The interactions between the mosquito-specific Mesonivirus, arboviruses, and the mosquito host

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“Life is like riding a bicycle. To keep your balance, you must keep moving.”

Albert Einstein

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Abbreviations

%	Percent
˚C	Degree celsius
AaIDV	Aedes albopictus densovirus
AalDV 2	Aedes albopictus densovirus 2
AeAV	Aedes Anphevirus
ABTV	Arboretum virus
Ago	Argonaute
AgDNV	Anopheles gambiae densovirus
AMIV	Anopheles minimus Iridovirus
ASALV	Agua Salud alphavirus
APRV	Aedes pseudoscutellaris reovirus
AUD	Alphavirus unique domain
BADUV	Badu virus
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BTV	Bluetongue virus
BNITM	Bernhard Nocht Institute for Tropical Medicine
bp	Basepair
BUNV	Bunyamwera virus
BUNV-NLuc	Bunyamwera virus with nano luciferase reporter
BVDV	Bovine viral diarrhea virus
C	Capsid
CAVV	Cavally virus
CALBOV	Calbertado virus
cDNA	Complementary DNA
CFAV	Cell-fusing agent virus

CHIKV	Chikungunya virus
CMDV	Cimodo virus
CPE	Cytopathic effect
CpDV	Culex pipiens densovirus
CppDV	Culex pipiens pallens densovirus
CT	Cycle threshold
cRNA	Complementary RNA
CuTLV	Culex originated Tymoviridae-like virus
CxNV1	Culex narnavirus 1
CxFV	Culex Flavivirus
CYV	Culex Y virus
CVR	Centre for Virus Research
DAAD	German Academic Exchange Service/Deutsche Akademische Austauschdienst
DF	Dengue fever
Dcr	Dicer
DAPI	4', 6-diamidino-2-phenylindole
DENV	Dengue virus
DKV	Dianke virus
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dpi	Days post infection
dsRNA	Double-stranded RNA
E	Envelope
eGFP	Enhanced green fluorescent protein
EDTA	Ethylendiamin tetraacetic acid
EEEV	Eastern equine encephalitis virus
EIP	Extrinsic incubation temperature
EILV	Eilat virus

ESV	Espirito Santo virus
ER	Endoplasmic reticulum
FBS/FCS	Fetal bovine serum/fetal calf serum
FERV	Ferak virus
FHV	Flock House virus
Fig.	Figure
FLuc	Firefly luciferase
g	Grams
GFP	Green fluorescent protein
GMEM	Glasgow Minimum Essential Medium
h	Hour/Hours
HCL	Hydrochloric acid
Hel	Helicase
hpi	Hours post infection
HVD	Hypervariable domain
HANKV	Hanko virus
IMD	Immune deficiency
ISV	Insect-specific virus
JAK-STAT	Janus Kinase-Signal Transducer, Activator of Transcription
JEV	Japanese encephalitis virus
kb	Kilobase
KCl	Potassium chloride
KRV	Kamiti river virus
KH_2PO_4	Potassium dihydrogen phosphate
KIBV	Kibale virus
L	Large
LACV	La cross encephalitis virus
LacZ	Bacterial beta-galactosidase gene
LTNV	La Tina virus
m	Minute/Minutes

M	Membrane
ml	Millilitre
mm	Millimetre
mRNA	Messenger RNA
miRNA	Micro RNA
Mac	Macro domain
MAFFT	Multiple Alignment using Fast Fourier Transform
MAYV	Mayaro virus
MeSV	Mesonivirus
MEGA	Molecular Evolutionary Genetics Analysis
MERDV	Merida virus
MOI	Multiplicity of infection
MOUV	Moussa virus
MoNV	Mosinovirus
MSV	Mosquito-specific virus
miRNA	Micro RNA
MMLV	Moloney Murine Leukemia virus
Mtr	Methyl transferase
MRV	Menghai rhabdovirus
MVEV	Murray Valley encephalitis virus
MEM	Minimal essential medium
$MgCl_2$	Magnesium chloride
ML	Maximum Likelihood
MWAV	Mwinilunga alphavirus
n	Number
N	Nucleocapsid
NABU	Nature Conservation Union Germany
NaCl	Sodium chloride
NGS	Next generation sequence
NCBI	National Center for Biotechnology Information

NCR	Non-coding region
NEAA	Non-essential amino acids
ng	Nanogram
N/A	Not applicable
nm	Nanometre
ns	Nonsignificant
nt	Nucleotide
NS	Nonstructural
NseV	Nse virus
NLuc	Nano luciferase
NHUV	Nhumirim virus
NRUV	Ninarumi virus
OdV	Odorna virus
ORF	Open reading frame
ONNV	O'nyong'nyong virus
p	P-value
pp	Polyprotein
Pro	Protease
PEP	Pairwise evolutionary distance
PLB	Passive lysis buffer
PCLV	Phasi Charoen-like virus
PBS	Phosphate buffered saline
PBT	Phosphate buffered saline with Triton-X100
PCR	Polymerase chain reaction
PCV	Palm creek virus
PFU	Plaque forming units
pH	Potential of hydrogen
pmol	Picomole
preM	Premembrane protein
piRNA	Piwi-interacting RNA

Piwi	p-element induced wimpy testis
qRT-PCR /qPCR	quantitative real-time polymerase chain reaction
RT	Reverse transcription
RLU	Relative luciferase unit
RNA	Ribonucleic acid
RNAi	RNA interference
RVFV	Rift valley fever virus
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
rpm	Rounds per minute
rRNA	Ribosomal ribonucleic acid
s	Second or Sense
sp	Signal peptide
S	Small
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SBV	Schmallenberg virus
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
siRNA	Small interfering RNA
SFV	Semliki Forest virus
SFV-FLuc	Semliki Forest virus with firefly luciferase reporter
SLEV	St Louis encephalitis virus
ssRNA	Single-stranded RNA
sgRNA	subgenomic RNA
sRNA	Small RNA
SINV	Sindbis virus
SHTV	Shinobi tetravirus
SWKV	Sarawak virus
TAE	TRIS-acetate-EDTA buffer
$TCID_{50}$	50 % tissue culture infective dose

T_m	Melting temperature
TM	Transmembrane domain
TRIS	*Tris* (hydroxymethyl) aminomethane
TBEV	Tick-borne encephalitis virus
UTR	Untranslated region
UV	Ultraviolet
USUV	Usutu virus
µg	Microgram
µl	Microliter
µm	Micrometre
vDNA	Viral DNA
vRNA	Viral RNA
vsiRNA	Viral siRNA
vpiRNA	Viral piRNA
V	Volt
VSR	Viral suppressors of RNA
viRNA	Viral specific small interfering RNA
VEEV	Venezuelean equine encephalitis virus
WALV	Wallerfield virus
WEEV	Western equine encephalitis virus
WNV	West Nile virus
WUR	Wageningen University and Research
YCV	Yichang virus
YF	Yellow fever
YFV	Yellow Fever virus
ZnHel	Zinc-binding helicase
ZIKV	Zika virus

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1 Introduction

1.1 A brief background of the study

Mosquito-specific virus (MSV) per this thesis is a virus isolated from mosquitoes which is unable to replicate in vertebrates (cells) independent of its replication in other insect cells. Mesonivirus (MeSV) is a MSV which has been found to infect mosquito populations worldwide, including also species that can be vectors to arboviruses of public health concern (Wang et al. 2017).This highlights the possibility for mosquitoes to be coinfected with MeSV and an arbovirus. However, it is not clear, if MeSV infection triggers the antiviral immune response of the mosquito host, like the RNA interference (RNAi) pathways. Furthermore, it is unclear whether this response or any other interaction has an effect on potential coinfections with arboviruses, which can in turn influence vector competence of the mosquito host. Therefore, it is imperative to understand these interactions in vitro as it is a vital first step to exploring these possibilities in the mosquito as potential vector control strategy. The MeSV used was isolated at the Bernhard Nocht Institute for Tropical Medicine (BNITM) from a *Coquilletidia richiardii* mosquito pool, collected in Germany.

Usutu virus (USUV) on the other hand is the main arbovirus of concern in this study and mainly found in birds but also known to cause diseases in humans such as meningoencephalitis and neurological impairments (Clé et al. 2019). USUV is spreading in Germany since its first detection and showed an immense increase of infection detected in 2019 (NABU 2019). Besides, it was suggested that there could be a positive correlation between USUV and MeSV as they were often identified in the same vector pools, mainly *Culex* sp. mosquito pools from Germany (personal communication, Dr. Lühken, BNITM). Furthermore, this study was conceived as a result of immediate attention to these two (USUV, MeSV) emerging viruses in Germany. MeSV infection may change the competence for arboviruses like USUV as recently reported for other MSVs. Involving other mosquito-borne viruses, it is important to investigate the interaction between MeSV and arboviruses belonging to the main arbovirus families, including USUV (*Flaviviridae*), Semliki Forest virus (SFV, *Togaviridae*), and Bunyamwera virus (BUNV*, Peribunyaviridae*). The first isolate of SFV was from *Aedes abnormalis* mosquitoes in Uganda (Smithburn and Haddow 1944) and are known to cause mild symptoms in humans (Mathiot et al. 1990). Fascinating, SFV has been used as a model to study alphaviral life cycle and has a wide host range (Quetglas et al. 2010). Similary, BUNV was also first isolated from *Aedes* mosquitoes in Uganda (Smithburn, Haddow, and Mahaffy 1946). However, BUNV has a wider mosquito vector range than SFV and in addition to *Aedes* mosquitoes, *Culex* mosquitoes are also vectors (Tauro et al. 2015). BUNV causes febrile

illness and encephalitis in humans (Christopher, Colin, and Frederick 2016). Taken together, the interaction of these main arbovirus families with the newly isolated MeSV is imperative to provide a first hand report in relation to emerging and re-ermerging viruses of public health importance.

1.2 Arboviruses

Arthropod-borne viruses (Arboviruses) are viruses that are capable of being transmitted from an arthropod to a vertebrate host. Arboviruses require hematophagous arthropod vectors (mosquitoes, ticks, sandflies, and midges) to transmit viruses between vertebrate hosts (Moureau et al. 2015). A number of arboviruses are known to cause human diseases. Mosquito-borne viruses affecting humans are concentrated in three important families, the *Flaviviridae* (genus Flavivirus), *Togaviridae* (genus Alphavirus), and the *Peribunyaviridae* (primarily genus Orthobunyavirus but with a few important outliers such as the Phlebovirus Rift Valley Fever) (Braack et al. 2018).

Arboviruses are known to actively infect and replicate in their mosquito vector prior to transmission; however causing minimal pathologic changes and fitness costs (Putnam and Scott 1995; Styer et al. 2007). Studies revealed that over 500 different arboviruses are catalogued of which about 100 are known to be pathogenic to humans. The clinical manifestation of these pathogenic arboviruses includes: acute self-limiting fever, muscle and joint pain, hemorrhagic symptoms, and neurological disorders (Hayes et al 2008; Karabatsos 1985; Tsai and Chandler 2003).

1.2.1 General life cycle of arboviruses

Over 90% of arboviruses that cause human disease are vectored by mosquitoes (McGraw and O'Neill 2013). Vertebrate host and vector used by individual arboviruses varies considerably. In aggregate, arboviruses are transmitted in nature by three orders of haematophagous insects (Diptera, Anoplura and Hemiptera) and two families of ticks (*Argasidae* and *Ixodidae*) to all four classes of terrestrial or semi-terrestrial vertebrates on all seven continents (Althouse and Hanley 2015). A competent vector may become infected with an arbovirus when it imbibes viraemic blood from an infected vertebrate host. Vector competence, the ability of an arthropod to become infected with a pathogen and transmit it to a susceptible vertebrate host, depends upon many factors, including: (i) the genotype by genotype interaction between vector and virus (Lambrechts et al. 2009, 2013; Tabachnick 2013); (ii) the dose of virus ingested; (iii) basal immune activation, which is a product of, among other factors, the arthropod's genotype and the composition of its microbiome (Jupatanakul, Sim, and Dimopoulos 2014); and (iv) environmental conditions such as temperature and humidity (Jupatanakul et al. 2014). Prior or

concurrent infections with other viruses may also modulate vector competence (Jupatanakul et al. 2014; Kent, Crabtree, and Miller 2010a). When taken up by a competent vector species, an arbovirus infects and disseminates across the midgut to circulate, via the haemolymph, to the salivary glands, where it replicates to sufficient concentrations to enable transmission to a susceptible vertebrate host (Fig. 1.1). The period of time required to complete this process is termed the extrinsic incubation period (EIP). Once infection is established it generally persists for the life of the vector, although virus titre in tissues can vary (Kuno and Chang 2005).

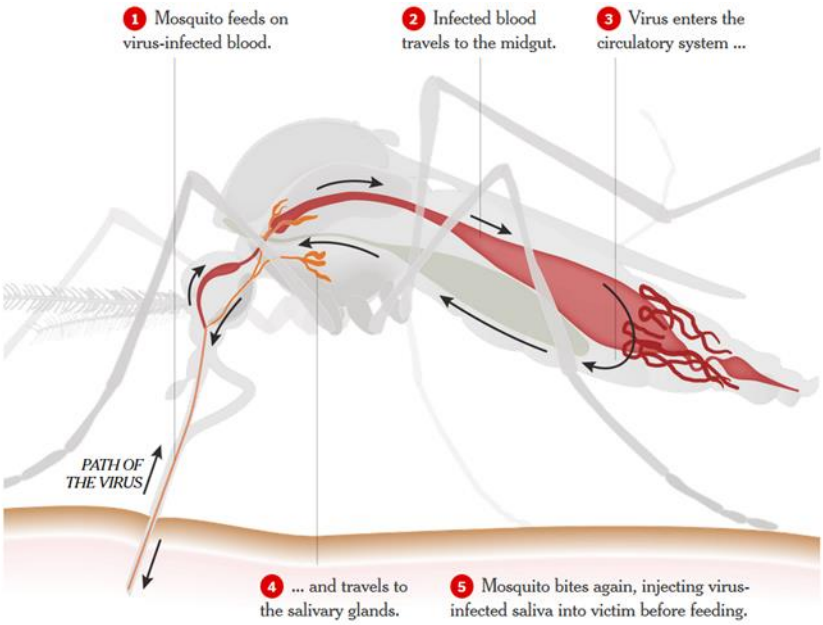

Fig. 1. 1: Schematic illustration of the path of an arbovirus in the mosquito.

Virus-infected blood is taken up by a mosquito via blood feeding, enters the the circulatory sytem after passing through the midgut. Reaching the salivary gland, virus-infected saliva is injected into the host during a mosquito bite. The figure was adapted from Sarah Almukhtor and Mika Grondahl; the anatomical life of the mosquito. http://virusvisions.blogspot.com/2016/08/1-mosquito-feeds-on-virus-infected-blood.html.

Most arboviruses are zoonotic and can occur in three different transmission cycles. Namely: urban epidemic cycle, enzootic sylvatic cycle, and rural epizootic cycle (Fig. 1.2). Arboviruses are maintained in natural transmission cycles in which they replicate in hematophagous arthropods and are transmitted to vertebrate hosts during a blood meal. Such arboviruses are initially maintained in an enzootic sylvatic cycle involving rodents, birds, or nonhuman primates as reservoir hosts. Spillover from enzootic cycle sometimes

occurs through incidental biting of humans by enzootic and/or bridge vectors. Some arboviruses may achieve further amplification in domestic animals within a rural epizootic cycle. Others may alter their host range, from nonhuman primate to humans, leading to urban epidemic transmission cycle (Weaver and Barrett 2004). There is a possibility of vertical transmission from an infected vector to its progeny regarding arboviral infections, but its importance in arbovirus outbreaks is not known yet (Lequime, Paul, and Lambrechts 2016; Lumley et al. 2017). Moreover, it was speculated that transmissions via co-feeding, transovarial, transstadial, and venereal are possibilities for some arboviral infections (Bente et al. 2013; Xia et al. 2016).

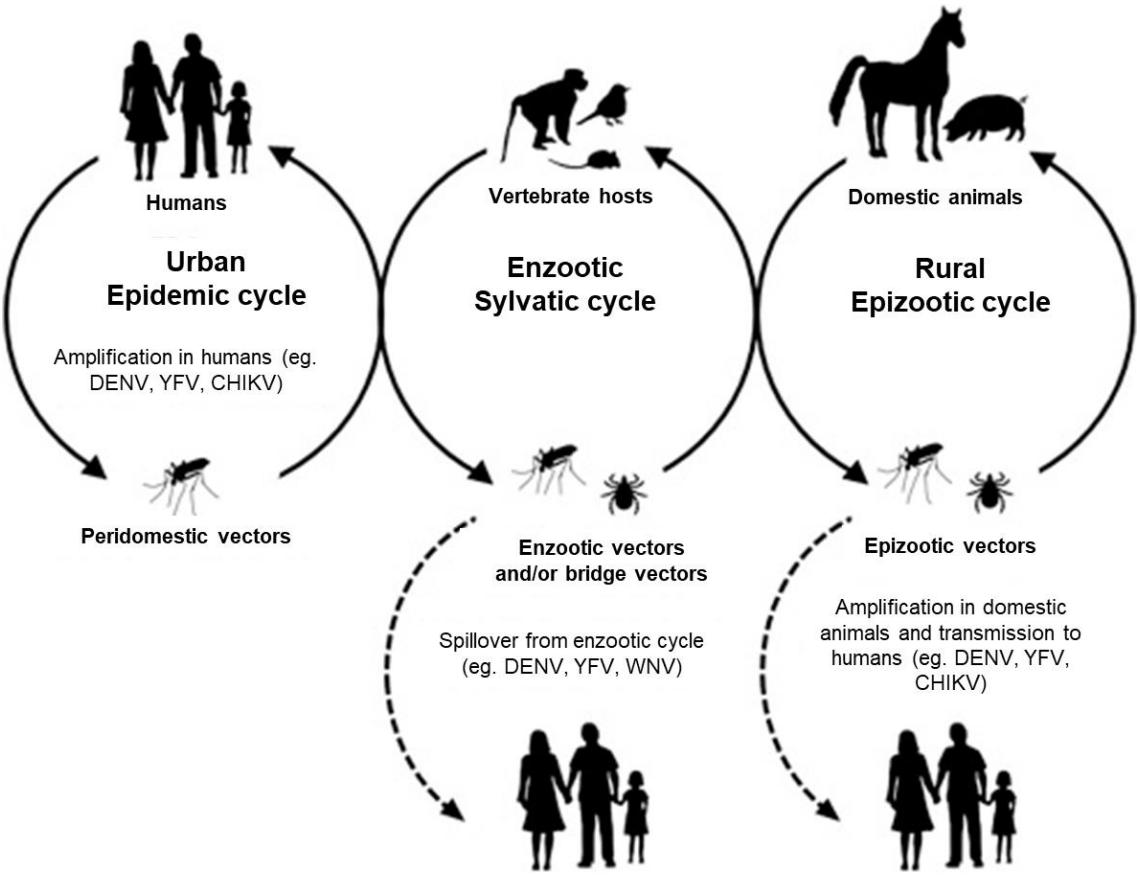

Fig. 1. 2: General arbovirus transmission cycles.

Arbovirus transmission occurring in three different cycles; epidemic cycle, sylvatic cycle, and epizootic cycle. Transmission between a domestic vector and the human population is via the epidemic cycle (For example; Dengue virus=DENV, Yello fever virus=YFV, and Chikungunya virus=CHIKV). The sylvatic cycle is basically between a vertebrate host in the wild and a vector (For example; DENV, YFV, and West Nile virus=WNV). Transmission between a domestic animal and a vector is via epizootic cycle (For example; Japanese encephalitis virus=JEV). Sylvatic and epizootic cycles may experience spill over to human population. Adapted from (Mueller and Cao-Lormeau 2018).

1.2.2 Flaviviruses *(Flaviviridae)*

The *Flaviviridae* is a family of small enveloped viruses, single-stranded with positive-sense RNA genomes of approximately 10-11kb econding a 5‘ untranslated region (5‘UTR), a single long open reading frame (ORF), and a 3‘UTR (Lindenbach, Thiel, and Rice 2013). Approximately 100 and 400-700 nucleotides (nts) made up the 5‘UTR and 3‘UTR respectively without a poly A tail and form conserved structures such as 5’m_7G cap and 3’OH for replication and translation. The ORF encodes a large polyprotein which is cleaved into the different viral proteins by host and viral proteases. It generates three structural (S) proteins (Capsid:C, premembrance/membrane:prM/M, and envelope:E) and at least seven nonstructural (NS) proteins, namely: NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B, and NS5 (Brinton and Basu 2015; Lindenbach et al. 2013; Rice et al. 1985). Fig. 1.3 below illustrates the structure of the genome.

Hematophagous arthropods and vertebrate hosts are responsible for the horizontal transmission of most flaviviruses. Examples of flaviviruses include: Dengue virus (DENV), Yellow Fever virus (YFV), Japanese Encephalitis virus (JEV), West Nile virus (WNV), Zika virus (ZIKV), Usutu virus (USUV), and tick-borne encephalitis virus (TBEV) (Cadar et al. 2017; Dobler 2010; Weaver and Barrett 2004; Wilder-Smith et al. 2017).

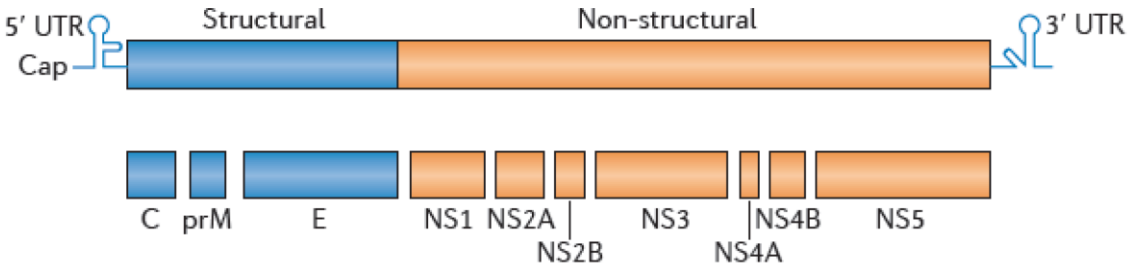

Fig. 1. 3: Flavivirus genome organization.

At the top is the viral genome with the structural and nonstructural protein coding regions and the 5′- and 3′-UTR. The 5’UTR has a conserved cap for translation initiation. Boxes below the genome indicate viral proteins generated by the proteolytic processing cascade. The three structural proteins include: Capsid (C), premembrane (prM), and Envelope (E). The non-structural proteins are made of NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. Taken from (Beck et al. 2013).

1.2.2.1 Usutu virus

Usutu virus (USUV) is a mosquito-borne flavivirus isolated for the first time in 1959 from *Culex neavei* mosquitoes in the Natal region of South Africa (McIntosh 1985). The virus has been isolated from mosquitoes and birds mainly in Africa and Europe. The following

countries had a record for USUV detection in mosquitoes and birds: Senegal, Central African Republic, Burkina Faso, Côte d'Ivoire, Morocco, Nigeria, Uganda, Austria, Hungary, Switzerland, Italy, Spain, Germany, Poland, Czech Republic, United Kingdom, and Israel (Ashraf et al. 2015; Bakonyi et al. 2007; Buckley et al. 2003; Busquets et al. 2008; Clé et al. 2019; Hubálek et al. 2008; Manarolla et al. 2010; Nikolay et al. 2011).

