Fibriansah G, Ng TS, Kostyuchenko VA, Lee J, Lee S, Wang J, et al. Structural changes in dengue virus when exposed to a temperature of 37 degrees C. J Virol. 2013;87(13):7585-92.

35. Hasan SS, Sevvana M, Kuhn RJ, Rossmann MG. Structural biology of Zika virus and other flaviviruses. Nature structural & molecular biology. 2018;25(1):13-20.

36. Rossignol ED, Peters KN, Connor JH, Bullitt E. Zika virus induced cellular remodelling. Cell Microbiol. 2017;19(8).

37. Cortese M, Goellner S, Acosta EG, Neufeldt CJ, Oleksiuk O, Lampe M, et al. Ultrastructural Characterization of Zika Virus Replication Factories. Cell Rep. 2017;18(9):2113-23.

38. de Armas-Rillo L, Valera MS, Marrero-Hernandez S, Valenzuela-Fernandez A. Membrane dynamics associated with viral infection. Rev Med Virol. 2016;26(3):146-60.

39. Perera R, Riley C, Isaac G, Hopf-Jannasch AS, Moore RJ, Weitz KW, et al. Dengue virus infection perturbs lipid homeostasis in infected mosquito cells. PLoS Pathog. 2012;8(3):e1002584.

40. Heaton NS, Perera R, Berger KL, Khadka S, Lacount DJ, Kuhn RJ, et al. Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis. Proc Natl Acad Sci U S A. 2010;107(40):17345-50.

41. Willard KA, Demakovsky L, Tesla B, Goodfellow FT, Stice SL, Murdock CC, et al. Zika Virus Exhibits Lineage-Specific Phenotypes in Cell Culture, in Aedes aegypti Mosquitoes, and in an Embryo Model. Viruses. 2017;9(12).

42. Goodfellow FT, Willard KA, Wu X, Scoville S, Stice SL, Brindley MA. Strain-Dependent Consequences of Zika Virus Infection and Differential Impact on Neural Development. Viruses. 2018;10(10).

43. Anfasa F, Siegers JY, van der Kroeg M, Mumtaz N, Stalin Raj V, de Vrij FMS, et al. Phenotypic Differences between Asian and African Lineage Zika Viruses in Human Neural Progenitor Cells. mSphere. 2017;2(4).

44. Sheridan MA, Balaraman V, Schust DJ, Ezashi T, Roberts RM, Franz AWE. African and Asian strains of Zika virus differ in their ability to infect and lyse primitive human placental trophoblast. PLoS One. 2018;13(7):e0200086.

45. Shao Q, Herrlinger S, Zhu YN, Yang M, Goodfellow F, Stice SL, et al. The African Zika virus MR-766 is more virulent and causes more severe brain damage than current Asian lineage and dengue virus. Development. 2017;144(22):4114-24.

46. Udenze D, Trus I, Berube N, Gerdts V, Karniychuk U. The African strain of Zika virus causes more severe in utero infection than Asian strain in a porcine fetal transmission model. Emerg Microbes Infect. 2019;8(1):1098-107.

47. Dowall SD, Graham VA, Rayner E, Hunter L, Atkinson B, Pearson G, et al. Lineage-dependent differences in the disease progression of Zika virus infection in type-I interferon receptor knockout (A129) mice. PLoS Negl Trop Dis. 2017;11(7):e0005704.

CHAPTER 6

CONCLUSIONS

Zika virus (ZIKV) is a mosquito-borne flavivirus. The primary route of transmission is through the bite of *Aedes* mosquitoes, but the virus can also be transmitted from mother to child, sexually and through blood transfusion. Only 20% of infected people will develop Zika fever which is characterized by mild fever, rash, conjunctivitis, and join pain. ZIKV is especially dangerous during pregnancy because it can cause severe birth defects such as microcephaly. These adverse pregnancy outcomes associated with ZIKV infection urged scientists on developing therapeutics, vaccines, and novel vector control strategies. Still, important ecological and molecular questions about key drivers of ZIKV transmission remain unanswered. We explored how the variation in viremia and temperature affect mosquito-virus interactions and ZIKV transmission, both of which are important for successfully implementing control strategies.

Specific aim 1: To determine the effect of viral concentration on ZIKV transmission risk. We hypothesized that increasing doses of ZIKV will result in higher mosquito infection prevalence and vector competence, shorter extrinsic incubation period (EIP) and overall higher transmission of the virus. To test this hypothesis, we exposed field-derived population of *Ae. aegypti* to four concentrations of ZIKV (10^3, 10^4, 10^5, 10^6 PFU/mL) representative of the potential variation in the field. We tested mosquito bodies, heads and saliva for the presence/absence of ZIKV and measured the effect of viral

concentration on infection prevalence, dissemination rates, vector competence, and EIP. We also tracked mosquito mortality to determine if the virus concentration influenced mosquito survival.