In 2001, the first case of USUV was confirmed in Europe after a considerable die-off of Eurasian blackbirds (*Turdus merula*) in Austria (Weissenböck et al. 2002). Although, USUV infection mainly affects birds, it is also known to be able to cause human disease such as meningoencephalitis and neurological impairments (Cavrini et al. 2009; Pecorari et al. 2009; Santini et al. 2014). USUV has been isolated from many mosquito species including *Culex pipiens pipiens*, *Cx. neavei*, *Cx. perexiguus*, *Cx. perfuscus*, *Aedes albopictus*, *Ae. caspius*, *Anopheles maculipennis*, *Coquillettidia aurites*, and *Mansonia africana* (Busquets et al. 2008; Calzolari et al. 2010, 2012, 2013; Hubalek 1994; Jöst et al. 2011; Tamba et al. 2011; Vázquez et al. 2011).

The natural life cycle of USUV involves mosquito-bird-mosquito cycles where mosquitoes act as vectors and birds as amplifying (reservoir) hosts (Steinmetz et al. 2011; Vázquez et al. 2011). Furthermore, the virus has been isolated from bats in Germany recently (Cadar et al. 2014). The isolation from bats raised the question of bats as reservoir hosts in Africa and mosquitoes as vectors. Mosquitoes are capable of transmitting the virus to humans, horses, and rodents deviating from the proposed enzootic cycle for USUV (Cavrini et al. 2009; Diagne et al. 2019; Pecorari et al. 2009; Savini et al. 2011).

Culex mosquitoes are considered to be the most common vectors for USUV and vector competence studies were recently done to confirm this. *Cx. pipiens* biotype *molestus* and *Cx. torrentium* mosquitoes from Germany proved competent for USUV. There is a high possibility that these two mosquito species played a historic role in the spread, maintenance, and introduction of USUV into Germany (Holicki et al. 2020). This current study aims to understand the interaction USUV with a mosquito-specific virus (*Mesoniviridae*) as key to providing vital information about its dynamics. USUV 491 strain was used in this current study, isolated in Germany from a Blackbird (*Turdus merula*).

1.2.3 Alphaviruses *(Togaviridae)*

Alphaviruses are enveloped and icosahedral viruses that possess a positive-sense single-strand RNA genome of approximately 11kb. The genome consist of a 5’ end non-coding region, one polyprotein encoding the non-structural proteins, a subgenomic promoter with a polyprotein encoding the structural proteins and a 3’ non-coding region with a poly(A) tail (Ahola and Kaariainen 1995; Strauss and Strauss 1994).

Ten different proteins are encoded by the genome with two polyproteins (non-structural and structural polyprotein) that are cleaved by host and viral proteases into the separate proteins. The non-structural proteins (nsP1-nsP4) are important for replicating the viral genome, and the structural proteins (C, E3, E2, 6K/TF, and E1) function in virus assembly (Strauss and Strauss 1994). To compare, the genome comprises two ORFs with a subgenomic promoter unlike Flaviviruses with only one ORF. Also, the genome has a poly(A) tail at the 3'UTR unlike Flaviviruses. However, similar 5'm_7G cap is seen in both virus families. The Fig. 1.4 below illustrates the genome organization for alphaviruses.

Most alphaviruses are transmitted between their vertebrate host and invertebrate vector with few exceptions. For many alphaviruses and flaviviruses dead-end hosts are incapable of developing sufficient viremia to infect mosquitoes. However, human-mosquito-human transmission has been associated with alphaviruses (such as Chikungunya virus, CHIKV) outbreak (Chen et al. 2018). SFV and Sindbis virus (SINV) are model alphaviruses as they are category 2 viruses and are easy to handle. Other medically important alphaviruses include: Onyongnyong virus (ONNV), Ross River virus (RRV), and Venezuelean equine encephalitis virus (VEEV) (Faragher et al. 1988; Kaariainen et al. 1987; Kinney et al. 1986; Strauss et al. 1988; Strauss, Rice, and Strauss 1984).

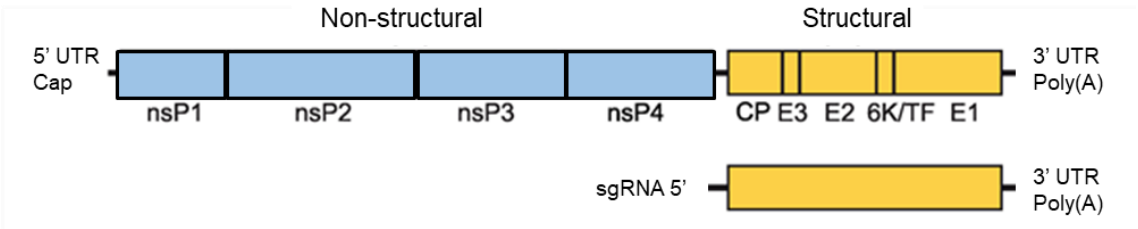

Fig. 1. 4: Alphavirus genome organization.

The viral RNA genome has two ORFs with structural and non-structural protein coding regions with 5'- and 3'-UTR. The 5'UTR and 3'UTR has a conserved cap and a poly(A) tail respectively. The non-structural proteins include: nsP1, nsP2, nsP3, nsP4. Structural proteins are made up of CP, E3, E2, 6K, and E1. The sequences encompassed by the subgenomic RNA (sgRNA) are also shown on the positive strand. Taken from (Chen et al. 2018).

1.2.3.1 Semliki forest virus

Semliki Forest virus (SFV) was first isolated from *Aedes abnormalis* mosquitoes in the Semliki Forest of Uganda in 1942 (Smithburn and Haddow 1944). It was found in eastern, central, and southern parts of Africa and are known to cause only mild symptoms in humans (Mathiot et al. 1990) and lethal encephalis in rodents (Atkins, Sheahan, and Dimmock 1985). SFV is spread mainly by mosquito bites and only one lethal (laboratory accident) human infection has been reported. Regarding this human infection, the patient

had been exposed to large amount of virus and predicted to be immunodeficient (Willems et al. 1979). Interestingly, the virus has been used extensively in biological research as a model to study viral life cycle and viral neuropathy. Due to its efficient replication in mosquito vectors and verbebrate hosts such as rodents, it has also been developed as a vector for genes and as a tool for gene therapy (Lundstrom 2003; Quetglas et al. 2010).

A variety of reporter based SFVs have been published over time, expressing for example fluorescent proteins or luciferase. SFV4 expressing Firefly luciferase expression (SFV-FLuc) was used in this current study. This reporter virus was previously constructed by inserting Firefly luciferase (FLuc) between duplicated nsP2-protease cleavage sites at the nsP3 and nsP4 protein junctions of the SFV genome (Schnettler et al. 2013; Varjak, Maringer, et al. 2017). It is therefore easy to investigate the interaction of mosquito-specific viruses with alphaviruses using SFV-FLuc as model reporter virus. Hence, this was one of the objectives of this current study.

1.2.4 Orthobunyaviruses *(Peribunyaviridae)*

Othobunyaviruses are enveloped, tripartite, single-stranded negative-sense RNA genome. Each genome segment has the same basic structure with the coding region flanked by UTRs at the 5′ and 3′ ends which partly bind to each to form a panhandle structure. There is no poly(A) tail, but a cap at the 5’ end of the mRNAs. The small (S) segment (~1kb) encodes the nucleocapsid (N) protein and the nonstructural protein NSs (Fuller, Bhown, and Bishop 1983). The medium (M) segment (~4.5kb) encodes a viral glycoprotein precursor (in the order Gn-NSm-Gc) for two envelope glycoproteins Gn, Gc that are co-translationally cleaved by host signalase and signal peptide peptidase and a nonstructural protein NSm (Fazakerley et al. 1988; Fazakerley and Ross 1989; Shi et al. 2016). The large (L) segment (~6kb) encodes the RNA-dependent RNA polymerase.This genome structure is generally reflected by most orthobunyaviruses with some differences for example in the presence or length of NSs (Elliott 2014). The Fig. 1.5 below illustrates the genome organization for orthobunyaviruses.

Othobunyaviruses are the largest and most diverse in the *Peribunyaviridae* and members occur globally in tropical, temperate, and arctic ecological niches. A wide range of arthropod and vertebrate hosts are described for the viruses and each posseses a restricted arthropod and host range limiting its geographic distribution (Beaty and Calisher 1991). Different vertebrate hosts have been associated with orthobunyaviruses including squirrels (La Crosse virus), bats (Mojuí dos Campos virus), rabbits (Snowshoe hare virus), ungulates (Akabane virus), sloths (Oropouche virus), and birds (Mermet virus). Most orthobunyaviruses are transmitted by mosquitoes. However, biting midges, bed bugs, and wingless bat flies are also known vectors (Watts et al. 1974). Orthobunyaviruses can cause human diseases including: encephalitides (La Crosse virus), febrile

illnesses (Bunyamwera virus), and viral haemorrhagic fever (Ngari virus garissa variant). Furthermore, adverse outcomes are recorded in livestock such as fetal abnormalities and abortion storms (Cache Valley and Schmallenberg viruses). Interesting, reassortment of genomic segments has been reported during coinfection with more than one orthobunyavirus with the possibility to give rise to more pathogenic reassorted viruses, compared to their parental viruses (Beaty et al. 1981; Bishop and Beaty 1988; Borucki et al. 1999; Briese, Kapoor, and Lipkin 2007; Cheng et al. 1999; Nunes et al. 2005; Yanase et al. 2012).

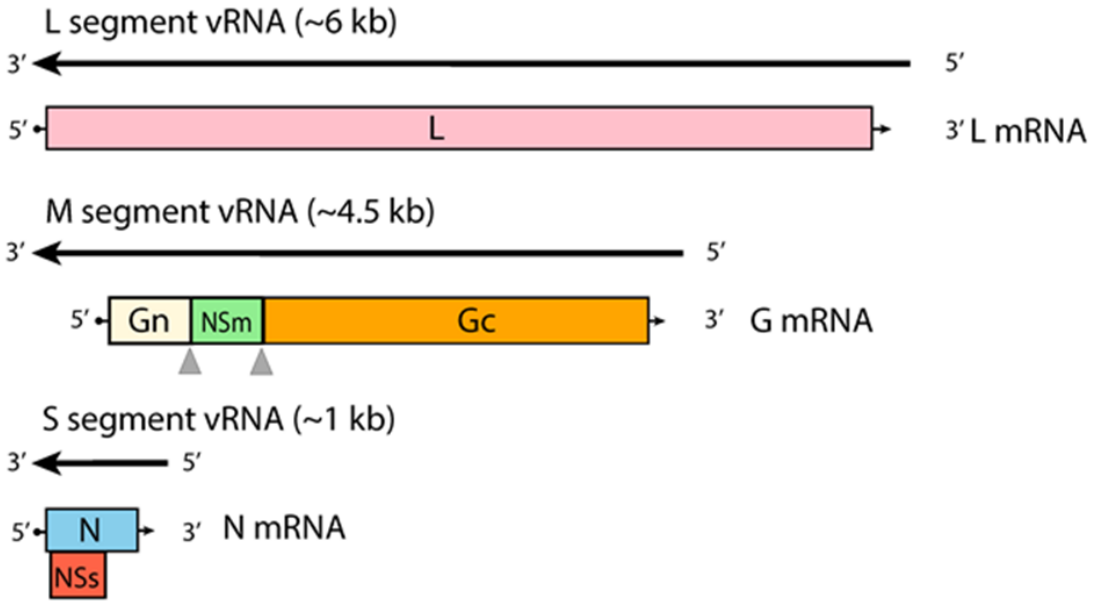

Fig. 1. 5: Orthobunyavirus genome organization.

The viral RNAs (vRNAs) are depicted in a 3'→5' direction and messenger RNAs (mRNAs) are depicted in a 5'→3' direction. The mRNAs depict ORFs that encode the N, nucleocapsid protein; Gn and Gc, external glycoproteins; L, large protein, as well as the non-structural proteins NSs and NSm. Taken from (Hughes et al. 2020).

1.2.4.1 Bunyamwera virus

Bunyamwera virus (BUNV) is the prototype virus of both the *Orthobunyavirus* genus and the family *Peribunyaviridae*. BUNV was originally isolated from *Aedes* mosquitoes in the Semliki Forest in Uganda in 1943 (Smithburn et al. 1946). However, BUNV has also been isolated in *Culex*, *Mansonia,* and *Ochlerotatus* mosquitoes (Tauro et al. 2015). BUNV infections cause febrile illness and encephalitis (rarely) in humans in sub-Saharan Africa with wild rodents likely to serve as amplifying reservoir. The virus has been isolated from

humans in Uganda, Nigeria, and South Africa and antibodies have been detected in humans in most of sub-Saharan Africa, with 82% prevalence recorded in some locations. Antibodies have been detected in domestic animals, non-human primates, rodents, and birds, and viral load capable of supporting mosquito transmission have been recorded in rodents, bats, and non-human primates (Christopher et al. 2016). However, the full natural history of the virus remains unresolved.

A variety of reporter based BUNVs have been published over time, expressing for example fluorescent proteins or luciferase. BUNV expressing Nano luciferase expression (BUNV-NLuc) was used in this current study. This reporter virus was previously constructed by replacing the NSm cytoplasmic domain of the Bunyamwera virus genome with Nano luciferase (NLuc) to generate BUNV-NLuc (Dietrich, Shi, et al. 2017). It is therefore easy to investigate the interaction of mosquito-specific viruses with orthobunyaviruses using BUNV-NLuc as model reporter virus. Hence, this was one of the objectives of this current study.

1.3 Mosquito-specific viruses

This section contains parts of my review article on mosquito-specific viruses, transmission and interaction (Agboli et al. 2019).

1.3.1 General overview

The class *insecta* of the arthropods are the largest group of animals and are main reservoir of enormous number of viruses. Through virus discovery, mosquitoes are known to harbour diverse range of viruses which are classified into two groups based on host-range. Those infecting mosquitoes and vertebrates are classified as the arboviruses including Yellow Fever (YFV) and Dengue (DENV) viruses; the second group is the mosquito-specific viruses (MSVs) with mosquito host restriction (Vasilakis and Tesh 2015). MSVs belong to the main virus families; *Flaviviridae* (+ssRNA genome), *Togaviridae* (+ssRNA genome), *Peribunyaviridae* (-sRNA genome), *Rhabdoviridae* (-sRNA genome), *Reoviridae* (dsRNA genome), *Mesoniviridae* (+ssRNA genome), and unclassified Negeviruses. Examples of MSVs reported in literature include: Cell Fusing Agent virus (CFAV), Kamiti River virus (KRV), and Palm Creek virus (PCV) belong to the *Flaviviridae*; Badu virus (BADUV), Kibale virus (KIBV), and Phasi Charoen-like virus (PCLV) belong to the family *Peribunyaviridae*; Aedes Anphevirus (AeAV), Merida virus (MERDV), and Arboretum virus (ABTV) belong to the family *Rhabdoviridae*. Most MSVs are RNA viruses (For example: Flaviviruses), but there are a few DNA forms (For example: Densoviruses). These viruses were isolated from a wide range of mosquitoes mainly *Aedes*, *Culex*, *Anopheles*, *Ochlerotatus*, and *Coquilletidia*. Also, these mosquitoes have a broad

geographical distribution. Although not well understood, the possible modes of transmission could be by vertical and horizontal means (Agboli et al. 2019). MSVs often infect mosquitoes and remain persistent for the remainder of their adult lives.

1.3.2 Discovery and identification of mosquito-specific viruses

As early as the 1960s, many virus families such as *Parvoviridae*, *Baculoviridae* and *Iridoviridae* causing apparent symptoms or mortality in mosquito larvae have been discovered and studied. During the past decade, the use of next generation sequencing, metagenomics, and phylogenetics have steered a new era of virus discovery. With the widespread of these techniques, many novel viruses have been discovered, documented, and exhibiting a restricted host-range (Fig. 1.6) (Junglen and Drosten 2013; Marklewitz et al. 2013; Vasilakis et al. 2013, 2014).This has also sparked questions concerning the transmission of these viruses and interactions with their mosquito hosts and its microbiome. The Table 1.1 below illustrates a few examples of MSVs and their families (Agboli et al. 2019).

The first MSV isolated was Cell Fusing Agent virus (CFAV), isolated 45 years ago from *Aedes aegypti* cells and failed to replicate in mammalian cell lines (BHK-21 and Vero cell lines) (Stollar and Thomas 1975). The second MSV, Kamiti River virus (KRV) was isolated from *Aedes macintoshi* mosquitoes collected in Kenya and found to be related to CFAV (Crabtree et al. 2003; Sang et al. 2003). After these discoveries, a lot of MSVs were discovered in a variety of mosquitoes belonging to known and new virus families and genera.

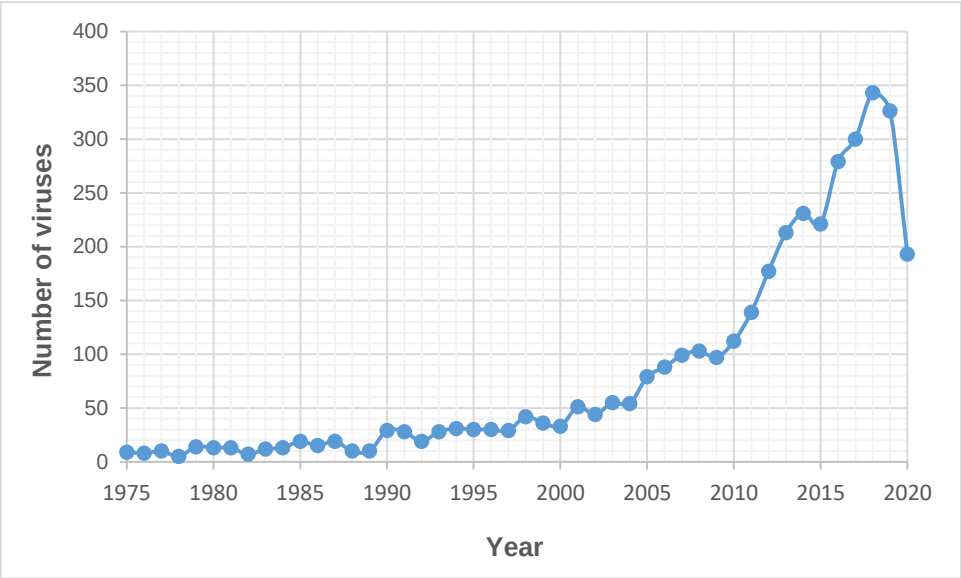

Fig. 1. 6: Mosquito-specific viruses reported in literature.

Increasing trend of mosquito-specific virus discovery and research. A search at the NCBI database (https://www.ncbi.nlm.nih.gov) by entry of the syntax 'mosquito specific viruses'. Last search date: 13th August, 2020.

Table 1. 1: Examples of mosquito-specific viruses

MSVs are discovered and confirmed to grow extensively on insect cell iines. At least 3 examples for each family containing late and most recent discovery; unless the family has less MSVs. The year in the table signifies the date of mosquito collection from the field except for cell lines reporting the year the result was published. Table adapted from (Agboli et al. 2019).

MSV (Acronym)	Year	First Mosquito Host	Country isolated	Family	Reference
			Flaviviridae		
Cell-fusing agent virus (CFAV)	1975	*Aedes aegypti* cell line	USA		(Stollar and Thomas 1975)
Kamiti River virus (KRV)	1999	*Aedes macintoshi*	Kenya		(Sang et al. 2003)
La Tina virus (LTNV)	1996	*Aedes scapularis*	Peru		(Guzman et al. 2018)
			Togaviridae		

Eilat virus (EILV)	1982	*Anopheles coustani*	Israel		(Nasar et al. 2012)
Agua Salud alphavirus (ASALV)	2013	*Culex declarator*	Panama		(Hermanns et al. 2020)
Mwinilunga alphavirus (MWAV)	2016	*Culex quinquefasciatus*	Zambia		(Torii et al. 2018)
			Peribunyaviridae		
Phasi Charoen virus (PCLV)	2007	*Aedes aegypti*	Thailand		(Yamao et al. 2009)
Ferak virus (FERV)	2004	*Culex* sp.	Côte d'Ivoire		(Marklewitz et al. 2015)
Badu virus (BADUV)	2003	*Culex* sp.	Australia		(Hobson-Peters et al. 2016)
			Rhabdoviridae		
Moussa virus (MOUV)	2004	*Culex decens*	Côte d'Ivoire		(Quan et al. 2010)
Merida virus (MERDV)	2007	*Culex quinquefasciatus*	Mexico		(Charles et al. 2016)
Aedes Anphevirus (AeAV)	2018	*Aedes albopictus* cell line RML-12	USA		(Parry and Asgari 2018)
			Mesoniviridae		
Nse virus (NseV)	2004	*Culex nebulosus*	Côte d'Ivoire		(Zirkel et al. 2013)
Yichang virus (YCV)	2015	*Culex* sp.	China		(Wang et al. 2017)
Dianke virus (DKV)	2013	*Aedes vexans*	Senegal		(Diagne et al. 2020)
			Tymoviridae		
Culex originated *Tymoviridae*-like virus (CuTLV)	2005	*Culex* sp.	China		(Wang et al. 2012)
			Birnaviridae		
Culex Y virus (CYV)	2010	*Culex pipiens (s.l.)*	Germany		(Marklewitz et al. 2012)

Espirito Santo virus (ESV)	2012	*Aedes albopictus* C6/36 cells	Brazil		(Vancini et al. 2012)
Negeviruses					
Negev virus	2008	*Culex coronator*	USA		(Vasilakis et al. 2013)
Wallerfield virus (WALV)	2009	*Culex portesi*	Trinidad & Tobago		(Auguste et al. 2014)
Mayapan virus	2007	*Psorophora ferox*	Mexico		(Charles et al. 2018)
Nodaviridae					
Mosinovirus (MoNV)	2004	*Culicidae* mosquitoes	Côte d'Ivoire		(Schuster et al. 2014)
Reoviridae					
Aedes pseudoscutellaris reovirus (APRV)	2005	*Aedes pseudoscutellaris* mosquito cells	France		(Attoui et al. 2005)
Cimodo virus (CMDV)	2008	*Culex nebulosus*	Côte d'Ivoire		(Hermanns et al. 2014)
Ninarumi virus (NRUV)	2009	*Ochlerotatus fulvus*	Peru		(Sadeghi et al. 2017)
Parvoviridae					
Culex pipiens pallens densovirus (CppDV)	2008	*Culex pipiens pallens*	China		(Zhai et al. 2008)
Culex pipiens densovirus (CpDV)	2000	*Culex pipiens pallens*	France		(Jousset, Baquerizo, and Bergoin 2000)
Aedes albopictus densovirus 2 (AalDV 2)	1993	*Aedes albopictus* C6/36 cells	France		(Jousset et al. 1993)
Iridoviridae					
Anopheles minimus Iridovirus (AMIV)	2010	*Anopheles minimus*	China		(Huang et al. 2015)

			Permutotetraviridae		
Sarawak virus (SWKV)	2013	*Aedes albopictus*	Malaysia		(Sadeghi et al. 2017)
Shinobi tetravirus (SHTV)	2018	*Aedes albopictus* C6/36 cells	Japan		(Fujita et al. 2018)
			Iflaviridae		
Aedes vexans ifla-virus	2017	*Aedes vexans arabiensis*	Senegal		(Parry et al. 2020)
Armigeres iflavirus	2010	*Armigeres mos-quitoes*	Philippine		(Kobayashi et al. 2017)
			Orthomyxoviridae		
Sinu virus	2013	Adult mosquito pool	Colombia		(Contreras-Gutiérrez et al. 2017)
			Totiviridae		
Omono river virus	2007	*Culex inatomii*	Japan		(Isawa et al. 2011)

1.3.2.1 *Mesoniviridae*

The order *Nidovirales* is made of a genetically diverse group of viruses in four main families: *Arteriviridae*, *Coronaviridae*, *Roniviridae* and newly established *Mesoniviridae* (Lauber et al. 2012). Though mesoniviruses do not cause disease in humans or livestock, they are of interest because of their association with other members of the nidoviruses in terms of structural and genetic similarities. In addition, the position of coronaviruses in relation to mesoniviruses in phylogenetic analyses made scientists to suggest that *Coronaviridae* may have evolved in arthropods (Junglen et al. 2009; Nga et al. 2011; Zirkel et al. 2011, 2013). Approximately 80 virus species of nidoviruses with host variety from crustaceans to mammals has been established. Mesoniviruses are hosted by insects, coronaviruses by insects and vertebrates, arterviruses by vertebrates, and roniviruses by crustaceans (Adams et al. 2016; Wang et al. 2017). However, the first report of a MeSV in invertebrates other than mosquitoes was the detection in an Aphid species *Aphis citricidus* (Hemiptera: *Aphididae*) in China (Chang et al. 2020).

Mesoniviridae is a recently established family in the order *Nidovirales* comprising a group of linear positive-sense, single-stranded RNA [(+)ssRNA] mosquito-specific viruses with

a 5’ cap structure and 3’ poly(A) tail (Lauber et al. 2012). The actual genomic size of mesoniviruses varies depending on the species. However, the family has an average genomic size of 20kb. Yichang virus genome has a size of 20,893 bp excluding the poly(A) tail and it is regarded as the largest genome of the *Mesoniviridae* (Wang et al. 2017). *Mesoniviridae* viral particles are enveloped, spherical, about 60–80 nm diameter with club-shaped surface spikes (Zirkel et al. 2013). The genome is organized in seven major open reading frames (ORFs) as ORF1a, ORF1b, ORF2a, ORF2b, ORF3a, ORF3b, ORF4 (Wang et al. 2017; Warrilow et al. 2014). Two polymerase polyproteins (pp) are predicted to be encoded by ORF1a and ORF1b, while the ORFs in the 3’-end encode structural proteins such as the spike (S) glycoprotein (ORF2a), the nucleocapsid (N) protein (ORF2b), and two proteins with membrane-spanning regions (ORF3a and -3b) (Nga et al. 2011; Zirkel et al. 2011) (Fig. 1.7)

Notwithstanding considerable differences in genome size and gene composition, all *Nidovirales* (mesoniviruses) have similar genome organizations and replication strategies (Kuwata et al. 2013). The large 5′ part of the genome encodes two partially overlapping ORFs, which are designated as ORF1a and 1b. Translation of ORF1a yields a polyprotein called pp1a. ORF1b is translated as a fusion with the ORF1a product, to form polyprotein pp1ab, by a putative −1 ribosomal frameshift (RFS) at the overlap region (ORF1a/1b), which contains a specific slippery sequence just upstream of a pseudoknot structure. Thus, a frameshift just upstream of the ORF1a termination codon mediates the production of a C-terminally extended polyprotein jointly encoded by ORF1a and ORF1b. The large polyproteins pp1a and pp1ab are proteolytically processed to yield the replicase subunits, including a 3C-like (3CL) protease flanked by transmembrane (TM) domains, which is encoded by ORF1a, and both an RNA-dependent RNA polymerase (RdRp) and a superfamily 1 helicase (HEL1) encoded by ORF1b. Combined, these markers form a distinctive constellation: 3CLpro_RFS_RdRp_HEL1 typical for mesoniviruses (Gorbalenya et al. 2006; Lauber et al. 2012). The region downstream of ORF1b contains multiple smaller ORFs, the number of which varies among mesoniviruses, and which encode a set of viral structural proteins (for example, nucleocapsid protein and spike glycoprotein). Translation of these structural proteins occurs through a nested set of 3′-coterminal subgenomic mRNAs that are controlled by leader transcription-regulating sequences (TRSs) (Pasternak, Spaan, and Snijder 2006).

Spatiotemporal distribution of *Mesoniviridae* over a wide geographic regions and broad species host range in mosquitoes (*Culex* spp, *Aedes* spp, *Coquillettidia* spp, *Uranotaerina* spp.) with *Culex* mosquitoes as the main host has been observed. This suggests that mesoniviruses may be common in mosquito populations worldwide. The species of mosquitoes are from Africa (Côte d’Ivoire), Asia (Vietnam, Thailand, Korea, Indonesia, and China), Australia (Northern Territory), and North America (USA) (Hang et al. 2016; Kuwata et al. 2013; Liu et al. 2013; Nga et al. 2011; Thuy et al. 2013; Vasilakis et al. 2014; Warrilow et al. 2014; Zirkel et al. 2011, 2013). At the moment, about 13 virus

strains (For example: Nse, Meno, Hana, Cavally, and Yichang viruses) appear to represent the family *Mesonivridae* with 9 virus species (*Alphamesonirus 1-9*) (Amoa-Bosompem et al. 2020; Vasilakis et al. 2014) (Fig. 1.8). At the moment, all other mesoniviruses have only been detected in one mosquito host and Dianke virus is the only one where the same virus has been found in different mosquitoes (Diagne et al. 2020).

To the best of my knowledge, mesoniviruses were not reported in Germany until recently when Dr. Börstler during her PhD studies identified MeSV 8345 in *Coquillettidia richiardii* mosquitoes. Dr. Börstler performed field entomological survey from 2013 to 2015 in Germany and collected different mosquito species (Börstler 2016). Furthermore, the available sequence reveals its relatedness to Yichang virus isolated in China (Wang et al. 2017) (Fig. 1.9). Little characterization was done for mesoniviruses in general and MeSV 8345 specifically. Therefore, it is vital to further characterize MeSV 8345 to better understand its interaction with the mosquito host.

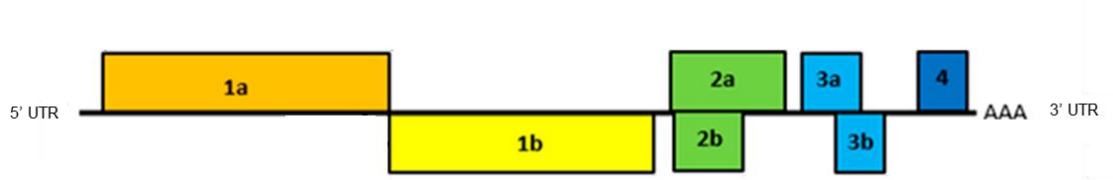

Fig. 1. 7: Genomic structure of Mesoniviridae.

The genomic structure is representative for mesoniviruses. The coding and 5′- and 3′-untranslated regions (UTRs) of the genome are represented by the horizontal lines. Poly(A) tail is located at the 3‘ end of the genome. Open reading frames (ORFs) are shown as open rectangles and depicted as ORF1a, -1b,-2a, -2b, -3a, -3b, and -4. Adapted from (Amoa-Bosompem et al. 2020).

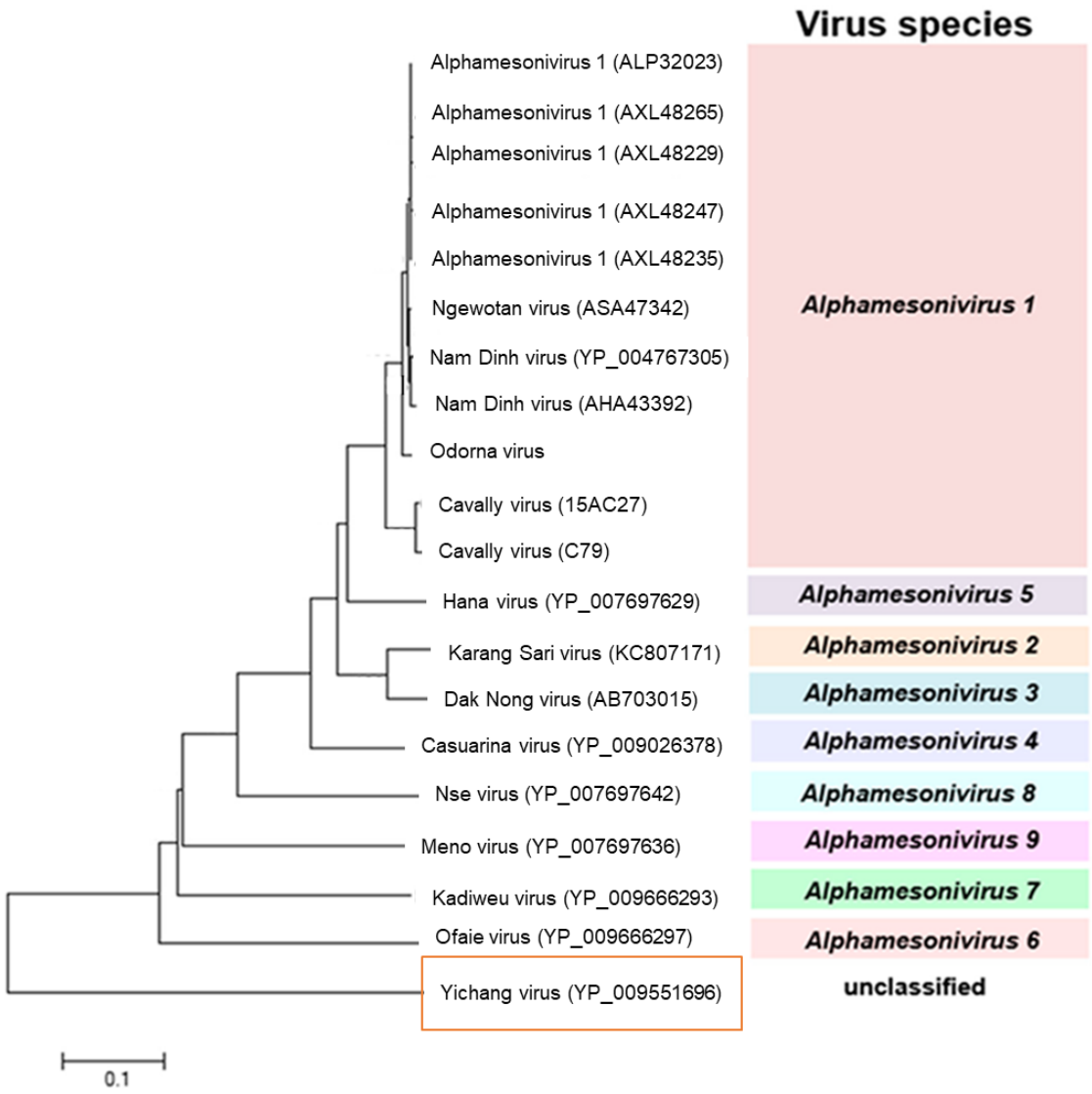

Fig. 1. 8: Maximum likelihood phylogenetic tree of Mesonivirus species.

The phylogenetic tree is representative for mesoniviruses. The maximum likelihood tree constructed with conserved amino acid domains in genome sequences of the mesoniviruses, extraction done by using MAFFT 7 online version and the Gblocks program. Yichang virus is highlighted, a close relative for MeSV 8345 strain (not shown). Nine species are labelled as *Alphamesonivirus 1-9*. Figure adapted from (Amoa-Bosompem et al. 2020).

1.3.3 General life cycle of mosquito-specific viruses

The life cycle of MSVs is briefly described as the mosquito-only cycle where the virus circulates within the mosquito and does not involve vertebrates (Fig. 1.9). This illustrates the unique life cycle of MSVs in their ecological niche which matches their single host

tropism. This is different from the cycle of arboviruses involving vertebrate hosts in addition to the invertebrate vectors. It is important to note that for a lot of MSVs, the life cyle is not known and often only suggested.

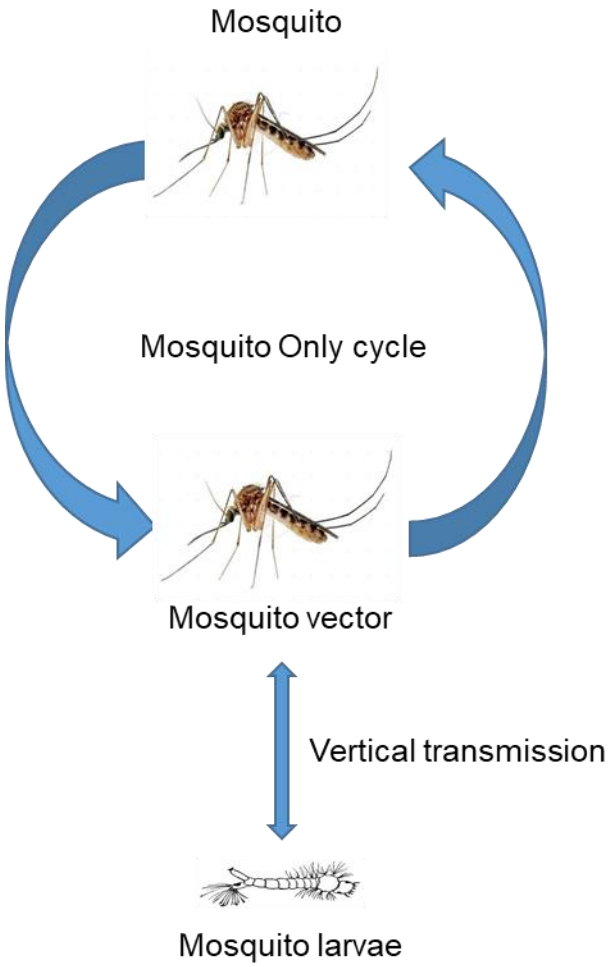

Fig. 1. 9: Illustration of the life cycle of mosquito-specific virus.

The life cycle of MSVs is restricted to the mosquito vector. Infected adult mosquito can transmit MSV to noninfected mosquito through food or water. It is possible that the virus can be passed on to the progeny (mosquito larvae) via vertical transmission (transovarially).

1.3.4 Mechanisms of transmission of mosquito-specific viruses

Viruses can be transmitted to the host vertically, from parents to the offspring or horizontally, from the environment or via a vector. Alternatively, viruses can adopt a mixed-mode transmission involving both horizontal and vertical transmission, which seems to be the most common form of transmission of microbiota, including viruses, in nature. Transmission routes can alter the outcome of the infection and play a defining role in the ecology

of the virus, their spread and their maintenance in nature (Cressler et al. 2016; Ebert 2013).

Mosquito-specific RNA viruses are often considered vertically transmitted. Indeed, several MSVs including Culex flavivirus (CxFV), Aedes flavivirus (AeFV), and Kamiti River virus (KRV) have been found in field-collected larvae, eggs or adult males. However, experimental evidence for their vertical transmission is rare and so far limited to the *Flaviviridae* family. Venereal transmission also occurred between CxFV infected and uninfected *Cx. pipiens* males and females bidirectionally. Moving away from RNA viruses, vertical transmission of mosquito-specific DNA viruses such as densoviruses is also a growing field of research. Aedes albopictus densovirus (AalDV2) can be transmitted to the offspring and the efficiency of transmission depends on the virus titre in *Aedes aegypti* females (Agboli et al. 2019).

In addition to the vertical transmission, horizontal transmission (e.g. from water to larvae and through feeding to larvae or adult mosquitoes) can also explain the observed presence of MSVs in male adults and larvae in nature. So far, mostly mosquito-specific DNA viruses have been studied for horizontal transmission during larval stage due to the easily observable disease symptoms and resulting mortality. Aedes albopictus densovirus (AalDV2) has experimentally shown to infect mosquito larvae when infected crushed larvae or cultured cells were added to the rearing water. For mosquito-specific RNA viruses, horizontal transmission during the larval stage is rarely studied experimentally and studies showed different results. KRV (*Flaviviridae)* was able to infect the larvae when added to rearing water, while CxFV (*Flaviviridae*) was not detected in the water where the infected larvae were reared (Agboli et al. 2019). Further, Yichang virus (YCV, *Mesoniviridae*) was able to infect larvae of *Culex quinquefasciatus* and *Aedes albopictus* mosquitoes when added to their breeding water (clean water and sewage water) (Ye et al. 2020). A few studies on the diversity of RNA viruses also indirectly suggested the importance of horizontal transmission in nature, as many mosquitoes from the same collection site had genetically close virus variants independently from the host species. Overall, the mixed-mode transmission including both horizontal and vertical transmission routes is likely to be the key for MSV persistence and dispersal in nature (Agboli et al. 2019).

Experimentally proven and hypothetical transmission routes for MSVs are summarized in Fig. 1.10. These routes are via adult mosquitoes feeding on plant nectars or from plant materials in the water. MSVs become persistent in the infected larvae or adults. Furthermore, parasitic mermithids are also agents for MSV transmission by entering the larvae (Agboli et al. 2019).

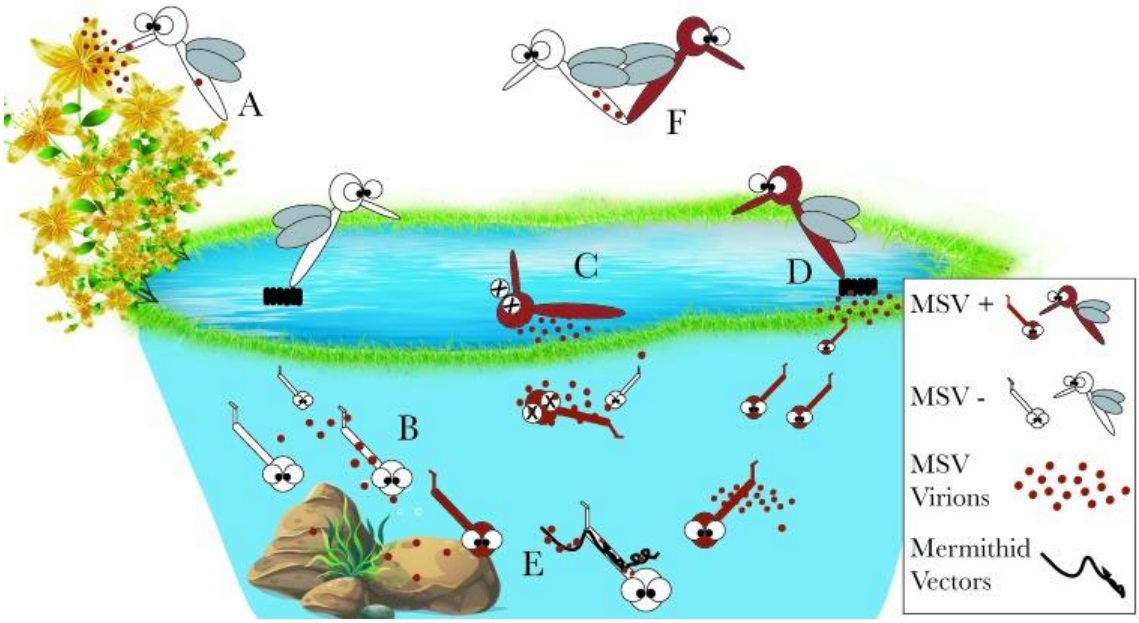

Fig. 1. 10: Potential transmssion routes for mosquito-specific viruses.

Potential transmission routes for MSVs: It is hypothesized that MSVs can be transmitted horizontally from environment to the adults through feeding on plant nectars [**A**] or to the larvae from plant material in the water or directly through the water [**B**]. Infected larvae and adults might become natural reservoir for some MSVs when they die (e.g. food source) [**C**]. The larvae that survive the infection can emerge as adults and potentially vertically transmit MSVs to their offspring either directly through the egg (transovarially) or indirectly by contaminating the egg surface (transovum) or the water [**D**]. Some MSVs have been shown to be horizontally transmitted by parasitic mermithids, where the latter carries the MSV when they enter the larvae [**E**]. MSVs can also be venereally transmitted between infected and uninfected adults in a low rate [**F**]. Adapted from (Agboli et al. 2019).

1.3.5 Acute and persistent infection of mosquito-specific viruses

Cell lines or mosquitoes could be infected by MSVs acutely or persistently infected. An acute infection is a new infection resulting in high virus replication and production. However, with a persistent infection the virus is at a lower replication and its production levels. Therefore, a persistent infection can occur if an acute infection is not cleared.

In nature, mosquitoes are frequently infected by one or several MSVs and this translates to their respective cell lines. Many reports indicate that invertebrate cell culture systems are persistently infected with MSVs (Bell-Sakyi and Attoui 2013, 2016; Wu et al. 2010). The extent to which invertebrate cell lines were infected with MSVs is unknown and the use of NGS has highly improved the knowledge of persistent MSV infection in mosquito-derived cell lines. *Aedes aegypti*-derived Aag2 cells were detected to be persistently infected with Phasi Charoen-like virus (PCLV) (Maringer et al. 2017; Schnettler et al. 2016) and Cell Fusing Agent virus (CFAV) (Schnettler et al. 2016; Scott et al. 2010).

MSVs were identified in mosquito-derived cell lines via small RNA deep sequencing confirming that MSVs are persistently present in the respective cell lines. Examples of confirmed persistent MSVs include: Calbertado virus (CALBOV) in *Culex tarsalis*-derived Ct cell lines, Merida virus (MERDV) in *Culex quinquefasciatus*-derived Hsu cell lines, Culex Y virus (CYV) and Aedes densovirus (AeDNV) in Aag2 cells (Franzke et al. 2018; Göertz, Miesen, et al. 2019; Rückert et al. 2019). *Aedes albopictus*-derived C6/36 cell lines are so far the only mosquito-derived cell line without reports of persistent MSV infection (Göertz, Miesen, et al. 2019). Together, persistent MSVs identified so far belong to different virus families and most of them are cell specific.

1.4 Mosquitoes

1.4.1 General overview

Mosquitoes are dipterans of the suborder Nematocera and all are placed within the family *Culicidae*. Mosquito vectors can transmit several pathogens, including arboviruses, protozoans, and filariae that cause infectious diseases of significant public health concern. Human and animal diseases such as Rift Valley fever (RVF), dengue fever (DF), yellow fever (YF), Zika (ZIK), and chikungunya (CHIK) are caused by viruses with mosquitoes as main vectors (Becker et al. 2010; Digoutte et al. 1995). It is known that the mosquito vector is not just tolerating the virus infection but actively fights it. However this is most of the time not sufficient for viral clearance, hence minimal pathological changes and fitness costs are encountered by the vector (Kean et al. 2015; Olson and Blair 2015; Styer et al. 2007).

Mosquito vectors of public health concern mainly belong to the three genera, *Anopheles*, *Aedes* and *Culex.* Globally, about 3500 mosquito speices in 44 genera are described. Approximately 460 species are recognized for the genera *Anopheles* with seven subgenera, *Culex* with 26 subgenera comprising 770 species, and *Aedes* with 70 subgenera including 927 species are the highest diversified species and most important for public health (Freitas et al. 2015; Laurito et al. 2017; Wilkerson et al. 2015).