We showed that increasing the concentration of ZIKV in the blood-meal increases the probability that mosquitoes will become infected, which increases the probability of mosquitoes disseminating infection and to become infectious. Although the mean proportion of mosquitoes with disseminated infection significantly increased with increasing dose, the effect of dose on virus dissemination and salivary gland infection is driven by the initial probability of becoming infected. Once the virus successfully escapes the midgut, the effect of dose is no longer evident. We have also observed the effect of dose on the rates of midgut escape and salivary gland invasion. At higher doses, the virus was detectable in mosquito bodies at all tested time points, while it took more than 4 days for virus to exit the midgut and disseminate and about 8 days to penetrate salivary glands. We did not see any significant differences in the daily probability of survival between the uninfected blood-fed control mosquitoes relative to those infected with ZIKV, and we observed no effects of increasing dose on mosquito survival among the infected mosquitoes.

In research and diagnostics, qPCR is widely used as a fast and sensitive way of detecting viral genome. We compared the performance of plaque assays and RT-qPCR to assess the infection status. While both methods gave similar numbers of positive samples, ZIKV genome was present in saliva much earlier than infectious virus particles suggesting that RT-qPCR assay is potentially over-estimating key transmission parameters such as EIP.

Lastly, we used these data to parameterize a mechanistic R_0 model and to estimate the number of infectious bites a human population would experience from a mosquito population of a given size. We showed that mosquito population feeding on increasing viral doses contribute more infectious bites and produce more secondary ZIKV infections. Increasing viremia from 10^4 to 10^6 PFU/mL increases relative R_0 3.8-fold. Variation in viremia, and the frequency distribution of hosts with different viremias should be accounted when estimating R_0 and in assessing the efficiency of arbovirus prevention strategies.

Specific aim 2: To investigate how changes in temperature affect ZIKV transmission potential, measured through changes in vector competence, extrinsic incubation period, and mosquito survival. Our hypothesis was that temperature variation will have a unimodal effect on overall transmission of ZIKV with extreme temperatures resulting in lower transmission and optimal temperatures in highest transmission potential in *Ae. aegypti* mosquitoes. We conducted experiments to estimate the thermal performance of ZIKV in field-derived *Ae. aegypti* across eight constant temperatures (16°C, 20°C, 24°C, 28°C, 32°C, 34°C, 36°C, 38°C). We collected and titrated mosquito bodies, heads, legs, and saliva to measure changes in proportion of infected mosquitoes, vector competence, and EIP. We tracked mosquito mortality at each temperature to assess the effect of temperature on mosquito survival.

We observed strong, unimodal effects of temperature on the number of mosquitoes infected, with disseminated infections, and that became infectious. While all three response variables dropped at both cool and warm temperatures, the extent of the decrease was more pronounced as the virus spread through the mosquito, suggesting

these traits exhibit different thermal sensitivities. Even though both low and high temperature resulted with the decreased likelihood of becoming infected or infectious, the cool temperatures limited midgut escape, while warmer temperatures caused high mosquito mortality. Temperature also had a very pronounced effect on the EIP. While no infectious mosquitoes were detected at the coolest temperature (16°C) in 21 days, as the temperature increased, the time required for mosquitoes to become infectious decreased, from 21 days at 20°C, to 3 days at 38°C. Overall, the daily probability of mosquito survival was highest for mosquitoes housed at 24°C and 28°C relative to cooler (16 – 20°C) and warmer (32 – 38°C) temperatures. Mosquito survival was lowest at the warmest temperature of 38°C, with no mosquitoes surviving past 3 dpi.

Trait thermal responses for vector competence, EIP, and mosquito lifespan were all unimodal. Vector competence and mosquito lifespan were symmetrical, peaking at 30.6°C and 24.2°C, respectively. Extrinsic incubation rate thermal response was asymmetrical with a peak at 36.4°C. We used these thermal responses to update an existing temperature-dependent model and infer temperature effects on ZIKV transmission. We found that ZIKV transmission was optimized at 28.9°C, and had a thermal range of 22.7°C - 34.7°C. Any changes in temperature due to climate change, urbanization, or seasonality toward the predicted thermal optimum of 29°C could cause ZIKV expansion north and into longer seasons. In contrast, areas that are near the thermal optimum could experience a decrease in overall environmental suitability.

Lastly, we demonstrated that predicted thermal minimum for ZIKV transmission is 5°C warmer than DENV, suggesting that current estimates on the global environmental suitability for ZIKV transmission are greatly over-predicting its possible range.