1.4.2 *Culex* and *Aedes* mosquitoes

Culex mosquitoes are vectors of arboviral infections such as West Nile virus, Usutu virus, Janapense encephalitis virus, and St. Louis encephalitis virus. They are also transmitters of human lymphatic filariasis (Harbach 2011; Nikolay et al. 2012; Talla et al. 2016; Tandina et al. 2016). The subgenus *Culex* Linnaeus contains the medically important complexes, *Cx. pipiens* and *Cx. vishnui* (Harbach 2020; Laurito et al. 2017). The *Cx. pipiens* complex includes: *Cx. pipiens pipiens* (with two forms, pipiens and molestus),

Cx. pipiens pallens, *Cx. quinquefasciatus, Cx. australicus,* and *Cx. globocoxitus* (Farajollahi et al. 2011). The *Cx. vishnui* complex includes: *Cx. vishnui*, *Cx. tritaeniorhynchus*, and *Cx. pesudovishnui* (Longbottom et al. 2017). Another common species is the *Cx. torrentium* which is considered to be a sister-taxon of *Cx. pipiens* (Vinogradova, Ivshina, and Shaikevich 2013). Lastly, *Cx. tarsalis* is also a medically important vector of arboviruses (Ayers et al. 2018). Geographically, the northern parts of the temperate zones have no *Culex* mosquitoes. However, across the tropical and subtroptical regions there is *Cx. quinquefasciatus* and *Cx. pipiens pipiens* covers the temperate zones, Africa, Middle East, and Asia. Two non-European and African species are located in Australia, *Cx. australicus* and *Cx. globocoxitus* (Farajollahi et al. 2011). The Fig. 1.11 below illustrates the geographical distribution of *Culex* mosquitoes.

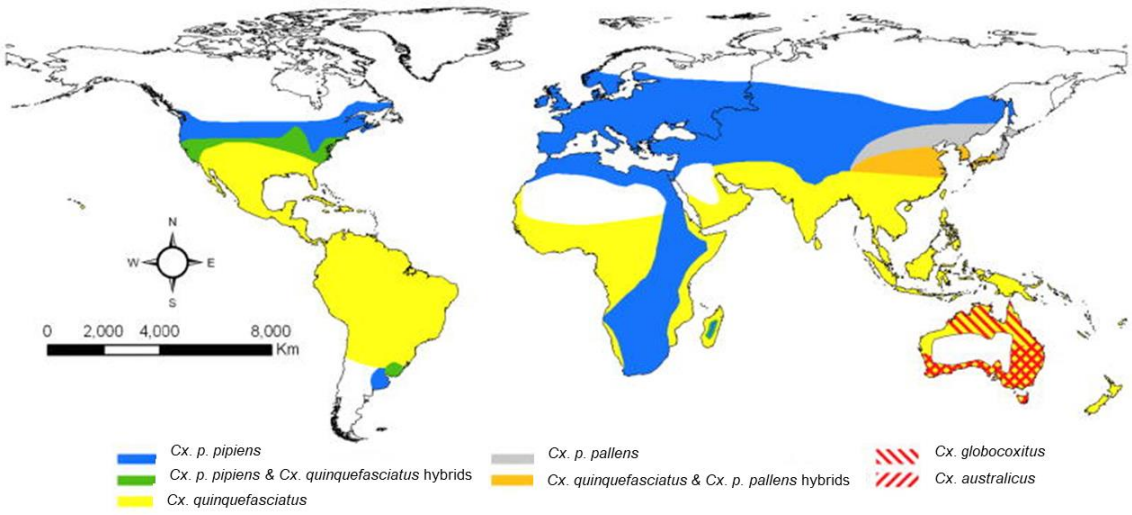

Fig. 1. 11: Global distribution of *Culex pipiens* complex mosquitoes.

Culex species are absent from extreme northern parts of temperate zones. *Cx. quinquefasciatus* is present mainly across tropical/subtropical regions. *Cx. pipiens pipiens* is present across temperate regions and into parts of the Middle East, Africa, and Asia. There is some overlap with *Cx. quinquefasciatus*. *Cx. australicus* and *Cx. globocoxitus* are restricted to Australia. Map adapted from (Farajollahi et al. 2011).

Aedes mosquitoes are the primary vectors of several medical important arboviruses, Fellow fever, Dengue fever, Chikungunya, and Zika fever. Human lymphatic filariasis is also transmitted by *Aedes* mosquito (Russell, Webb, and Davies 2005; Souza-Neto, Powell, and Bonizzoni 2019). This implies that both *Aedes* and *Culex* mosquitoes are vectors of infections of high public health importance (arboviruses and lymphatic filariasis). The most prolific medically important species of the genus *Aedes* are *Ae. aegypti* and *Ae. albopictus*. Other important vectors include: *Ae. aborigins*, *Ae. atlanticus*, *Ae. atropalpus*, *Ae. camptorhynchus*, *Ae. cantator*, *Ae. polynesiensis*, and *Ae. vexans*

(NIAID 2020; WRBU 2020). In the past, *Ae. aegypti* and *Ae. albopictus* were geographically resticted to the African continent and South East Asia respectively (Mousson et al. 2005; Scholte and Schaffner 2007). However, they have currently colonized almost all continents (Kraemer et al. 2015). Fig. 1.12 below illustrates the geographical distribution of *Aedes* mosquitoes.

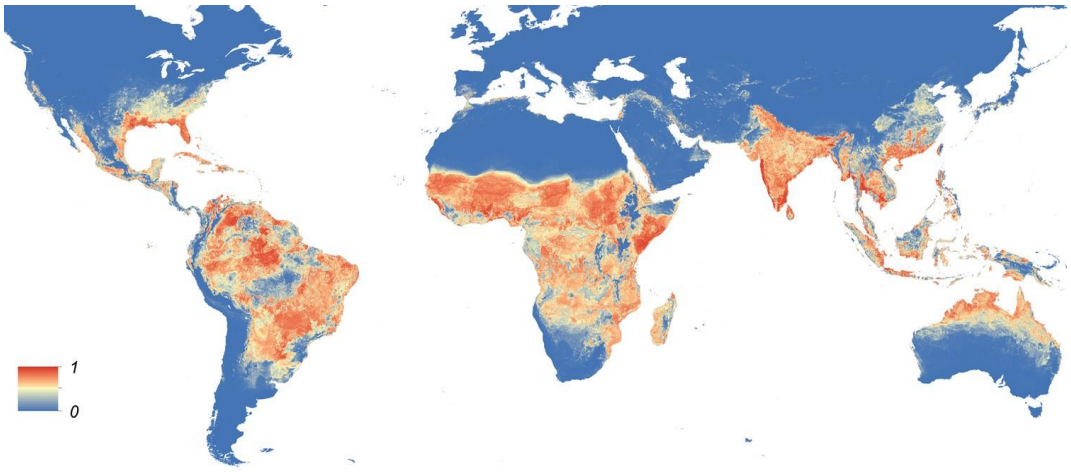

Fig. 1. 12: Global map of the predicted distribution of *Aedes aegypti*.

The map depicts the probability of occurrence (from 0 blue to 1 red). *Aedes aegypti* originated from Africa but are now seen across all continents. *Ae. aegypti* are present mainly across tropical/subtropical regions but can be found in more temperate regions such as the Northern United States of America. *Ae. Aegypti* are not found in the northernmost temperate zones. Map adapted from (Kraemer et al. 2015).

1.5 Interactions of mosquito-specific viruses with arboviruses

This section contains parts of my review article on mosquito-specific viruses, transmission and interaction (Table 1.2) (Agboli et al. 2019).

Mosquitoes can easily be infected with both MSVs and arboviruses simultaneously; although, MSVs have a strict tropism. However, it has been shown already that MSVs may affect the mosquitoes' ability to acquire, maintain and transmit these viruses (i.e. vector competence) (Agboli et al. 2019). In the recent years, a number of studies have povided information on the experimental interaction of MSVs and arboviruses in vitro and in vivo. The interaction leads to reduction or increasing the replication of the target arbovirus. At times, there is no effect of MSV on the target arbovirus.

It was shown that *Culex pipiens* mosquitoes persistently infected with mosquito-specific Culex Flavivirus (CxFV) suppresses the replication of West Nile virus (WNV). The results

also suggest that the presence of CxFV may impact the intensity of enzootic transmission of WNV and the risk of human exposure (Bolling et al. 2012a). In another experiment, CxFV had no effect on the replication of WNV using injected *Culex quinquefasciatus* mosquitoes (Kent, Crabtree, and Miller 2010b). Furthermore, CxFV-infection in *Culex pipiens* did not affect the transmission of Rift Valley Fever virus (RVFV). This implies that CxFV existing in field-collected *Culex pipiens* populations does not affect their vector competence for RVFV (Talavera et al. 2018). Overall, the effect of CxFV on investigated arboviruses replication depends on the infection route and mosquito species.

Mosquito-specific Palm Creek virus (PCV) suppressed the replication of WNV and Murray Valley Encephalitis Virus (MVEV) by 10–43 fold at 48 hours post-infection in *Aedes albopictus*-derived cells C6/36. Interestingly, no inhibitory effect of PCV infection was observed for the alphavirus Ross River virus (RRV), suggesting the possibility of superinfection exclusion between MSVs and arthropod-borne viruses belonging to the same family (Hobson-Peters et al. 2013). Other investigations with PCV also showed that persistent infection of PCV has no inhibitory effect on the replication of WNV in vivo (Hall-Mendelin et al. 2016).

Mosquito-specific Nhumirim virus (NHUV) suppressed the replication of WNV and also blocks the transmission in *Culex quinquefasciatus* mosquitoes (Goenaga et al. 2015; Kenney et al. 2014). The first investigation was performed by Kenney and colleagues using three arboviruses: WNV, St Louis Encephalitis virus (SLEV) and Japanese Encephalitis virus (JEV). These in vitro coinfection experiments showed that prior or concurrent infection of *Aedes albopictus* derived mosquito cells (C6/36) with NHUV resulted in a significant reduction in virus production of WNV, SLEV and JEV. The inhibitory effect was most effective against WNV (>10^6 fold peak titre) and SLEV (>10^4 fold peak titre) (Kenney et al. 2014). Another NHUV study involves CHIKV, Zika virus (ZIKV) and Dengue virus (DENV). In the study, NHUV suppressed the replication of ZIKV and DENV-2 but not CHIKV in *Aedes albopictus* derived cells. Significant reductions in ZIKV (10^5 fold) and DENV-2 (10^4 fold) were observed in cells concurrently inoculated with NHUV or pre-inoculated with NHUV. The authors suggest that NHUV can interfere with both midgut infection and salivary gland infection of ZIKV in *Aedes aegypti* (Romo et al. 2018).

Coinfection of CFAV and PCLV limits replication of arboviruses in *Aedes* mosquito cells. Schultz and colleagues found the growth of ZIKV to be consistent in *Aedes albopictus* cells but variable in *Aedes aegypti* cell lines. They linked this finding to the observation that PCLV was present in the ZIKV-growth-variable *Aedes aegypti* cell lines but absent in *Aedes albopictus* lines. Furthermore, PCLV infection of CFAV-positive *Aedes albopictus* cells inhibited the growth of ZIKV, DENV and La Crosse virus (LACV) (Schultz, Frydman, and Connor 2018). Data from the first CFAV study and the recent result suggest that persistently infected cell lines with MSV can impact arbovirus growth.

Active infection of *Aedes albopictus*-derived cells (C7/10) with mosquito-specific Eilat virus (EILV) reduced the titres of coinfecting viruses (SINV, VEEV, EEEV, WEEV and CHIKV) by approximately 10–10,000 fold and delayed the replication kinetics by 12–48 hours. Additionally, prior in vivo EILV infection of *Aedes aegypti* mosquitoes delayed dissemination of CHIKV for 3 days (Nasar et al. 2015).

Recently, the interference of Yichang virus (YCV, *Mesoniviridae*) with representative flaviviruses was assessed. YCV significantly inhibited the replication of medically important DENV-2 and ZIKV, in cell culture, and reduced transmission rate of DENV-2 in *Aedes albopictus* mosquitoes (Ye et al. 2020). To the best of my knowledge, this is the only mesonivirus-arbovirus interaction experimented so far and has focused on *Aedes* mosquitoes.

Altogether, most of these studies have been done with flaviviruses and if an effect is seen it is usually that the MSV and arbovirus belong to the same virus family. These interactions provide information on MSVs that could be studied in detail and use as a probable novel biological vector control tool for arbovirus infections of public health importance. However, more knowledge is needed and the best is a virus found that interferes with different virus families. A very limited information is available on mesonivirus-arbovirus interactions. Therefore, investigating these interactions is a vital first step to exploring the possibility of any effect which can in turn influence vector competence of the mosquito host. The current study seeks to unravel the possible effect MeSV 8345 will have on arboviruses from different families.

Table 1. 2: Literature review of experimental interaction of mosquito-specific viruses and arboviruses.

Families of various MSVs with their experimental target arboviruses indicated. The possible effect of MSV on the growth of arboviruses is grouped for individual MSVs. Adapted from (Agboli et al. 2019).

MSV (Family)	**Arbovirus**	**Experimental interaction**		**Effect on growth of arbovirus**	**Reference**
		In-vitro	**In-vivo**		
Flaviviridae					
CFAV	DENV-1 ZIKV LACV	Yes Yes Yes	Yes Yes No	Reduction	(Baidaliuk et al. 2019; Schultz et al. 2018; Zhang et al. 2017)

NHUV	ZIKV DENV CHIKV WNV SLEV JEV	Yes Yes Yes Yes Yes Yes	Yes No No Yes No No	Reduction No effect	(Goenaga et al. 2015; Kenney et al. 2014; Romo et al. 2018)
CxFV	RVFV WNV DENV, JEV JEV	No Yes Yes Yes	Yes Yes No No	Reduction No effect Increase	(Bolling et al. 2012a; Kent et al. 2010b; Kuwata et al. 2015; Talavera et al. 2018)
PCV	WNV WNV MVEV	No Yes Yes	Yes No No	Increase Reduction	(Hall-Mendelin et al. 2016; Hobson-Peters et al. 2013)
Togavridae					
EILV	SINV, VEEV, EEEV, WEEV, CHIKV	Yes No	No Yes	Reduction	(Nasar et al. 2015)
Peribunyaviridae					
PCLV	ZIKV, DENV LACV	Yes	No	Reduction	(Schultz et al. 2018)
Rhabdoviridae					
MRV	ZIKV	Yes	No	Reduction	(Fujita et al. 2018)
Parvoviridae					
AalDV	JEV, DENV-2	Yes	No	Reduction	(Burivong et al. 2004; Kanthong et al. 2008, 2010)
AgDNV	MAYV	Yes	Yes	Reduction	(Urakova et al. 2020)
Mesoniviridae					
YCV	DENV-2, ZIKV JEV	Yes Yes	Yes No	Reduction No effect	(Ye et al. 2020)
Unclassfied Anphevirus					

AeAV	DENV	Yes	No	Reduction	(Parry and Asgari 2018)
Unclassified Riboviria					
SHTV	ZIKV	Yes	No	Reduction	(Fujita et al. 2018)

AalDV: Aedes albopictus densovirus, **AeAV:** Aedes Anphevirus, **AgDNV:** Anopheles gambiae densovirus, **DENV:** Dengue virus, **CFAV:** Cell fusing agent virus, **CxFV:** Culex Flavivirus, **EILV:** Eilat virus, **EEEV:** Eastern Equine Encephalitis virus, **JEV:** Japanese Encephalitis virus, **LACV:** La Cross Encephalitis virus, **MAYV:** Mayaro virus, **MRV:** Menghai rhabdovirus, **MVEV:** Murray Valley Encephalitis virus, **NHUV:** Nhumirim virus, **PCLV:** Phasi charoen-like virus, **PCV:** Palm Creek virus, **RVFV:** Rift Valley Fever virus, **SHTV:** Shinobi tetravirus, **SINV:** Sindbis virus, **SLEV:** St Louis Encephalitis virus, **VEEV:** Venezuelan Equine Encephalitis virus, **WEEV:** Western Equine Encephalitis virus, **WNV:** West Nile virus, **YCV:** Yichang virus, **ZIKV**: Zika virus.

1.6 Antiviral RNA interference

This section contains parts of my review article on mosquito-specific viruses, transmission and interaction (Agboli et al. 2019).

1.6.1.1 General overview of RNA interference

Mosquitoes rely on innate immune response as they lack an adaptive immune system. Upon infection, mosquitoes are known to mount an innate immune response against microbial challenge (bacterial, fungal, and viral infections). The major mosquito immune signaling pathways that have been implicated in the antiviral defense are the Toll, immune deficiency (IMD), Janus kinase/signal transducers and activators of transcription (JAK-STAT) and are therefore called classical innate immune pathways. Additionally, there is RNA interference (RNAi) pathway which is not a classical innate immune pathway as the production of its key proteins is not virus-induced (Sim, Jupatanakul, and Dimopoulos 2014).

Among the antiviral defense pathways in mosquito vectors, RNAi is a key player in limiting arbovirus replication (Blair and Olson 2014). RNAi is a sequence-specific knockdown response and divided into three main pathways depending on the small RNA (sRNA) molecules involved. These include: micro (mi)RNA, small interfering (si)RNA, and Piwi-interacting (pi)RNA pathway (Kean et al. 2015). Mosquitoes do not just tolerate infection but actively fight it through RNAi; however this is most of the times not sufficient to clear the infection but suggested to be the driver to move the acute infection into a persistent infection. Most knowledge about the antiviral RNAi in insects comes from studies in the

model organism, *Drosophila melanogaster* with either insect-specific viruses or non-natural arbovirus infection.

The siRNA pathway can be exogenously (viral replication intermediates) or endogenously (transposable elements, viral DNA forms) induced. The inducer double stranded RNA (dsRNA) can come from the exogenous or endogenous sources as mentioned above. The dsRNA is recognized by a Dicer protein (Dcr-2), which causes a cleavage of the dsRNA to form 21 bp-long virus-derived siRNAs, also called viRNAs. After viRNAs are incorporated and unwound in the RNA-induced silencing complex (RISC) that harbours Argonaute 2 (Ago 2) as a catalytic domain, one strand of the viRNA is retained and used as a guide to find complementary viral RNA, which is then degraded. The exogenous siRNA pathway is known to demonstrate antiviral response in invertebrates. Hence, it was shown to act antiviral for a variety of arboviruses in mosquitoes (Khoo et al. 2010; McFarlane et al. 2014; Samuel et al. 2016; Sánchez-Vargas et al. 2009), and cell lines, mostly *Aedes aegypti* (Dietrich, Jansen, et al. 2017; Dietrich, Shi, et al. 2017; McFarlane et al. 2014; Schnettler et al. 2013; Varjak, Donald, et al. 2017; Varjak, Maringer, et al. 2017; Varjak, Dietrich, et al. 2018), but not MSV yet.

The piRNA pathway is a Dicer independent pathway and with small RNAs (sRNAs) with a broader size range (25-29 nt) than the siRNA pathway. The piRNAs harbour specific characteristics due to their so-called 'ping-pong' production pathway: A10 bias for sense small RNAs and U1 bias for anti-sense small RNAs, as well as 10 nts complementarity between the sense and antisense small RNAs (Miesen, Joosten, and van Rij 2016; Siomi et al. 2011; Siomi, Miyoshi, and Siomi 2010). In mosquitoes especially *Aedes*, the endogenous piRNA pathway consists of two distinct branches, primary biogenesis (intermediate) pathway and secondary biogenesis (mature) pathway. In the primary pathway, piRNAs are processed from single-stranded RNA precursors that are transcribed from genomic loci known as piRNA clusters. Primary piRNAs are typically antisense to viral/cellular elements, exhibit a strong bias for a 5'-uridine residue (U1) associated with Piwi protein complex. Primary piRNAs are then fed into the second pathway, the "ping-pong dependent" amplification cycle. In this pathway, after binding of the target transcript, cleavage occurs ten nucleotides upstream from the 5' end of the primary piRNA, resulting in secondary piRNAs with an adenine residue in position 10 (A10), which are Argonaute-3 (Ago3) associated. Secondary piRNAs then bind complementary targets resulting in cleavage at the A-U base-pairing, resulting in piRNAs identical (or very similar) to the initial primary piRNA, exhibiting a 5'-U1 residue. Cleavage of the target transcript occurs via the Slicer activity of Ago3. The observed nucleotide bias is a hallmark of endogenous piRNAs, and is the basis for the ping-pong dependent amplification model (Liu et al. 2019; Rückert et al. 2019; Senti and Brennecke 2010; Varjak, Leggewie, and Schnettler 2018). Overview of the siRNA and piRNA pathway is illustrated in Fig. 1.13 below.

In recent years, research of the antiviral RNAi and its interaction with arboviruses in the mosquito vector has been performed showing similarities and differences to the model organism, *D. melanogaster* (Blair 2011; Blair and Olson 2014; Donald et al. 2012; Sim et al. 2014). The knowledge and the role of piRNA pathway proteins in mosquitoes is increasing. In contrast to *D. melanogaster* which expresses Piwi, Aub, and Ago3, mosquitoes lack Aub but encodes Ago3 and an expansion of PIWI proteins (1-7PIWI proteins) (Campbell, Black, et al. 2008; Schnettler et al. 2013). The piRNA pathway in *D. melanogaster* is mainly responsible for the control of transposon expression and focused mainly on the germline and surrounding cells. However, piRNAs in mosquitoes have not only been concentrated on the germline but are also present in somatic tissue (Agboli et al. 2019). Moreover, piRNAs in *D. melanogaster* appear not to act antivirally (Petit et al. 2016).

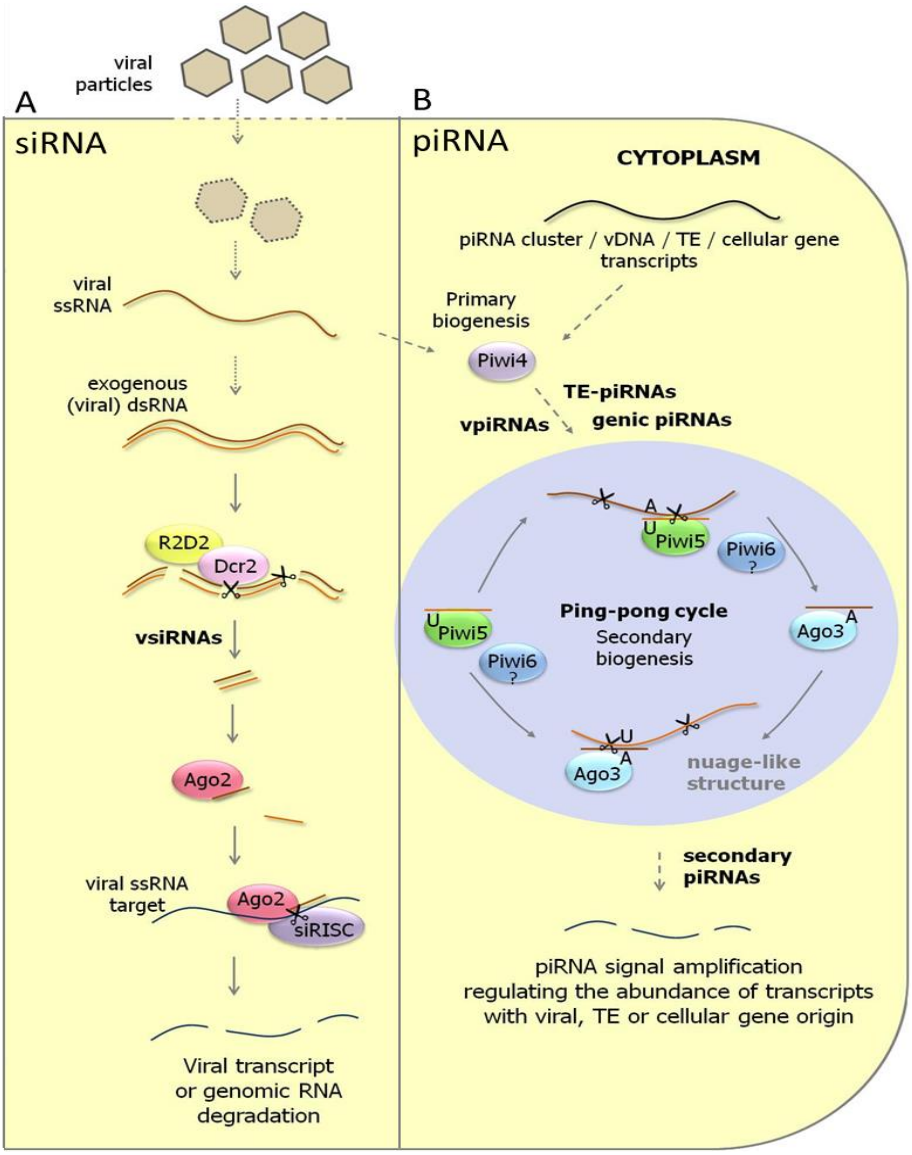

Fig. 1. 13: Overview of exogenous siRNA and piRNA pathways.

[**A**] **siRNA pathway**. Long dsRNA precursors that have exogenous (viral replication intermediates) origin are cleaved by Dicer-2 and its co-factor R2D2 to siRNAs and vsiRNAs. siRISC complexes containing Ago-2 subsequently scan parasitic RNA populations (transposon transcripts, viral transcripts and genomic RNAs, RNAs derived from viral integrations in genome) to trigger their destruction. [**B**] **piRNA pathway** in aedine mosquitoes. ssRNA precursors from various origins (transposable elements, viral mRNAs and genomic RNAs, transcripts from viral DNA forms, cellular gene transcripts) are processed to primary piRNAs by a Dicer-independent mechanism. While Piwi-4 does not directly interact with piRNAs, it was proposed that it acts as an important factor to activate the production of secondary piRNAs by the ping-pong mechanism. In the ping-pong cycle, piRNAs of antisense orientation (U1 bias) are mostly associated with Piwi-5 and possibly also with Piwi-6. On the other hand, piRNAs of sense orientation (A10 logo) are loaded by Ago-3. The piRNA ping-pong cycle is considered an important amplification mechanism to regulate the abundance of transcripts of transposon, viral or cellular origin. dsRNA, double stranded RNA; Dcr, Dicer; RISC, RNA-induced silencing complex; Ago, Argonaut; viRNA, viral specific small interfering RNA; siRNA, small interfering RNA; ssRNA, single stranded RNA. Adapted from (Liu et al. 2019).

1.6.1.2 Antiviral RNA interference and arboviruses

Studies have shown that arbovirus infection of mosquito cells, produced viral small RNAs, mainly siRNAs and piRNAs. This involves the arbovirus families *Togaviridae* (Chikungunyavirus, CHIKV; Semliki Forest virus, SFV; Sindbis virus, SINV), *Flaviviridae* (Dengue virus, DENV; West Nile virus, WNV; Zika virus, ZIKV), *Peribunyaviridae* (Bunyamwera virus, BUNV; La Crosse virus, LACV; Rift Valley Fever virus, RVFV; Schmallenberg virus, SBV), and *Reoviridae* (Blutongue virus, BTV). In general, viral small RNAs of piRNAs (23-30 nt) and siRNAs (21 nt) were produced and were distributed between genomic and antigenomic strands (Liu et al. 2019). These studies suggest the possibility of RNAi pathway acting antiviral.

During SINV infections of Dcr-2-incompetent C6/36 cells, mainly viral small RNAs of piRNA size (23–28 nt) were produced and were distributed unequally between genomic (70%) and antigenomic (30%) strands (Brackney et al. 2010). Hot spots of piRNA-sized small RNAs were observed in the subgenomic region that encodes the structural genes. Similarly, during infection of C6/36 and C7-10 cells with CHIKV, only vpiRNAs of 23–30 nt size were detected that showed a clear ping-pong amplification (1U antisense, A10 sense) signature (Goic et al. 2016; Morazzani et al. 2012). By contrast, both vsiRNAs and vpiRNAs were produced during CHIKV infection in Dcr-2-competent U4.4 cells (Goic et al. 2016; Morazzani et al. 2012).

Viral small RNAs following infection was abundant and observed to be much lower in *Culex* than *Aedes* mosquitoes (Dietrich, Jansen, et al. 2017). Here, the infection status or virus-specific effects could be the cause. Low amounts of vpiRNAs were produced during infection of *Culex* mosquitoes with RVFV (Dietrich, Jansen, et al. 2017) whereas they were not observed during WNV or USUV infection (Brackney, Beane, and Ebel 2009; Fros et al. 2015; Göertz et al. 2016) or SINV infection (Miesen, Joosten, et al. 2016). While the importance of the contribution still needs detailed investigation, the detection of RVFV-specific vpiRNAs suggests that the possibility of contribution of the piRNA pathway to the antiviral defense can be widend to *Culex* mosquitoes (Liu et al. 2019).

The knockdown or knockout of the key siRNA pathway proteins (Dcr-2 or Ago2) in whole mosquitoes and derived cell lines results in an increase in replication of a variety of arboviruses from different families. These include: DENV, CHIKV, SFV, SINV, ZIKV, BUNV, and Onyongnyong virus (ONNV) (Campbell, Keene, et al. 2008; Keene et al. 2004; McFarlane et al. 2014; Sánchez-Vargas et al. 2009; Schnettler et al. 2013; Waldock, Olson, and Christophides 2012). Similar knockdown studies involving CHIKV were reported for Ago3 and Piwi-related genes. In addition, the antiviral effect for Piwi4 was reported for the tested viruses, CHIKV, SFV, ZIKV, and BUNV (Varjak, Dietrich, et

al. 2018; Varjak, Donald, et al. 2017). The knockdown of respective RNAi proteins is not limited to cell lines but also mosquitoes. However, this is less studied. A study with *Aedes aegypti* mosquitoes reported an increase in CHIKV titers in the midgut and head after Ago2 knockdown. Another study revealed an increased replication of YFV in Dcr-2 mutant mosquito line (*Aedes aegypti* mosquitoes). Dcr-2 mutant mosquito line for *Aedes aegypti* was recently introduced and considered to be more efficient cell line for RNAi-mediated knockdown compared to earlier studies which involved the use of Ago2 and Dcr-2 (Samuel et al. 2016). The results of the knockdown of these proteins/pathways in both cell lines and mosquitoes proved their antiviral activity and also suggest their involvement in the production of viral specific small RNAs.

Taking the reports together, there are proofs of interactions of arboviruses with the RNAi machinery both in cell lines and mosquitoes. Viral specific small RNAs are produced during RNAi-arbovirus interactions. However, the production is not applicable to all viruses and not for all mosquito-virus combinations.

1.6.1.3 Antiviral RNA interference and mosquito-specific viruses

RNAi does not only interfere with arboviruses but also MSVs. Small RNAs for different MSVs have been reported in mosquitoes and derived cell lines for different virus families (Table 1.3). Most data concerns persistent infections in mosquitoes or cells and the viruses are grouped into the following families: *Flaviviridae* (Cell Fusing Agent virus, CFAV; Calbertado virus, CALBOV; Palm Creek virus, PCV), *Birnaviridae* (Culex Y virus, CYV), *Phenuiviridae* (Phasi Charoen-like virus, PCLV)*, Reoviridae* (Aedes pseudoscutellaris reovirus)*, Nodaviridae* (Flock House virus, FHV)*, Rhabdoviridae* (Merida virus, MERDV), and *Parvoviridae* (Aedes albopictus densovirus 2 (Dipteran brevidensovirus 2); Aedes densovirus (Dipteran brevidensovirus 1)) (Agboli et al. 2019).

The first report of small RNA profiling in MSVs was generated by CFAV infected Aag2 and C6/36 cell lines by Scott and colleagues (Scott et al. 2010). They found that CFAV-specific small RNAs were detected in the Aag2 cell culture samples, but only a few CFAV-matching reads in *Aedes aegypti* mosquitoes. Also, CFAV-specific small RNAs were discovered in C6/36 cells. In summary, both vsiRNAs in Aag2 cells and vpiRNAs in C6/36 cells were observed in CFAV infections of which the latter showed a component of ping-pong signature for adenine (A10), which is also a characteristic of piRNAs bound by Ago3 (Scott et al. 2010).

Compared to other MSVs, CYV was thoroughly studied for small RNA molecules. Franzke and colleagues detected CYV-specific small RNAs in Aag2, U4.4, and C7-10 cells suggesting an actively replicating virus infection in these cells (Franzke et al. 2018). Further, the results indicated that the antiviral RNAi-based response to persistent CYV infection in Aag2 and U4.4 cells was based on the siRNA pathway, although piRNA-like

molecules without ping pong signatures were produced but at a low level (Franzke et al. 2018). In addition to screening infected Aag2 and U4.4 cells, Ct cells were also investigated to produce CYV-derived siRNAs (van Cleef et al. 2014; Göertz, Miesen, et al. 2019).

Infections of *Aedes aegypti* cells with the negative-strand Aedes anphevirus (AeAV) produced low amount of vsiRNAs and high concentration of vpiRNA with a clear ping-pong pattern for A10 sense with U1 antisense (Parry and Asgari 2018). In a different study, infection of *Culex*-derived cell lines with CALBOV and MERDV showed different patterns of viral small RNAs. The MERDV-derived piRNAs showed a positive strand-bias and ping-pong signature (Rückert et al. 2019).

Small RNA profiles of MSVs in *Culex tarsalis*-derived Ct cells revealed the production of vsiRNAs and vpiRNAs (Göertz, Miesen, et al. 2019). In this experiment, large numbers of 21 nt vsiRNAs aligned to the (+, genomic) and (-, antigenomic) strands of CALBOV, FHV, and novel Culex Narnavirus (CxNV1) while the PCLV has the alignment to the (-, genome; +, antigenomic) strands. Unlike FHV where the viral siRNAs were found to map predominantly to specific hot spots on the viral genome, PCLV, CALBOV, and CxNV1 had their siRNAs distributed all along the viral genome. Furthermore, no evidence of viral piRNAs was found for CALBOV, FHV, nor CxNV1 in the Ct cells except for PCLV L, M, and S segments. The PCLV L, M, and S segments displayed 25-30 nt putative viral piRNAs with A10 (+, genome) and U1 (-, antigenome) biases in combination with a 10 nt overlap, suggestive of ping-pong amplification of viral piRNA production (Göertz, Miesen, et al. 2019). This shows that formation of viral piRNA is extremely virus-specific for MSVs. Therefore similar to arboviruses, it seems that the production of small RNA depends on virus-mosquito pairing.

Taken together, studies on small RNAs (mainly siRNA and piRNA) induced by MSV infection in mosquitoes, so far highlighted the interaction between the mosquito RNAi pathways and MSVs, although whether the interaction is antiviral has not yet been thoroughly investigated.

Table 1. 3: Small RNA profiles of mosquito-specific viruses.

Small RNA profiles detected through small RNA deep sequencing for in vivo and in vitro experiments. Mosquito-specific viruses, their families, and size of the genomes indicated. Taken from (Agboli et al. 2019)

MSV	Family	Genome	Small RNAs	in vivo/in vitro	Species	Reference
Cell fusing agent virus (CFAV)	*Flaviviridae*	+ssRNA	piRNAs and siRNAs	in vitro	Aag2 (*Aedes aegypti*) C6/36 (*Aedes albopictus*)	(Schnettler et al. 2016; Scott et al. 2010)
			Small RNA**	in vitro	Aag2	(Franzke et al. 2018)
Calbertado virus	*Flaviviridae*	+ssRNA	siRNAs	in vitro	Ct (*Culex tarsalis*)	(Göertz, Miesen, et al. 2019; Rückert et al. 2019)
Palm Creek virus (PCV)	*Flaviviridae*	+ssRNA	siRNAs and piRNA-like	in vivo	*Aedes aegypti*	(Lee et al. 2017)
Culex Y virus (CYV)	*Birnaviridae*	dsRNA	piRNA-like	in vitro	Aag2 C7/10 (*Ae. albopictus*) U4.4 (*Ae. albopictus*)	(Franzke et al. 2018)
			siRNAs	in vitro	Ct Aag2 U4.4	(van Cleef et al. 2014; Franzke et al. 2018; Göertz, Miesen, et al. 2019)
Phasi Charoen-like virus (PCLV)	*Phenuiviridae*	-ssRNA	Small RNAs**	in vitro	Aag2 (*Ae. aegypti*) C7/10	(Franzke et al. 2018)
			siRNAs and piRNAs	in vitro	Aag2 Ct	(Göertz, Miesen, et al. 2019; Schnettler et al. 2013)

			siRNAs and piRNAs	in vivo	*Ae. aegypti*	(Aguiar et al. 2015)
Aedes pseudoscutellaris reovirus	*Reoviridae*	dsRNA	Small RNAs**	in vitro	*Aedes*	(Franzke et al. 2018)
Flock House virus	*Nodaviridae*	+ssRNA	siRNA	in vitro	Ct	(Göertz, Miesen, et al. 2019)
Culex narnavirus 1	Narna-like***	+ssRNA***	siRNA	in vitro	Ct	(Göertz, Miesen, et al. 2019)
Aedes Anphevirus (AeAV)	n/a*	-ssRNA	piRNA and siRNA	in vitro	Aag2.*w*MelPop-CLA (*Wolbachia* strain *w*MelPop-CLA infected Aag2)	(Parry and Asgari 2018)
Aedes albopictus densovirus 2 (Dipteran brevidensovirus 2)	*Parvoviridae*	ssDNA	Small RNAs**	in vitro	Aag2	(Franzke et al. 2018)
Aedes densovirus (Dipteran brevidensovirus 1)	*Parvoviridae*	ssDNA	siRNA	in vitro	Aag2	(Göertz, Miesen, et al. 2019)
Merida virus (MERDV)	*Rhabdoviridae*	-ssRNA	siRNAs and piRNAs	in vitro	Hsu	(Rückert et al. 2019)

*Family as of yet unassigned. Order: Mononegavirales; **Small RNAs have been isolated and mapped against the virus. The type of small RNA has not been identified. ; ***Based on classification of (Göertz, Miesen, et al. 2019).

1.6.1.4 Suppressors of antiviral RNAi pathway

Recently, some arboviruses and MSVs were known to encode proteins that could interfere with the antiviral RNAi response. These proteins are called viral suppressors of RNAi (VSR). Although, not all MSVs encode potent VSRs, the situation is less clear for arboviruses (Aliyari et al. 2008; Nayak et al. 2010).The mechanisms differ even for related viruses of the same family and it often involves binding of key molecules (For example: siRNA, dsRNA, Ago2) of the antiviral RNAi pathway (O'Neal et al. 2014). VSR activity can be host-specific and the degree of interference can vary immensely between different VSRs (van Mierlo et al. 2014).

Numerous proteins of arboviruses have been reported acting as VSRs. For example: NS4b and NS3 of DENV (Kakumani et al. 2013, 2015), nsp2 and nsp3 of CHIKV (Mathur

et al. 2016). However, their activity is controversial and highly discussed. The capsid protein of flaviviruses (YFV, ZIKV, WNV, and DENV) acts by binding dsRNA and hence inhibiting the activity of Dcr-2 (Samuel et al. 2016). Schnettler and colleagues have shown that that the 3′-untranslated region-derived RNA molecule known as subgenomic flavivirus RNA (sfRNA) efficiently suppressed siRNA- and miRNA-induced RNAi pathways in both mammalian and insect cells. This novel role for sfRNA as a nucleic acid-based regulator of RNAi pathways is a strategy that may be conserved among flaviviruses (Schnettler et al. 2012). Conversely, other studies reported the lack of VSR activity with these arboviruses (Li and Ding 2005; Schnettler et al. 2012; Varjak, Donald, et al. 2017). This may be due to the variability and sensitivity of the VSR assays used in the experimental design. Also, the genotype and strain for both virus and mosquito species could be a factor. Indeed, VSR results for arboviruses are controversial.

Currently described VSRs, mostly from plant or insect infecting viruses, have been shown to employ a selection of targets. However, the best characterized VSRs are RNA binding proteins, which are able to shield viral dsRNA from Dicer processing and subsequent RISC assembly. Examples here include the B2 proteins of nodaviruses (including Flock House virus, Wuhan nodavirus and Nodamura virus; *Nodaviridae*) as well as the 1A protein of the Drosophila C virus (*Dicistroviridae*), although the latter does not bind to short dsRNA such as siRNAs (Berry et al. 2009; Chao et al. 2005; Nayak et al. 2010; Qi et al. 2011, 2012; van Rij et al. 2006; Sullivan and Ganem 2005). Interestingly, some B2 proteins of nodaviruses might also directly interact with Dicer to inhibit its function (Qi et al. 2012; Singh et al. 2009). Targets of other VSRs can include direct interaction with Ago2 (thus blocking efficient target cleavage) or even degradation of dsRNA to prevent the formation of a mature RISC complex (Hussain, Abraham, and Asgari 2010; van Mierlo et al. 2012, 2014; Nayak et al. 2010).

In comparison to other insects, relatively little data is available on VSRs in mosquitoes. Up until now, a few MSVs have only been investigated for the presence of VSR. Similar to the other viruses of the *Nodaviridae* family mentioned previously, the Mosinovirus encodes for a B2 protein that is capable of binding long dsRNA and thus inhibits processing of the dsRNA into siRNAs by Dicer (Schuster et al. 2014). The Culex Y virus (*Birnaviridae*) VP3 protein has been shown to exhibit similar properties. In addition, the VSR was able to also bind siRNAs, presumably preventing the efficient take up into the RISC complex (van Cleef et al. 2014; Fareh et al. 2018). Similar to most recent identified MSVs, there is no data describing a VSR for mesoniviruses, neither in mosquitoes nor other insects. The Table 1.4 below illustrates RNAi suppresssors encoded by insect-specific viruses and arboviruses (Agboli et al. 2019; Gammon and Mello 2015).

Table 1. 4: RNAi suppressors encoded by insect-specific viruses and arboviruses.

[a]Experimental data obtained using human Dicer, inhibition of Dicer-2 in insects is presumed.
[b]Presumed function based on similarity to WNV sfRNA and ability to inhibit RNAi in insect cell assays.
Adapted from (Agboli et al. 2019; Gammon and Mello 2015)

Virus	Family	RNAi sup-pressor	Mechansism of suppression	Reference
RNA virus				
Flock House virus	*Nodaviridae*	B2	Binding long dsRNA prevents cleavage by Dicer-2; Binding siRNA prevents incorporation into RISC; Dicer-2 binding	(Aliyari et al. 2008; Chao et al. 2005; Li et al. 2004; Li, Li, and Ding 2002; Singh et al. 2009)
Nodamura-virus	*Nodaviridae*	B2	Binding of long dsRNA prevents cleavage by Dicer-2; Binding siRNA prevents incorporation into RISC; inhibition of Dicer-2 activity[a]	(Aliyari et al. 2008; Li et al. 2004; Sullivan and Ganem 2005)
Wuhan Nodavirus	*Nodaviridae*	B2	Binding long dsRNA prevents cleavage by Dicer-2; Binding siRNA prevents incorporation into RISC; Dicer-2 binding	(Qi et al. 2011, 2012)
Mosinovi-rus	*Nodaviridae*	B2	Binding long dsRNA prevents cleavage by Dicer-2	(Schuster et al. 2014)
Drosophila C virus	*Dicistroviri-dae*	1A	Binding long dsRNA prevents cleavage by Dicer-2	(Nayak et al. 2010; van Rij et al. 2006)
Cricket pa-ralysis vi-rus	*Dicistroviri-dae*	1A	Inhibition of Ago2 slicer (endonucle-ase) activity	(Nayak et al. 2010; Wang et al. 2006)
Drosophila X virus	*Birnaviridae*	VP3	Binding long dsRNA prevents cleavage by Dicer-2; Binding siRNA prevents incorporation into RISC	(van Cleef et al. 2014; Valli et al. 2012)
Culex Y vi-rus	*Birnaviridae*	VP3	Binding long dsRNA prevents cleavage by Dicer-2; Binding siRNA prevents incorporation into RISC	(van Cleef et al. 2014)
Nora virus	Unassigned	VP1	Inhibition of Ago2 slicer (endonucle-ase) activity	(van Mierlo et al. 2012, 2014)

Dimm Nora-like virus	Unassigned	VP1	Inhibition of Ago2 slicer (endonuclease) activity	(van Mierlo et al. 2014)
Dengue virus	*Flaviviridae*	NS4B	Inhibition of Dicer-2 activity [a]	(Kakumani et al. 2013)
West Nile virus	*Flaviviridae*	sfRNA	Inhibition of Dicer-2 activity [a]	(Schnettler et al. 2012)
Dengue virus	*Flaviviridae*	sfRNA	Inhibition of Dicer-2 activity [b]	(Schnettler et al. 2012)
DNA virus				
Heliothis virescens ascovirus-3e	*Ascoviridae*	Orf 27 (RNase III)	Degradation of siRNA	(Hussain et al. 2010)
Invertebrate iridescent virus type 6	*Iridoviridae*	340R	Binding long dsRNA prevents cleavage by Dicer-2; Binding siRNA prevents incorporation into RISC	(Bronkhorst et al. 2014)

1.7 Study objectives

1.7.1 Aim of the study

Mosquito-specific viruses (MSVs) are known to inhibit or increase the replication of arboviruses. Therefore, MSVs have the potential to be developed into novel biological vector control agents, since vaccines and antivirals are not present for most arboviruses. MeSV is a newly identified MSV and understanding its interaction with the mosquito host and other arboviruses could help to identify its potential to be used as a novel vector control tool in the future. Furthermore, antiviral RNAi response is known to induce virus resistance in mosquitoes. The study hypothesized that MeSV can suppress the replicative ability of arboviruses and also interfere with the antiviral RNAi response in the mosquito. Therefore, the overall aim of the study is to investigate the newly identified MeSV in more detail regarding its interaction with the mosquito host as well as with coinfected arboviruses.