Specific aim 3: To determine the mechanism responsible for inhibition of ZIKV replication at sub-optimal temperatures. We hypothesized that sub-optimal temperatures inhibit ZIKV replication by altering cellular environment and host factors necessary for viral replication or/and by preventing a viral function required to complete the viral replication cycle and produce progeny virus. We conducted a series of experiments in mosquito cells to define the block in virus replication cycle.

Similar to our *in vivo* study, ZIKV replication was diminished at cool temperatures, while both DENV and CHIKV replicated better at 20°C. We observed the same phenotype when ZIKV was added to cells adapted to grow at 20°C suggesting that the reduced ZIKV replication was not a result of acute cellular stress brought on by the temperature shift. If we established ZIKV infection in cells prior to cool temperature exposure, there was no difference in virus yields over five days, suggesting late stages of the virus replication cycle, including RNA production, protein production and cleavage, virus assembly and maturation, were not inhibited by the low temperature. When we examined virus spread in cells at 20°C, we found a reduction in viral yields, suggesting the cool temperatures is decreasing the efficiency of establishing a productive infection in the cell. Time of addition experiments with inhibitors that block ZIKV internalization and fusion suggested ZIKV enters C6/36 cells within the first two hours of infection at the optimal temperature. However, keeping the cells at 20°C for 2, 4, or 6 hours post-infection did not affect virus titers in comparison to the cells maintained at 28°C, suggesting that cool conditions do not block viral binding, internalization, nor fusion. Using the reverse approach, we determined the cells needed to be incubated at 28°C for at least the first 8 hours in order to detect virus particles in the supernatant when shifted to

20°C. Taken together, these data suggest that temperature impairs virus replication cycle after fusion yet before genome replication. We also demonstrated that this phenotype is specific to the Asian-lineage and is not observed among African-lineage strains of ZIKV.

This dissertation provided a better understanding on the effects of environmental variation on vector-virus interactions and transmission in the ZIKV system. The study presented in this thesis was the first to incorporate the variation in viremia in modeling ZIKV transmission. As majority (70%) of ZIKV infections are asymptomatic, it is crucial to characterize the role asymptomatic infections play in transmission. Assessing how virus concentration in the blood meal affects the probability of mosquitoes to become infected and infectious is the first step towards achieving that goal. However, the only way to truly assess the importance of asymptomatic patients in transmission is to involve them in the studies. Therefore, future studies should directly measure the probability of mosquitoes becoming infectious after feeding on patients with different viremia in endemic regions.

This dissertation was also the first to characterize the effect of temperature on ZIKV transmission in a comprehensive study with eight constant temperatures. Beyond the establishment of ZIKV transmission dynamics across a 22°C-temperature range, we defined a thermal optimum and range for transmission, and generated a thermal suitability map. While these findings have an important implication for vector control, this is only the first step in fully characterizing ZIKV transmission. Mosquitoes and the pathogens they transmit live in a variable world, and after exploring the main effect of temperature on transmission, we can further explore these dynamics in fluctuating environment. Moreover, there are other sources of variation in the environment such as

humidity, rainfall, availability of oviposition, resting habitats, and competition that need to be explored. As we know that the exposure during larval stages might not have the same effect as exposure during the adult stages, due to carry-over effects, it is important to assess different sources of variation across all stages of mosquito life cycle. Even further, all these responses might vary across different mosquito populations and strains of viruses, therefore future studies should characterize genetics of the pathogen (G_P) x genetics of the host (G_H) interactions and how environmental variation can alter the interactions (G_P x G_H x E).

The study presented in the last chapter was also the first to evaluate how temperature affects virus replication *in vitro*. Our data suggest that 20°C is limiting the efficiency of ZIKV establishing infection within the cell. Early event including binding, internalization, and fusion do not appear to be limiting and later stages in replication also occur efficiently if the cells were previously infected. This suggests that 20°C is blocking the establishment of viral replication sites on internal membranes. Future experiments should fully characterize the mechanism. Knowing that this phenotype is specific for Asian-lineage strains, opens the possibility in using the chimeric virus in elucidating the mechanism. Another approach is to passage the virus at cool temperatures and observe if the selective pressure is strong enough to induce virus adaptation to sub-optimal temperatures. If the virus can be adapted to replicate at cool temperatures, using sequencing and bioinformatic tools, we can further identify the regions of the virus genome responsible for limited replication at cool temperatures.

Collectively, this dissertation elucidated some of the important knowledge gaps of how ZIKV transmission potential is affected by variation in viremia and temperature. We

generated new models that account for those variations and rely on the experimentally assessed data, and we further investigated the mechanism. Taken together, this dissertation greatly enriched the field and enhanced our ability to more accurately determine temperature suitability and predict the number of people at risk, as well as assess the efficacy of intervention strategies.