1.7.2 Specific objectives

To answer the above mentioned aim, the following specific objectives are investigated:

a. Characteristics of MeSV and USUV infection.
b. Effect of MeSV infection on different arbovirus kinetics.
c. Interaction of MeSV with the mosquito antiviral RNAi response.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

Table 2. 1: Used equipment

Procedure	Technical equipment	Manufacturer/Supplier/Service provider
Luminescence	GloMax Navigator	Promega Corp. (Madison, WI, USA)
DNA/RNA extraction	NanoPhotometer® P 300	Implen (Munich, Germany)
	NanoDrop™ 2000 spectrophotometer	ThermoScientific (Wilmington, DE, USA)/PeQLab Biotechnologie GmbH (Erlangen, Germany)
Agarose gel electrophoresis	ChemiDoc Touch Imaging System	Bio-Rad Laboratories GmbH (Munich, Germany)
	Agarose gel chamber	Bio-Rad Laboratories GmbH (Munich, Germany)
	PowerPac Universal	Bio-Rad Laboratories GmbH (Munich, Germany)
	UV-transducers	Bio-Rad Laboratories GmbH (Munich, Germany)
	Agarose gel Tank	PeQLab Biotechnologie GmbH (Erlangen, Germany)
PCR and qRT-PCR	Flex Cycler	Analytic Jena AG (Eisfeld, Germany)
	LightCycler 2.0 Cool rack	Roche Diagnostics International AG (Risch-Rotkreuz, Switzerland)
	LightCycler 480	Roche Diagnostics International AG (Risch-Rotkreuz, Switzerland)
Immunostaining	The EVOS FL Auto Imaging System (Immunfluorescence microscope)	Life Technologies (Carlsbad, CA, USA)
Mosquito rearing	Air conditioner (Temperature&Humidity)	Lennox International (Richardson, Richardson, TX, USA)/ Lennox (Ratingen, Germany)
	Hemotek membrane feeding systems	Hemotek Ltd (Blackburn, UK)
	Collapsible Insect Rearing cage (BugDorm)	MegaView Science Co. Ltd. (Taipei, Taiwan)
	Drummond Nanoject II	Drummond Scientific Company (Broomall, PA, USA)

Mosquito infection	Cold light MLC-150C	Motic Deutschland GmbH (Wetzlar, Germany)
	Stereomicroscope SMZ 168	Motic Deutschland GmbH (Wetzlar, Germany)
Auxiliary equipment	Biosafety Cabinet II	Heraeus Company Inc (Hanau, Germany) Kendro Laboratory Products (Hamburg, Germany)
	Biosafety Cabinet II	ThermoScientific (Waltham, MA, USA)/ Schnakenberg Medizin and Labortechnik GmbH (Bremen, Germany)
	Clean bench HERAsafe	Heraeus Instruments (Hanau, Germany)
	Fume cupboard	Wesemann GmbH (Syke, Germany)
	New Brunswick Ultra-Low Temperature freezer C660 HEF™ (-80 °C)	Eppendorf AG (Hamburg, Germany)
	Fridges (-20 °C; + 4 °C); Premium and Comfort	Liebherr (Bulle, Switzerland)
	CO_2 Incubator (37 °C)	Heraeus Instruments (Hanau, Germany)
	Incubator (28 °C)	ThermoScientific (Waltham, MA, USA)
	CO_2 Incubator (37 °C)	New Brunswick Scientific (Edison, NJ, USA)
	Mini see-saw rocker SSM4	Bibby Scientific Limited (Staffordshire, UK)
	Heat block MBT 250	ETG GmbH (Ilmenau, Germany)
	Vortex Genie 1 and 2	Scientific Industries Inc. (Bohemia, NY, USA)
	Vortex mixer	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
	Centrifuge (Refrigerated)	Hitachi Koki Co Ltd (Japan)/Schnakenberg Medizin and Labortechnik GmbH (Bremen, Germany)
	Magnetic stirrer Stir bars	Hanna Instruments Deutschland GmbH (Vöhringen, Germany) neoLab Migge Laborbedarf-Vertriebs GmbH (Heidelberg, Germany)
	Microwave	Severin Elektrogeräte GmbH (Sundern, Germany)
	Scout® Pro Electronic balance	Ohaus Cooperation (Parsippany, NJ, USA)
	Microplate Centrifuge	Benchmark Scientific (Edison, NJ, USA)

Mechanical Aspirator	Maglite Mag Instrument (Ontario, Canada)
Plastic trays	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Sleeper	Inject+matic (Geneva, Switzerland)
Forceps neoLab-Dumont	neoLab Migge Laborbedarf-Vertriebs GmbH (Heidelberg, Germany)
Plastic trays	Bürkle (Bad Bellingen, Germany)
Pump	Vacuubrand GmbH & CO. KG (Wertheim, Germany)
Pistilles	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Water bath	Memmert GmbH & Co. KG (Schwabach, Germany)
Centrifuge (Refrigerated)	ThermoScientific (Waltham, MA, USA)/ Schnakenberg Medizin and Labortechnik GmbH (Bremen, Germany)
Centrifuge (Sigma 1-15)	Sigma Laborzentrifugen GmbH (Osterode am Harz, Germany)
Cool rack	BioCision (San Rafael, CA, USA)
Light microscope AE2000	Motic Deutschland GmbH (Wetzlar, Germany)
Multichannel pipette (Eppendorf Research® Plus; 50-300 µl)	Eppendorf AG (Hamburg, Germany)
Multichannel pipette (Eppendorf xplorer; 0.5-10 µl)	Eppendorf AG (Hamburg, Germany)
pH meter (SCHOTT instruments)	SI Analytics GmbH (Mainz, Germany)
Pipet aid pipetus®	Hirschmann Laborgeräte GmbH & Co. KG (Eberstadt, Germany)
Pipettes (Eppendorf Research® Plus)	Eppendorf AG (Hamburg, Germany)
Pipettes (Gilson PIPENTMAN®)	Gilson, Inc. (Middleton, WI, USA)
Thoma cell counting chamber	Paul Marienfeld GmbH & Co. KG (Lauda-Königshofen, Germany)
VWR MiniStar Silverline Microcentrifuge	Profcontrol (Shonwalde-glien, Germany)
Photometer	Berthold Technologies GmbH & Co.KG (Baden Württemberg, Germany)
Thermal Mixer	ThermoScientific (Waltham, MA, USA

2.1.2 Consumables

Table 2. 2: Used consumables

Material	Manufacturer/Supplier
Human blood (blood group 0)	Bloodbank University Hospital Hamburg Eppendorf (Hamburg, Germany)
Conical tubes (50 ml, 15 ml)	Sarstedt AG & Co. (Nümbrecht, Germany)
Cotton balls/pads	various vendors
Cover slip	R. Langenbrinck Labor-und Medizintechnik (Emmendingen, Germany)
Fish food (Tropical Tablets)	ASTRA Aquaristic GmbH (Hameln, Germany)
LightCycler® Capillaries (20 µl)	Roche Diagnostics International AG (Risch-Rotkreuz, Switzerland)
Microtubes (1.5 ml; safe-seal and standard)	Sarstedt AG & Co. (Nümbrecht, Germany)
Omnifix-F Tuberculin syringes (single use; 1 ml)	B. Braun Melsungen AG (Melsungen, Germany)
Pasteur pipette (disposable)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Petri dishes (92x16 mm)	Sarstedt AG & Co. (Nümbrecht, Germany)
Pipette (serological; 5/10/25 ml)	Sarstedt AG & Co. (Nümbrecht, Germany)
Pipette tips (with filter and standard; 1000/200/10 µl)	Sarstedt AG & Co. (Nümbrecht, Germany)
Plastic vials (175 ml)	Greiner Bio-One GmbH (Frickenhausen, Germany)
Plugs for plastic vials (Ø 52 mm)	K-TK e. K. (Retzstadt, Germany)
Multiply-ul Strip (PCR tubes)	Sarstedt AG & Co. (Nümbrecht, Germany)
qPCR seal	4titude (Surrey, UK)
Rotilabo® syringe filters (0.2 µm)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Specimen slide (Menzel-Gläser Superfrost® Plus)	Gerhard Menzel GmbH (Braunschweig, Germany)
StericapPlus Bottle-Top Vaccum Filtration Device	Merck Millipore (Billerica, MA, USA)
STRIP Tubes (0.1 ml)	LTF-Labortechnik GmbH & Co. KG (Wasserburg, Germany)
TC Flask T25/T75, standard, vent. cap	Sarstedt AG & Co. (Nümbrecht, Germany)
TC Plate 96 well, standard, round/flat bottom	Sarstedt AG & Co. (Nümbrecht, Germany)

Urine cups	neoLab Migge Laborbedarf-Vertriebs GmbH (Heidelberg, Germany)
Cups with Cotton plugs	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Weighing tray	neoLab Migge Laborbedarf-Vertriebs GmbH (Heidelberg, Germany)
Wooden spatula	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Zip-lock bags	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Filter paper	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Disposable bags	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Multiply-ul strip (PCR tubes)	Sarstedt AG & Co. (Nümbrecht, Germany)

2.1.3 Chemicals

Table 2. 3: Used chemicals

Chemicals	Manufacturer/Supplier
DharmaFECT 2	GE Healthcare Dharmacon Inc (Colorado, USA)
OptiMEM	Life Technologies Corp. (Grand Island, NY, USA)
2-Propanol	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
4-Aminobenzoic acid	Sigma-Aldrich (Steinheim am Albuch, Germany)
Agarose Standard (Roti®garose)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
6X DNA Loading Dye	ThermoScientific (Waltham, MA, USA)
D(-)-Fructose	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Disodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
GMEM	Life Technologies Corp. (Grand Island, NY, USA)
DMEM	PAN-Biotech GmbH (Aidenbach, Germany)
Leibovitz's L15 Medium (L-15)	PAN-Biotech GmbH (Aidenbach, Germany)
Ethanol (70 %)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)

Ethanol (≥ 99.5 %)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Ethidium bromide solution (0.5 %)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Ethylendiamin tetraacetic acid (EDTA)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
FBS/FCS (fetal bovine/calf serum) normal and Gold	PAN-Biotech GmbH (Aidenbach, Germany) PAA Laboratories GmbH (Pasching, Austria)
Formaldehyde (37 %)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Glacial acetic acid	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Hydrochloric acid (HCl)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
L-glutamine (200 mM)	PAN-Biotech GmbH (Aidenbach, Germany)
Non-essential amino acids (100X; MEM NEAA)	PAN-Biotech GmbH (Aidenbach, Germany)
Passive Lysis Buffer (PLB)	Promega Corp. (Madison, WI, USA)
Penicillin/Streptomycin	Gibco® Thermo Fisher (Carlsbad, CA, USA)
Potassium chloride (KCl)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Potassium dihydrogen phosphate (KH_2PO_4)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Schneider's Drosophila Medium	PAN-Biotech GmbH (Aidenbach, Germany)
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Sodium pyruvate (100 mM)	PAN-Biotech GmbH (Aidenbach, Germany)
Tetracyclin	Sigma-Aldrich (Steinheim am Albuch, Germany)
Tris(hydroxylmethyl)aminomethane (TRIS)	Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
Triton® X-100	MP Biomedicals, LLC. (Santa Ana, CA, USA)
Trypsin/EDTA solution (0.05/0.02 % in PBS)	PAN-Biotech GmbH (Aidenbach, Germany)
Luciferase assay substrate	Promega Corp. (Madison, WI, USA)
Stop and Glo substrate	Promega Corp. (Madison, WI, USA)
TRIzol reagent	Carlsbad, CA, USA

TRIzol LS reagent	Carlsbad, CA, USA

2.1.4 Recipes: buffers, media and solutions

Table 2. 4: Used buffers (A), media (B) and solutions (C)

A

Buffer	Recipe
Passive lysis buffer (1x) (from the kit)	5X Passive lysis buffer (1 volume) Distilled water (4 volumes)
PBT buffer (1X)	1X PBS 0.5 % Triton-X100
Phosphate buffered saline (PBS) (10X)	for 1 litre (in ddH_2O): 80 g NaCl 2 g KCl 26.8 g $Na_2HPO_4 \cdot 7H_2O$ 2.4 g KH_2PO_4 → pH 7.4
TRIS-acetate-EDTA (TAE) buffer (50X)	for 1litre (in ddH_2O): 242 g TRIS 57.1 ml glacial acetic acid 100 ml 50 mM EDTA

B

Medium	Recipe
Supplemented Leibovitz's L15 Medium	Leibovitz's L15 Medium 10 % FCS 1X Penicillin/Streptomycin 10 % Tryptose broth
Supplemented Dulbecco's Modified Eagle Medium (DMEM)	DMEM 10 % FCS (normal cell culture) 5 % FCS (virus propagation and infection assays) 1X Penicillin/Streptomycin 10 % Tryptose broth
Supplemented Glasgow Minimum Essential Medium (GMEM)	GMEM 10 % FCS (normal cell culture 5 % FCS (virus propagation and infection assays) 1X Penicillin/Streptomycin 10 % Tryptose broth
Supplemented Schneider's Drosophila Medium	Schneider's Drosophila Medium 10 % FCS 1X Penicillin/Streptomycin 10 % Tryptose broth

C

Solution	Recipe
Luciferase assay reagent	Luciferase assay buffer Luciferase assay substrate
Stop & Glo reagent	Stop & Glo buffer Stop & Go substrate
10 % SDS	10 % w/v SDS = 100 g + 1000 ml water
Blocking solution	1X PBT 10 % FCS
PBT	0.5 % v/v TrotonX-100 1X PBS
Blood meal (blood feed infection assay)	Human blood (blood group 0) 10 % FCS 50 % fructose solution (8 %) virus stock (1-1.6x10^7 PFU)
Blood meal (mosquito rearing)	Human blood (blood group 0) 50 % FCS 0.5 % fructose solution (8 %)
Fixation solution	1X PBS 4 % formaldehyde
Fructose solution (8 %)	for 1 litre (in H_2O): 80 g fructose 0.2 g 4-aminobenzoic acid
Tetracycline solution	8 % fructose solution 0.5 mg/ml tetracycline
1 % Agarose gel	0.4 g Agarose 40 ml 1% TAE

2.1.5 Kits and markers

Table 2. 5: Used kits and markers

Name	Manufacturer/Supplier
QuantiTect SYBR Green RT-PCR Kit (200)	QIAGEN (Hilden, Germany)
QuantiTect SYBR Green PCR Kit	QIAGEN (Hilden, Germany)
Dual Luciferase Reporter Assay System Kit	Promega Corp. (Madison, WI, USA)
Nano-Glo Luciferase Assay Kit	Promega Corp. (Madison, WI, USA)
Luciferase Assay System Kit	Promega Corp. (Madison, WI, USA)
MycoAlert Mycoplasma detection Kit	Lonza (Rockland, ME, USA)

MEGAscript RNAi Kit	Thermo Fischer Scientific (Vilnius, Lithuania)
MEGAscript T7 Kit	Ambion Inc. (Austin, TX, USA)
Dneasy Blood & Tissue Kit	QIAGEN (Hilden, Germany)
QIAamp Viral RNA Mini Kit	QIAGEN (Hilden, Germany)
QuantiTect Probe PCR Kit	QIAGEN (Hilden, Germany)
HotStarTaq Master Mix Kit	QIAGEN (Hilden, Germany)
Plasmid DNA purification Kit	MACHEREY-NAGEL GmbH & Co. KG (Düren, Germany)
CloneJET PCR Cloning Kit	ThermoScientific (Waltham, MA, USA)
GeneRuler 1 kb DNA Ladder	ThermoScientific (Waltham, MA, USA)
GeneRuler 100 bp DNA Ladder	ThermoScientific (Waltham, MA, USA)
Superscript III One-Step RT-PCR Kit	ThermoScientific (Waltham, MA, USA)
NucleoSpin Gel and PCR Clean-up Kit,	Macherey Nagel GmbH & Co. KG. (Duren, Germany)

2.1.6 Enzymes, antibodies, and stains

The enzyme listed in the following table was purchased individually. All other enzymes utilised were purchased as part of a kit (Table 2.5).

Table 2. 6: Used enzyme (A), antibody (B), and stain (C).

A

Name	Manufacturer/Supplier
GoTaq DNA Polymerase	Promega Corp. (Madison, WI, USA)
KOD DNA Polymerase	ThermoScientific (Waltham, MA, USA)
M-MLV Reverse Transcriptase	Promega Corp. (Madison, WI, USA)

B

Name	Source/Species	Manufacturer/Supplier
Hybridoma 3G1.1 dsRNA antibody	mouse	Roy Hall, Queensland University (Australia): https://eshop.uniquest.com.au/mozzy-mabs/
4G2 NS1 Flavivirus antibody	mouse	Roy Hall, Queensland University (Australia): https://eshop.uniquest.com.au/mozzy-mabs/

Alexa Fluor 488 anti-mouse IgG	Goat	Thermo Fisher Scientific (Waltham, MA, USA)

C

Name	Manufacturer/Supplier
diamidino-2-phenylindole (DAPI)	Thermo Fisher Scientific (Darmstadt, Germany)
Crystal violet	Merck KGaA (Darmstadt, Germany)

2.1.7 Oligonucleotides

Table 2. 7: Used oligonucleotides

ID	Sequence (5' → 3')	Target gene and product size [bp]	Utilisation	Origin / Reference
USUV_F8	CTGAGAAGGGAGGAAAAG	80bp	USUV detection PCR, Forward	Arbovirology, BNITM
USUV_R8	GCCACAATGAGTGTTATG	80bp	USUV detection PCR, Reverse	Arbovirology, BNITM
E-523	CTTGCTGGTAGTGGGAGTGG	108bp	USUV detection PCR, Forward	Self-Designed. USUV BNI-491 (KY113092.1)
E-524	AGAGCACTCTGTGGTTGACG	108bp	USUV detection PCR, Reverse	Self-Designed. USUV BNI-491 (KY113092.1)
E-384	TGGHGATKCRGAATTCATGCG	270bp	Pan MeSV PCR, Forward	Arbovirology, BNITM
E-385	ATCCCAACCRCCRTATTGTGC	270bp	Pan MeSV PCR, Reverse	Arbovirology, BNITM
E-433	ATTGCCTCCACCAAGAGAGC	459bp	MeSV PCR. Close to 3'UTR, Forward	Self-Designed for MeSV-8345

E-434	ATGGCGTCTAGAGTCTCGGT	459bp	MeSV PCR. Close to 3'UTR, Reverse	Self-Designed for MeSV-8345
E-435	AAGCTCAGGCAGACCAACTC	472bp	MeSV PCR. Close to 5'UTR, Forward	Self-Designed for MeSV-8345
E-436	GCCTGTGGCGGAAATGAATC	472bp	MeSV PCR. Close to 5'UTR, Reverse	Self-Designed for MeSV-8345
E-439	GCAGAAACTTACGGCTATGCAG	76bp	MeSV qRT-PCR, Forward	Self-Designed for MeSV-8345
E-440	GGAGAGCTGATGTTCAAGCG	76bp	MeSV qRT-PCR, Reverse	Self-Designed for MeSV-8345
E-492	gta ata cga ctc act ata ggg CCTCTAATCAACGAGAACCC	T7 for dsRNA, 3' end, 320bp	MeSV PCR, Forward	Self-Designed for MeSV-8345
E-493	gta ata cga ctc act ata ggg GGCCAGTAGGTCTTGAAAC	T7 for dsRNA, 3' end, 320bp	MeSV PCR, Reverse	Self-Designed for MeSV-8345
E-494	gta ata cga ctc act ata ggg GATCGTAAGAACCCCTTAGC	T7 for dsRNA, Middle, 320bp	MeSV PCR, Forward	Self-Designed for MeSV-8345
E-495	gta ata cga ctc act ata ggg GCCATTAGCATAACCGTAGG	T7 for dsRNA, Middle, 320bp	MeSV PCR, Reverse	Self-Designed for MeSV-8345
E-496	gta ata cga ctc act ata ggg CTCTGAAGGAGACACCTACATC	T7 for dsRNA, 5' end, 320bp	MeSV PCR, Forward	Self-Designed for MeSV-8345
E-497	gta ata cga ctc act ata ggg GGGTGTCTAATATCCCTTTCGG	T7 for dsRNA, 5' end, 320bp	MeSV PCR, Reverse	Self-Designed for MeSV-8345
E-552	gta ata cga ctc act ata ggg CGACAACTCTATCCAGCTCCG	T7 for dsRNA, Middle, 320bp	MeSV PCR, Forward	Self-Designed for MeSV-8345
E-553	gta ata cga ctc act ata ggg CCCTGCTTAATGCTTGCGG	T7 for dsRNA, Middle, 320bp	MeSV PCR, Reverse	Self-Designed for MeSV-8345

E-554	gta ata cga ctc act ata ggg ATAATGACCCCGGAGAAGCC	T7 for dsRNA, 5' end, 320bp	MeSV PCR, Forward	Self-Designed for MeSV-8345
E-555	gta ata cga ctc act ata ggg AGGTGAGACTATAGCCCGCTC	T7 for dsRNA, 5' end, 320bp	MeSV PCR, Reverse	Self-Designed for MeSV-8345
E-415	CTT GGG ACA GGA TCA TTC GGA	Protein Kinase A (PKA), 534bp	Housekeeper gene PKA detection in *Culex quinquefasciatus*, Forward	Molecular Entomology, BNITM
E-416	AAC ACC TAA TGC CCA CCA GT	Protein Kinase A (PKA), 534bp	Housekeeper gene PKA detection in *Culex quinquefasciatus*, Reverse	Molecular Entomology, BNITM
E-393	GACTGGTGGGCATTAGGTGTTC	Protein Kinase A (PKA)	PKA/CN-Forward	(Martins et al. 2017)
E-394	TCAGCAAAAAAAGGTGGATATCC	Protein Kinase A (PKA)	PKA/CN-Reverse	(Martins et al. 2017)
E-395	ROX-GTGTACGAGATGGCAGC-BHQ2	Protein Kinase A (PKA)	PKA Probe	(Martins et al. 2017)
OSM-328	GAGTAGCTAGTGGCTCCACTATTACC	350bp	Culex Y virus (CYV/ESV) detection PCR, Forward	Molecular Entomology, BNITM
OSM-329	CTGCCTCCTGAGGGATTCC	350bp	Culex Y virus (CYV/ESV) detection PCR, Reverse	Molecular Entomology, BNITM
E-233	CAGGTGGTAAACCATCCGAT	NS3, 500bp	Cell fusing agent virus (CFAV) detection PCR, Forward	Molecular Entomology, BNITM
E-234	AAGTTGGGAAACTAATGGATTGG	NS3, 500bp	Cell fusing agent virus (CFAV) detection PCR, Reverse	Molecular Entomology, BNITM
E-286	CAGTTAAAGCATTTAATCGTATGATAA	724bp	Phasi Charoen-like phasivirus	Molecular Entomology, BNITM

			(PCLV) detection PCR, Forward	
E-287	CACTAAGTGTTACAGCCCTTGGT	724bp	Phasi Charoen-like phasivirus (PCLV) detection PCR, Reverse	Molecular Entomology, BNITM
E-290	ATAGTGTGGGACGAGGAGGG	71bp	PCLV qPCR, Forward	Molecular Entomology, BNITM
E-291	AGGTGCCAACAGGAAACACT	71bp	PCLV qPCR, Reverse	Molecular Entomology, BNITM
E-411	CATCTGGACGACCTCGCTAC	586bp	Agua Salud alphavirus (ASALV) detection PCR, Forward	Molecular Entomology, BNITM
E-412	CTGGCCCGTTAATCGCAATG	586bp	Agua Salud alphavirus (ASALV) detection PCR, Reverse	Molecular Entomology, BNITM
E-356	CTTCTCCACCACAGCCAATG	*Aedes aegypti*-Piwi4	SYBR Green qPCR, *Aedes aegypti*-Piwi4-Forward	Molecular Entomology, BNITM (Dietrich, Shi, et al. 2017)
E-357	GTCCAATCTGCCTGTTCTCCA	*Aedes aegypti*-Piwi4	SYBR Green qPCR, *Aedes aegypti*-Piwi4-Reverse	Molecular Entomology, BNITM (Dietrich, Shi, et al. 2017)
E-358	CAGTTTTGGAAGACAGAGTTGGA	*Aedes aegypti*-Piwi5	SYBR Green qPCR, *Aedes aegypti*-Piwi5-Forward	Molecular Entomology, BNITM (Dietrich, Shi, et al. 2017)
E-359	CCTGCCGTCACTTTGTAATTTTC	*Aedes aegypti*-Piwi5	SYBR Green qPCR, *Aedes aegypti*-Piwi5-Reverse	Molecular Entomology, BNITM (Dietrich, Shi, et al. 2017)
E-360	TCCGACGTTTTCAAGTTTTGGA	*Aedes aegypti*-Piwi6	SYBR Green qPCR, *Aedes*	Molecular Entomology, BNITM

			aegypti-Piwi6-Forward	(Dietrich, Shi, et al. 2017)
E-361	CACTTTACACTGATCCTGCTCG	*Aedes aegypti*-Piwi6	SYBR Green qPCR, *Aedes aegypti*-Piwi6-Reverse	Molecular Entomol-ogy, BNITM (Dietrich, Shi, et al. 2017)
E-362	TGCTCCAGACGACGGTTTTG	*Aedes aegypti*-Ago3	SYBR Green qPCR, *Aedes aegypti*-Ago3-Forward	Molecular Entomol-ogy, BNITM (Dietrich, Shi, et al. 2017)
E-363	GGGTCAATATAACGGCTCCCAG	*Aedes aegypti*-Ago3	SYBR Green qPCR, *Aedes aegypti*-Ago3-Reverse	Molecular Entomol-ogy, BNITM (Dietrich, Shi, et al. 2017)
E-364	GGCTGCTCACCCAATGTATCAAGA	*Aedes aegypti*-Ago2	SYBR Green qPCR, *Aedes aegypti*-Ago2-Forward	Molecular Entomol-ogy, BNITM (McFarlane et al. 2014)
E-365	AACCGTTCGTTTTGGCGTTGAT	*Aedes aegypti*-Ago2	SYBR Green qPCR, *Aedes aegypti*-Ago2-Reverse	Molecular Entomol-ogy, BNITM (McFarlane et al. 2014)
E-366	GTACGATGCGTCGTAAGTAC	*Aedes aegypti*-Ago1	SYBR Green qPCR, *Aedes aegypti*-Ago1-Forward	Molecular Entomol-ogy, BNITM (McFarlane et al. 2014)
E-367	GTACTTGTCGAG-GAAGTATTTGG	*Aedes aegypti*-Ago1	SYBR Green qPCR, *Aedes aegypti*-Ago1-Reverse	Molecular Entomol-ogy, BNITM (McFarlane et al. 2014)
E-368	CCAGGCTATCCTGGAGTTG	*Aedes aegypti*-S7	SYBR Green qPCR, *Aedes aegypti*-S7-Forward	Molecular Entomol-ogy, BNITM (McFarlane et al. 2014)
E-369	GACGTGCTTGCCGGAGAAC	*Aedes aegypti*-S7	SYBR Green qPCR, *Aedes aegypti*-S7-Reverse	Molecular Entomol-ogy, BNITM (McFarlane et al. 2014)

E-342	GTA ATA CGA CTC ACT ATA GGG CCG-TATATCCGAAAAAGTGCTG	T7-dsRNA for *Aedes aegypti*-Piwi4.	dsRNA production against *Aedes aegypti*-Piwi4-PCR-Forward	Molecular Entomology, BNITM (Dietrich, Shi, et al. 2017)
E-343	GTA ATA CGA CTC ACT ATA GGG AGAGTCCAC-TCGATGTGTTTCA	T7-dsRNA for *Aedes aegypti*-Piwi4.	dsRNA production against *Aedes aegypti*-Piwi4-PCR-Reverse	Molecular Entomology, BNITM (Dietrich, Shi, et al. 2017)
E-344	GTAATACGACTCACTATAGGG CGTAATGTTGCTGTTTCGAATG	T7-dsRNA for *Aedes aegypti*-Piwi5.	dsRNA production against *Aedes aegypti*-Piwi5-PCR-Forward	Molecular Entomology, BNITM (Dietrich, Shi, et al. 2017)
E-345	GTAATACGACTCACTATAGGG GATTTGGAACAATTAGAGGTG	T7-dsRNA for *Aedes aegypti*-Piwi5.	dsRNA production against *Aedes aegypti*-Piwi5-PCR-Reverse	Molecular Entomology, BNITM (Dietrich, Shi, et al. 2017)
E-346	GTA ATA CGA CTC ACT ATA GGG ACCAGAAATAGTG-CAAACCCG	T7-dsRNA for *Aedes aegypti*-Piwi6.	dsRNA production against *Aedes aegypti*-Piwi6-PCR-Forward	Molecular Entomology, BNITM (Dietrich, Shi, et al. 2017)
E-347	GTA ATA CGA CTC ACT ATA GGG CATGTCGGTTGA-TAAGGTTGAA	T7-dsRNA for *Aedes aegypti*-Piwi6.	dsRNA production against *Aedes aegypti*-Piwi6-PCR-Reverse	Molecular Entomology, BNITM (Dietrich, Shi, et al. 2017)
E-352	GTA ATA CGA CTC ACT ATA GGG ACAGGTTTCAC-TGTTCAACCT	T7-dsRNA for *Aedes aegypti*-Ago1.	dsRNA production against *Aedes aegypti*-Ago1-PCR-Forward	Molecular Entomology, BNITM (McFarlane et al. 2014)
E-353	GTA ATA CGA CTC ACT ATA GGG GGTTTGAC-CGTTTTCTAGCTGC	T7-dsRNA for *Aedes aegypti*-Ago1.	dsRNA production against *Aedes aegypti*-Ago1-PCR-Reverse	Molecular Entomology, BNITM (McFarlane et al. 2014)
E-354	GTAATACGACTCACTATAGGG GCCCTCAACAAGAAACACC	T7-dsRNA for *Aedes*	dsRNA production against *Aedes aegypti*-	Molecular Entomology, BNITM

		aegypti-Ago2.	Ago2-PCR-Forward	(McFarlane et al. 2014)
E-355	GTAATACGACTCACTATAGGG GCGTTGATCTTGAGCCA	T7-dsRNA for *Aedes aegypti*-Ago2.	dsRNA production against *Aedes aegypti*-Ago2-PCR-Reverse	Molecular Entomology, BNITM (McFarlane et al. 2014)
E-348	GTA ATA CGA CTC ACT ATA GGG GAGTTAC-CTCATCAATGACGCG	T7-dsRNA for *Aedes aegypti*-Ago3.	dsRNA production against *Aedes aegypti*-Ago3-PCR-Forward	Molecular Entomology, BNITM (Dietrich, Shi, et al. 2017)
E-349	GTA ATA CGA CTC ACT ATA GGG GGCACTTAAATCCTG-TAGGTACCTT	T7-dsRNA for *Aedes aegypti*-Ago3.	dsRNA production against *Aedes aegypti*-Ago3-PCR-Reverse	Molecular Entomology, BNITM (Dietrich, Shi, et al. 2017)
E-373	TAATACGACTCAC-TATAGGGGTCGCCAGCGGCAC-CGCGCCTTTC	T7-ßGal-LacZ.	dsRNA production-LacZ control-PCR-Forward	Molecular Entomology, BNITM (Varjak et al. 2020)
E-374	TAATACGACTCAC-TATAGGGCCGG-TAGCCAGCGCGGATCATCGG	T7-ßGal-LacZ.	dsRNA production-LacZ control-PCR-Reverse	Molecular Entomology, BNITM (Varjak et al. 2020)
E-525	ACAATTTGGCTGTTGCGGAC	328bp	Culex Narnavirus detection PCR, Forward	Self-Designed for CxNV(GenBank: MK628543.1)
E-526	TCTCGAGTAAGGAGGGGTGG	328bp	Culex Narnavirus detection PCR, Reverse	Self-Designed for CxNV(GenBank: MK628543.1
E-527	CGTGCGCTATTTCCGAAAGG	452bp	Calbertadovirus detection PCR, Forward	Self-Designed for CALBOV (GenBank: KX669689.1)

E-528	GCTTGCGGTTCGAAACAAGT	452bp	Calbertado-virus detection PCR, Reverse	Self-Designed for CALBOV (GenBank: KX669689.1)
E-529	GTGCCGGAAAGTGAAGGACAA	318bp	Meridavirus detection PCR, Forward	(Weger-Lucarelli et al. 2018)
E-530	GTGCGGGGATGAATCAATCTC	318bp	Meridavirus detection PCR, Reverse	(Weger-Lucarelli et al. 2018)
E-564	AACACCTAGCCATTGCCCTC	138bp	Meridavirus detection qPCR, Forward	Self-Designed for Merida virus (GenBank: MH310083.1)
E-565	ACAGGGGCACATAGAACAGC	138bp	Meridavirus detection qPCR, Reverse	Self-Designed for Merida virus (GenBank: MH310083.1
E-566	CCTGGAGAAGGACGACACAC	85bp	Calbertado-virus detection qPCR, Forward	Self-Designed for CALBOV (GenBank: KX669689.1)
E-567	CATCTCAACTCCCACGCTGT	85bp	Calbertado-virus detection qPCR, Reverse	Self-Designed for CALBOV (GenBank: KX669689.1)
E-568	GTCTTTGTCCTCTCCCCGTG	137bp	Culex Narna-virus detection qPCR, Forward	Self-Designed for CxNV(GenBank: MK628543.1
E-569	AGGAAGGCATAAGGGCGAAC	137bp	Culex Narna-virus detection qPCR, Reverse	Self-Designed for CxNV(GenBank: MK628543.1

E543	ACCCTACTGGTTAACGTGCG	121bp	Flock House virus detection qPCR/PCR, Forward	Self-Designed for FHV1 (GenBank: NC004146.1)
E544	CCCAATGAGTGCATCGACCT	121bp	Flock House virus detection qPCR/PCR, Reverse	Self-Designed for FHV1 (GenBank: NC004146.1)
pJET1.2	CGACTCACTATAGGGAGAGCGGC	310-332bp position	Colony DNA insert detection PCR, Forward	CloneJET PCR Cloning Kit (Michelsen 1995)
pJET1.2	AAGAACATCGATTTTCCATGGCAG	428-405bp position	Colony DNA insert detection PCR, Reverse	CloneJET PCR Cloning Kit (Michelsen 1995)

2.1.8 Infectious agents, cells and organisms

Table 2. 8: Used infectious agent (A), cell line (B) and mosquito (C)

A

Infectious agent	**Strain**	**BSL level**	**Reference/ Accession#**	**Origin**
Usutu virus (USUV)	491 (Isolated from Blackbird *Turdus merula*)	2	(Cadar et al. 2017) KY426758	BNITM (Hamburg): Prof Dr Dr Schmidt Chanasit, Arbovirology group
Usutu virus (USUV)	490 (Isolated from Blackbird *Turdus merula*)	2	(Cadar et al. 2017) KY426757	BNITM (Hamburg): Prof Dr Dr Schmidt Chanasit, Arbovirology group
Usutu virus (USUV)	1477 (Isolated from *Culex pipiens pipiens* mosquito)	2	(Jöst et al. 2011) JF330418	BNITM (Hamburg): Prof Dr Dr Schmidt Chanasit, Arbovirology group

Mesonivirus (MeSV)	8345 (Isolated from *Coquilletidia richiardii* mosquito pool)	2	(Börstler 2016)	BNITM (Hamburg): Prof Dr Dr Schmidt Chanasit, Arbovirology group
Semliki Forest virus with firefly reporter SFV4(3H)-FFLuc (SFV-FLuc used for the thesis)	SFV4	2	(Schnettler et al. 2013)	BNITM (Hamburg): Prof Dr Esther Schnettler, Molecular Entomology group
Bunyamvera virus with nano luciferase reporter (BUNV-NLuc)	BUNV	2	(Dietrich, Shi, et al. 2017)	BNITM (Hamburg): Prof Dr Esther Schnettler, Molecular Entomology group

B

Name	**Source/Species**	**Type of cell line**	**Origin/ Supplier/ Reference**
Vero ATTC	African green monkey (*Chlorocebus sp.*); kidney	Mammalian (vertebrate)	BNITM (Hamburg), ATCC (LGC, Germany)
BHK-12	Baby hamster kidney cells	Mammalian (vertebrate)	BNITM (Hamburg) (Stoker and Macpherson 1964)
A549-Npro	Human lung epithlial cells, stably expressing the Bovine Viral Diarrhoea virus (BVDV) N protein	Mammalian (vertebrate)	Richard Randall, St. Andrews, UK. (Carlos et al. 2009; Hilton et al. 2006)
Hsu	*Culex quinquefasciatus*	Insect (invertebrate)	Nijmegen (Netherlands) (Hsu, Mao, and Cross 1970a)
Ct	*Culex tarsalis*	Insect (invertebrate)	Nijmegen (Netherlands) (Chao and Ball 1976)
Aag2	*Aedes aegypti*	Insect (invertebrate)	CVR, Glasgow, UK

			(Grace 1966; Lan and Fallon 1990; Peleg 1968)
C6/36	*Aedes albopictus*	Insect (invertebrate)	CVR, Glasgow, UK (Singh 1967)

C

Name	Strain	Origin
Culex quinquefasciatus	Malaysia	Bayer (Leverkusen, Germany)
Culex pipiens molestus	S	Field collection, Heidelberg, Germany

2.1.9 Plasmids

Table 2. 9: Used plasmids

Name of plasmid	Usage/Construct	Source/Reference
pIZ-Fluc	Firefly luciferase expression under baculovirus OpIE2 promoter: RNAi suppressor assays	Prof Dr Esther Schnettler, (Ongus et al. 2006; Varjak, Maringer, et al. 2017)
pAcIE1-Rluc	Renilla luciferase expression under baculovirus AcIE1 promoter: RNAi suppressor assays	Prof Dr Esther Schnettler, (Ongus et al. 2006; Varjak, Maringer, et al. 2017)

2.1.10 Databases and programmes

Table 2. 10: Used databases and programmes

Database/Programme	Utilisation
MySample 2.0	Cell and virus inventory
NCBI http://www.ncbi.nlm.nih.gov/	literature database and (Primer-) BLAST
GraphPad Prism 7	statistical analysis and image design
LightCycler Software Release 1.5.0 SP3	qRT-PCR analysis

LightCycler Software 4.05	qRT-PCR analysis
Primer3Plus http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi	Primer design
Geneious Prime (Biomatters, Inc.) https://www.geneious.com/prime/	Bioinformatics (ORF determination)
MAFFT 7 https://mafft.cbrc.jp/alignment/software/	Bioinformatics (Realignment of protein sequences) (Katoh, Rozewicki, and Yamada 2019)
MEGA X https://www.megasoftware.net	Bioinformatics (Realignment of protein sequences and phylogenetic tree construction) (Kumar et al. 2018)
SeaView https://bio.tools/seaview	Bioinformatics (Phylogenetic tree construction) (Gouy, Guindon, and Gascuel 2010)

2.2 Methods

2.2.1 Cells

Both mammalian and invertebrate cell lines were used in in the experimental designs. Vero ATTC (African green monkeys kidney cells), BHK-12 (Baby hamster kidney cells), and A549-Npro (human lung epithelial cells) were the mammalian cells deployed whilst Hsu (*Culex quinquefasciatus*), Ct (*Culex tarsalis*), Aag2 (*Aedes aegypti*), and C6/36 (*Aedes albopictus*) cells were the invertebrate cells. Additional information about these cell lines including their origin are indicated in Table 2.8.B. Besides, information about cell culture medium are indicated in Table 2.4.B.

Maintaining the cells and keeping them viable is by way of splitting them regularly. Splitting invertebrate cells: fresh medium was pipetted into a new flask depending on the splitting ratio. The cells were detached from the flask bottom into the medium using cell scrappers and resuspended by pipetting gently up and down and transferring a part (depending on the splitting ratio) to the new flask. Afterwards, the cells were incubated at 28 °C (no CO_2 buffering needed).

To split mammalian cells: the cell medium was removed and gently washed with PBS. Trypsin was pipetted onto the cells and incubated at room temperature until the cells

detach. The cells were resuspended in complete medium by pipetting gently up and down, followed by transferring part of the cell suspension (depending on splitting ratio) to a new flask. The flask was gently swirled to distribute the cells and later incubated at 37 ºC (in 5 % CO_2 incubator).

2.2.2 Viruses

2.2.2.1 Usutu virus and Mesonivirus

Three different strains of USUV (490, 491, and 1477) were available at the time of the study. USUV 490 and 491 were isolated from Blackbird (*Turdus merula*) whilst USUV 1477 was isolated from *Culex pipiens pipiens* mosquito. These viruses were produced in different cell lines in order to choose a suitable strain which could produce a consistent cytopathic effect (CPE) for onward experiments. After vigorous investigation to get a high concentration, USUV 491 was chosen for the experiments in this thesis. USUV 491 was passaged on Vero cells and virus titer determined by Median Tissue Culture Infectious Dose ($TCID_{50}$) using Vero ATTC cell lines. Mesonivirus (MeSV) 8345 on the other hand was isolated at the BNITM from a *Coquilletidia richiardii* mosquito pool and passaged on C6/36 cell lines. MeSV titer was determined by $TCID_{50}$ using C6/36 cell lines. Additional information regarding these viruses are indicated in Table 2.8.A.

2.2.2.2 Semliki Forest and Bunyamwera reporter viruses

SFV-FLuc and BUNV-NLuc were derived reporter viruses expressing Firefly luciferase and Nano Luciferase respectively. These viruses were produced from relative virus stocks, passaged on BHK-12 cells and their titers determined by $TCID_{50}$ using the same cell lines. Firefly luciferase (FLuc) was inserted between duplicated nsP2-protease cleavage sites at the nsP3 and nsP4 protein junctions of the SFV genome to generate SFV-FLuc reporter virus as previously described (Schnettler et al. 2013; Ülper et al. 2008; Varjak, Maringer, et al. 2017), Fig. 2.1.

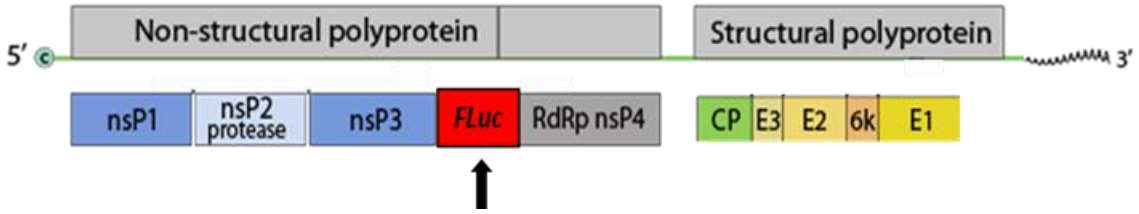

Fig. 2. 1: SFV-FLuc genome.

Outline of SFV-FLuc during which Firefly luciferase (FLuc) was inserted between duplicated nsP2-protease cleavage sites at the nsP3 and nsP4 protein junctions of the SFV4 genome (Schnettler et al. 2013).

For BUNV-NLuc, 62 residues of the NSm cytoplasmic domain (residues 395 to 455) was replaced by the Nano luciferase (NLuc) coding region between residues 394 and 456, resulting in a chimeric NSm-NLuc fusion protein (Fig. 2.2) (Dietrich, Shi, et al. 2017).

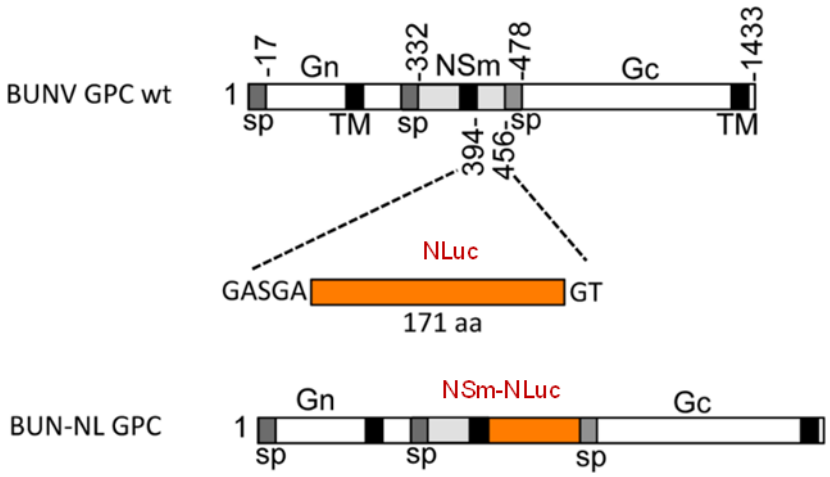

Fig. 2. 2: BUNV-NLuc genome.

Outline of BUNV-NLuc in which the coding region of the NSm cytoplasmic tail (residues 395 to 455) was replaced by that of Nano luciferase (NLuc); NSm-NLuc chimeric protein cleaved between Gn and Gc proteins. The fused NLuc is shown in orange, signal peptide (sp) in the grey box and transmembrane domain (TM) in the black box. The amino acid positions at the boundary of each protein are marked on top of Wt BUNV GPC ((Dietrich, Shi, et al. 2017).

2.2.2.3 Virus propagation and maintenance

USUV and MeSV were grown and titered on Vero ATTC and C6/36 cells respectively. BHK-21 cells were used to grow and titer SFV-FLuc and BUNV-NLuc virus stocks. As a general rule, cells were seeded 24-48 h before infection and should reach 75 % confluence before inoculation with a virus. Cell density of $2.1x10^6$ cells/flask were seeded in T75 cell culture flask with 20 ml complete medium. DMEM (Vero ATTC), GMEM (BHK-

21), and Leibovitz's L15 (C6/36) media with supplements were used (Table 2.4 B). Uninfected flask was also prepared as a negative control. The next day, the medium was changed and inoculated with 20-50 µl of initial virus stock. Afterwards, the cells were incubated under corresponding conditions (Mammalian cells: 37 ^{o}C in CO_2; Invertebrate cells: 28 ^{o}C). The cells were monitored over time and cell supernatants were collected when CPEs were observed. This collection time depends on the virus and the cell type. Generally, USUV on Vero ATTC cells and MeSV on C6/36 cells takes 7-8 days and 3-4 days respectively. Additionally, SFV and BUNV on BHK-21 takes 4-6 days. After collection, supernatants were centrifuged (10 min, 1000 rpm) to remove cell debris and stored in small aliquots at -80^{o}C. End point titration, $TCID_{50}$ was done to determine the titer of the viruses for onward experiments. Procedure for $TCID_{50}$ is described under section 2.2.16.

2.2.3 Plasmids

The plasmids used in the study were generated in different laboratories and reported in other studies. Detailed information about the plasmids are illustrated in Table 2.9 and were provider by Prof. Dr. Esther Schnettler for the study. SFV-FLuc reporter virus was constructed from the plasmid pCMV-SFV4(3H)-FFLuc whilst BUNV-NLuc was constructed from pTVT7BUNM-NL, pTV4BUNS, and pTVT7BUNL in a different laboratory (Dietrich, Shi, et al. 2017; Varjak, Maringer, et al. 2017). The plasmids pIZ-Fluc and pAcIE1-Rluc were used in RNAi suppressor assays to express Firefly and Renilla luciferase respectively (Ongus et al. 2006).

2.2.4 Mosquito rearing and maintenance

Culex quinquefasciatus and *Culex pipiens molestus* mosquitoes were reared in the insectary at 27-28 °C, 75-80 % humidity; 12 h day/night intervals. Approximately 100-200 adult mosquitoes were kept per a cage (Bugdorm, MegaView Science). Eight percent Fructose soaked filter paper/cotton pads were used as food via ad lib feeding for female and male adult mosquitoes. Cotton pads/filter paper used for blood feeding adult *Culex* mosquitoes and the developing larvae were fed with a Fish meal (Astra Aquaristic).

2.2.5 Extraction of RNA using TRIzol

TRIzol Reagent (Carlsbad) is a ready-to-use reagent, designed to isolate high quality total RNA (as well as DNA and proteins) from cell and tissue samples. RNA isolation from cell pellets was performed with TRIzol Reagent (Carlsbad) following the manufacturer's instruction. To do this, 1 ml of TRIzol was added to cell pellets and then lysed by pipetting gently up and down. For phase separation, 200 µl Chloroform was added to 1 ml of TRIzol, vortexed 15 s and centrifuged 15 min (10500 rpm, 4 ^{o}C). Then the aqueous phase was transferred into a new tube and 1 µl RNAse free glycogen (10 mg/ml; to

increase yield) was added as carrier with 500 µl Isopropanol per 1 ml of TRIzol to precipitate the RNA. The tube was incubated 10 min at room temperature and centrifuged 15 min (12000 rpm, 4 °C). The RNA was washed with 500 µl 70 % Ethanol and centrifuged 10 min (12000 rpm, 4 °C). The RNA was redissolved in 20 µl of RNAse free water after drying the pellet 1-2 h at room temperature. Finally, the concentration was measured using NanoDrop Spectrophotometer (ThermoScientific).

2.2.6 Extraction of RNA using TRIzol LS

This extraction method has the same approach using the TRIzol reagent (Carlsbad) above except the TRIzol LS (Carlsbad) is basically used for extracting RNA out of mosquito homogenate or supernatants. To start, each mosquito was smashed thoroughly in 500 µl medium without supplements in 1.5 ml eppendorf tube, centrifuged for 5 min at 1000 rpm (4 °C). TRIzol LS (600 µl) was added to 200 µl homogenate (3:1/TRIzol LS: suspension), mixed well by pipetted gently up and down. 160 µl Chloroform (per 600 µl TRIzol) was added to the suspension, vortexed for 15 s and incubated for 10 min at room temperature, centrifuged for 15 min at 12000 rpm (4 °C). To precipitate the RNA, 1 µl glycogen (10 mg/ml) was added as carrier together with 400 µl 100 % Isopropanol (per 600 µl TRIzol LS), centrifuged for 10 min at 12000 rpm (4 °C). 800 µl 70 % Ethanol (per 750 ml TRIzol) was added to wash the RNA, centrifuged for 5 min at 7500 rpm (4 °C). The RNA was redissolved in 20 µl of RNAse free water after drying the pellet 1-2 h at room temperature. Finally, the concentration was measured using NanoDrop Spectrophotometer (ThermoScientific).

2.2.7 Extraction of viral RNA with QIAamp Viral RNA Mini Kit

The Spin Protocol of the QIAamp Viral RNA Mini Kit (Qiagen) was used for isolation of viral RNA from virus stock and cell culture supernatants. Manufacturer's instruction in the QIAamp Viral RNA Mini Kit was used for the extraction process. Therefore, this was used for extraction of RNA from virus stocks used in the study. In summary, two main reagents were prepared to start the extraction process: Carrier RNA-AVE followed by AVL Buffer containing carrier RNA-AVE. For a single sample, 560 µl prepared Buffer AVL containing carrier RNA was pipetted into a 1.5 ml microcentrifuge tube and 140 µl cell-free sample (virus stock, cell culture medium) was added to the Buffer AVL–carrier RNA in a microcentrifuge tube. 560 µl Ethanol (96–100%) was added to the sample and the resulting solution was carefully applied to the QIAamp Mini column, centrifuged at 6000 x g (8000 rpm) for 1 min. 500 µl Buffer AW1 was added to the QIAamp Mini column and centrifuged at 6000 x g (8000 rpm) for 1 min. Again, 500 µl Buffer AW2 was added to the column and centrifuged for 3 min at full speed (20,000 x g; 14,000 rpm). Afterwards, the column was placed in a clean 1.5 ml microcentrifuge tube and 60 µl elution Buffer AVE was added, kept at room temperature for 1 min and later centrifuged for 1

min at 6000 x g (8000 rpm). Finally, the concentration was measured using NanoDrop Spectrophotometer (ThermoScientific).

2.2.8 Synthesis of cDNA with Moloney Murine Leukemia Virus Reverse Transcriptase

Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega) is an RNA-dependent DNA polymerase that can be used in First-Strand synthesis of cDNA. Manufacturer's instruction was used for the synthesis. Thermal Flex Cycler (Analytic Jena) was used for the cDNA synthesis.

Firstly, 1-2 µg of RNA, 1 µl of oligo dT (50 µM) or 1 µl of random hexamers primers were mixed in a sterile RNAse-free microcentrifuge tubes and double distilled water was added to complete the total reaction volume to 15 µl. The reaction mix was incubated for 5min at 70 ^{o}C to melt secondary structure within the template. The tube was cooled immediately for 1 min on ice to prevent secondary structure from reforming and then spun briefly to collect the solution at the bottom of the tube. The components in Table 2:11 were added to the annealed primer/template in the first reaction mix. Flicking the tube gently, the reaction was mixed and incubated for 60 min at 42°C or 37°C for oligo dT and random hexamers primers respectively.

Table 2. 11: Reaction master mix to the annealed primer/template

Component	Amount [µl]
5x M-MLV buffer	5
dNTPs (10mM)	1.25
RNAse inhibitor	1
Nuclease free water	1.75
M-MLV (200U/µl)	1

2.2.9 Polymerase chain reaction (PCR)

2.2.9.1 GoTaq DNA Polymerase PCR protocol

The GoTaq DNA polymerase (Promega) was used for end-point PCR. In general, 5x GoTaq buffer, primer sets (10 pmol/µl), dNTPs (10 mM), GoTaq polymerase, and cDNA template were mixed in a sterile RNAse-free microcentrifuge tubes and double distilled water was added to complete the total reaction volume to 50 µl (Table 2.12.A). The

microcentrifuge tubes containing the mix were loaded into a Thermal Flex Cycler (Analytic Jena) for amplification at the cycling conditions illustrated below in the Table 2.12.B. The annealing temperature varies and it is normally 2-3 ºC lower than the Tm of the lowest primer. In addition, the elongation or extension time is dependent on the size of the expected product. For example, 60 or 30 s for ~1kb or ~100bp respectively. Finally, the resulting PCR product was visualized using agarose gel electrophoresis.

Table 2. 12: GoTaq PCR protocol. (A) PCR master mix per a reaction and (B) PCR conditions.

GoTaq DNA polymerase was utilized. Primer pair information is summarized in Table 2.7. X varies based on the size of the PCR product. ∞ signifies infinity.

A

Component	Volume [µl]
5x GoTaq buffer	10
Forward primer (10pmol/ µl)	2.5
Reverse primer (10pmol/ µl)	2.5
dNTPs (10 mM)	1
GoTaq DNA polymerase	0.5
cDNA template	2.5
Nuclease-free water	31
Total mix	50

B

PCR step	Temperature [ºC]	Time	# Cycles
Initial denaturation	95	2 min	1
Denaturation	95	30 s	35
Annealing	Tm - 2 ºC	30 s	
Elongation	72	X s	
Final elongation	72	7 min	1
Cooling/End	4	∞	1

2.2.9.2 KOD DNA Polymerase PCR protocol

KOD DNA Polymerase is a hot start proofreading DNA polymerase that generates blunt ended PCR products. In general, 10x buffer, primer sets (10 pmol/µl), dNTPs (2 mM), KOD DNA polymerase, $MgCl_2$ or $MgSO_4$ (25 mM), and cDNA template were mixed in a sterile RNAse-free microcentrifuge tubes and double distilled water was added to complete the total reaction volume to 50 µl (Table 2.13.A). The microcentrifuge tubes containing the mix were loaded into a Thermal Flex Cycler (Analytic Jena) for amplification at the cycling conditions illustrated below in the Table 2.13.B. The annealing temperature varies and it is normally 2-3 ^oC lower than the Tm of the lowest primer. In addition, the elongation or extension time is dependent on the size of the expected product. For example, 10 s, 15 s, and 20 s for <500bp, 500-1000bp, 1000-3000bp respectively. Finally, the resulting PCR product was visualized using agarose gel electrophoresis.

Table 2. 13: KOD PCR protocol. (A) PCR master mix per a reaction and (B) PCR conditions.

KOD DNA polymerase was utilized. Primer pair information is summarized in Table 2.7. X varies based on the size of the PCR product. ∞ signifies infinity.

A

Component	Volume [µl]
10x buffer	5
Forward primer (10 pmol/µl)	1.5
Reverse primer (10 pmol/µl)	1.5
dNTPs (2 mM)	5
KOD DNA polymerase	1
$MgCl_2$/$MgSO_4$ (25 mM)	3
cDNA template	2.5
Nuclease-free water	30.5
Total mix	50

B

PCR step	Temperature [^oC]	Time	# Cycles
Initial denaturation	95	2 min	1

Denaturation	94	20 s	35
Annealing	Tm - 2 °C	10 s	
Elongation	70	X s	
Final elongation	70	7 min	1
Cooling/End	4	∞	1

2.2.9.3 SuperScript III one-step RT PCR protocol

The SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase (ThermoScientific) is designed for convenient end-point detection and analysis of RNA molecules by one-step RT-PCR using Thermal Flex Cycler (Analytic Jena). All components for cDNA synthesis and PCR are combined in a single tube with gene-specific primers and target RNA. Reverse transcription automatically follows PCR cycling without additional steps. It has a built-in hot start Platinum Taq DNA polymerase. The generic protocol and PCR conditions are illustrated in Table 2.14 below. Finally, the resulting PCR product was visualized using agarose gel electrophoresis.

Table 2. 14: SuperScript III one-step RT-PCR protocol. (A) PCR master mix per a reaction and (B) PCR conditions.

SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase was utilized. Primer pair information is summarized in Table 2.7. The volume of master mix per a reaction and sample per well are 21.5 µl and 3.5 µl respectively (25 µl total volume/reaction).

A

Component	Stock concentration	Volume/Reaction [µl]
Water	N/A	2.78
2x reaction mix	2x	12.00
$MgSO_4$	25 mM	0.86
BSA	1 mg/ml	0.86
Forward primer	10 pmol/µl	2.0
Reverse primer	10 pmol/µl	2.0
Enzyme mix	N/A	1
cDNA template	N/A	3.5
Total mix		25

B

PCR step	Temperature [ºC]	Time	# Cycles
Reverse Transcription (RT)	50	50 min	1
Initial denaturation	94	2 min	1
Denaturation	94	20 s	45
Annealing	55	45 s	
Elongation	68	60 s	
Final elongation	68	7 min	1
Cooling/End	4	∞	1

2.2.10 PCR clean-up and gel extraction

DNA extraction from agarose gel protocol using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) was used. The manufacturer's instructions with the kit was duly implemented.

To start, a cleaned scalpel was used to excise DNA fragment from an agarose gel. The weight of the gel was determined and transferred into a clean 1.5 ml test tube. Afterwards, 200 µl Buffer NTI was added to 100 mg of agarose gel, incubated for 5–10 min at 50 °C, and vortexed briefly every 2-3 min until gel was completely dissolved. To bind DNA, NucleoSpin Gel and PCR Clean-up column was placed into a Collection tube (2 mL) and 700 µl sample loaded, centrifuged for 30 s at 11,000 x g. The silica membrane was then washed by adding 700 µL Buffer NT3 to the NucleoSpin Gel and PCR Clean-up Column, centrifuged for 30 s at 11,000 x g. Afterwards, the silica membrane was dried by centrifuging for 1 min at 11,000 x g to remove Buffer NT3 completely. The DNA was eluted by placing NucleoSpin Gel and PCR Clean-up Column into a new 1.5 ml microcentrifuge tube, 15–30 µL Buffer NE added, incubated at room temperature (18–25 °C) for 1 min, and centrifuged for 1 min at 11,000 x g. Finally, the concentration of the DNA was measured using NanoDrop Spectrophotometer (ThermoScientific) and confirmation via Sanger sequencing at LGC Genomics (Berlin).

2.2.11 Sample preparation for Sanger sequencing

Sanger sequencing is a chain-termination sequencing technique based on the detection of labelled chain-terminating nucleotides that are incorporated by a DNA polymerase

during the replication of a template (nucleotide sequence of DNA determination). To prepare samples, PCR product or plasmid (10 µl) was pipetted into 1.5 ml test tube. If using a gel as a product, the band was cut out and purified. Either forward or reverse primer [4 µl (5 µM)] for the target product was added to the PCR product/plasmid and sent for sequencing (LGC sequencing company).

2.2.12 Verification of knockdowns using SYBR Green qPCR

A relative quantification (delta-delta CT method) of RNAi transcripts (Ago1-3, Piwi 4-6) per S7 housekeeping transcript was performed to verify knockdowns in Aag2 cells. The amplification was done using LightCycler 480 (Roche). RNA was extracted using TRIzol (Carlsbad) and cDNA synthesized with oligo dT primer using Thermal Flex Cycler (Analytic Jena). The synthesized cDNA was used as the PCR template. The analysis and quantification was done using delta-delta CT ($\Delta\Delta$CT) method (Livak and Schmittgen 2001).

$$RQ = 2^{-\Delta\Delta CT}$$

RQ = relative quantity; CT = cycle threshold of transcripts.

The generic protocol and SYBR green PCR protocol conditions are illustrated in Table 2.15 below.

Table 2. 15: SYBR Green qPCR protocol. (A) PCR master mix per a reaction and (B) PCR conditions.

QuantiTect SYBR Green PCR kit was utilized (Table 2.5). Primer pair (S7, Ago1-3, Piwi4-6) information is summarized in Table 2.7. The volume of master mix reaction and sample per well were 9 µl and 1 µl respectively (10 µl total volume/reaction). The acquisition modes at 72 ^{o}C/30 s cycling and 95 ^{o}C/1 s melting curve were set at single and continuous respectively.

A

Component	Stock concentration	Volume/Reaction [µl]
Nuclease free Water	N/A	3.4
2X QuantiTect Master Mix	2x	5
Forward primer	10 pmol/µl	0.3
Reverse primer	10 pmol/µl	0.3
cDNA template	N/A	1
Total mix		10

B

PCR step	Temperature [°C]	Time	# Cycles
Initial heat activation and denaturation	95	15 min	1
Denaturation	94	15 s	45
Annealing	60	30 s	
Elongation/Extension (single step)	72	30 s	
Melting curve	95	5 s	1
	50	15 s	
	95	1 s	
Cooling/End	40	30 s	1

2.2.13 Luciferase assay systems

The luciferase assay system was used for reporter quantitation in cell cultures. Using 24 well plate, $1.8x10^5$ cells were seeded per well in triplicates for various time points and then incubated at 28 °C. After 24 h, the seeded cells were inoculated with target virus/plasmid/dsRNA. The cells were incubated at 28 °C after inoculation and lysed with passive lysis buffer (1x). Luciferase activities were determined using luciferase kits (Promega) and GloMax luminometer (Promega) following manufacturer's instructions. Depending on the experiment, Luciferase assay kit (Firefly luciferase activity), Nano luciferase kit (Nano luciferase activity), or Dual luciferase assay kit (Firefly luciferase/Renilla luciferase activity) was used.

2.2.14 Immunofluorescence assay for Usutu virus and Mesonivirus

To visualize virus presence in the cells, an immunofluorescence assay was performed. For this, specific primary antibody targeting Flavivirus (Arbovirus)-specific protein NS1 and nonspecific virus replicative intermediate dsRNA primary antibody were used. NS1 was used for detection of USUV while dsRNA was used for MeSV.

For the immunofluorescence assay, cells (C6/36, Vero ATTC) were seeded in a 24 well plate (~ 1.8×10^5 cells/well), infected with the target virus and incubated. Following, supernatant was removed and cells were fixed in 4 % formaldehyde for 30 min, followed by 0.1% SDS/PBS and incubated for 10 min. For permeabilization, PBT was added and incubated for 30 min at room temperature. The plate was incubated for 30 min at room

temperature after adding blocking solution (PBT+ 10 % FCS) and primary antibodies were added to the wells after preparing dilutions in blocking solution (3G1.1 dsRNA antibody 1:100, NS1 antibody 1:100 dilution). Afterwards, the plate was incubated on a soft shaker 1-2 h at room temperature and washed three times with PBT at 2-3 min, followed by adding secondary antibody (anti-mouse alexa 488, 1:1000) together with DAPI (1:2000 dilution). The plate was covered with aluminium foil and incubated 1 h, followed by a washing step with PBT at 2-3 mins. The fluorescence was detected immediately with the EVOS FL Auto Imaging System (Life Technologies) and the corresponding filters.

2.2.15 Virus growth kinetics

For virus growth curves in vitro, corresponding cells were plated in a 6 well or 12 well plate (Total volume of 2 ml/well in a 12 well plate and 4 ml/well in a 6 well plate). The number of invertebrate cells (i.e. Ct, Hsu, C6/36, Aag2) plated was 4.0×10^5 or 1.0×10^6 cells per well in a 12 or 6 well plates respectively. In case of vertebrate cells (i.e. Vero ATTC), 1.5×10^5 or 3.75×10^5 cells per well were seeded in a 12 or 6 well plates respectively. Triplicate wells were plated for each growth kinetic. After 24 h and observing a good monolayer of cells, the growth media was replaced with a virus-media with the required MOI of the target virus. Thus, 12 and 6 well plates received 500 µl/well and 1 ml/well virus-media respectively. After the inoculation with the target virus, plates were incubated for 1h at 28 ^oC (invertebrate cells) or 37 ^oC with 5 % CO_2 (vertebrate cells). The medium was then removed and the cells washed gently 1-2 min with 1x PBS, followed by replacing the medium. Thereafter, cell supernatants (200 µl) were taken at different time periods (0, 6, 10, 24, 48, and 72 hpi) and stored at -80^oC. The titer of the collected supernatants were determined by Median Tissue Culture Infectious Dose ($TCID_{50}$) on the corresponding cells.

2.2.16 Median tissue culture infectious dose ($TCID_{50}$)

Median Tissue Culture Infectious Dose ($TCID_{50}$) assay was performed to determine virus concentrations. $TCID_{50}$ was performed on C6/36 and Vero ATTC cell lines with MeSV and USUV respectively. BHK cell lines were used for SFV-FLuc and BUNV-NLuc reporter viruses.

Depending on the virus, $4x10^4$ cells per well were seeded in a 96 well plate with 180 µl cell specific medium per well and were allowed to grow overnight. 20 µl virus per well was pipetted into the first column of the 96 well plate, followed by a 10x serial dilution, changing the pipette tips at each dilution (Fig. 2.3). The plate was fixed in 4 % formaldehyde for at least 1 h and crystal violet dye was used to stain the plate for at least 30 min.

The plate was dried after washing with tap water and $TCID_{50}$ determined by the Spearman and Kärber algorithm as described by Hierholzer and Killington (Hierholzer, J.C. & Killington, R.A. 1996).

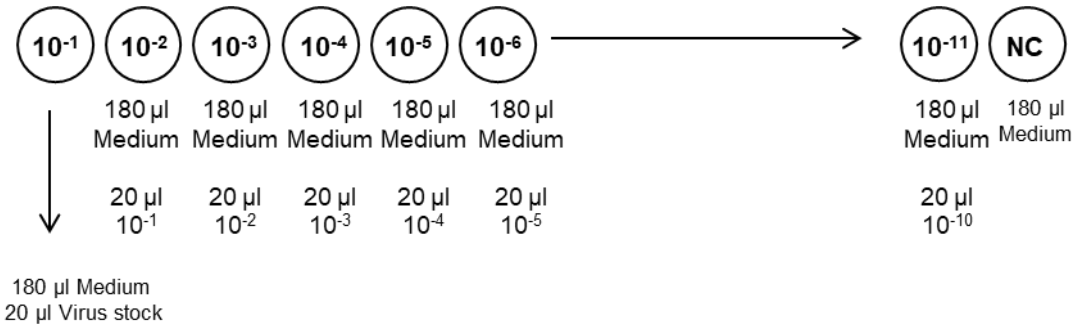

Fig. 2. 3: Titration for virus concentration determination via $TCID_{50}$.

Diagram showing the dilution steps in a 96 well plate. 180 µl cell specific medium per well and 20 µl target virus per well was pipetted into the first column. 10x serial dilution (10^{-1} to 10^{-11}) and a negative control well (NC).

2.2.17 Cloning and purification of plasmid DNA

To determine the sequence of PCR products, amplicons were cloned, purified and sequenced. CloneJET PCR Cloning Kit (ThermoScientific) was used following the manufacturer's instructions. To start the procedure, either the blunt-end cloning protocol for cloning blunt-end PCR products generated by proofreading DNA polymerases or sticky-end cloning protocol for cloning PCR products with 3'-dA overhangs generated by Taq DNA polymerase. The manufacturer's instruction was followed with the chosen protocol depending on the PCR product.

The ligation reaction was set and the mix was transformed using chemically competent DH5 alpha *E.coli*, plated on ampicillin resistant agar plates. A colony PCR was performed to determine the successful ligation, using 10x Taq buffer (2.0 µl), 2mM dNTP (2.0 µl), 25 mg $MgCl_2$ (1.2 µl), pJET1.2 primers (0.4 µl each), Nuclease free water (13.9 µl), and DNA polymerase (0.1 µl) to produce 20 µl total reaction volume. A sample of an individual colony was picked and resuspended in 20 µl PCR master mix. Afterwards, the PCR was performed under the following conditions: 95 °C, 3 min; 94 °C, 30 s, 60 °C, 30 s, 72 °C at 1 min/kb; 25 cycles. The resulting PCR product was visualized using agarose gel electrophoresis. After identifying the presence of the insert, the plasmid was isolated via miniprep using NucleoSpin Plasmid EasyPure protocol by Macherey Nagel and sent for Sanger sequencing.

2.2.18 Plasmid DNA isolation and purification

Plasmid DNA products were isolated and purified via NucleoSpin Plasmid EasyPure protocol using Plasmid DNA purification kit (Macherey-Nagel) following manufacturer's instructions.

Firstly, saturated *E. coli* culture (2-10 ml) was centrifuged for 30 s at > 12,000 x g to pellet cells. Afterwards, the cells were lysed by adding 150 µl Buffer A1, 250 µl Buffer A2, incubated at room temperature (18–25 °C) for 2 min, 350 µl of Buffer A3 was then added to the mix. The lysate was centrifuged for 3 min at full speed (> 12,000 x g) for clarification. To bind the DNA, Nucleospin Plasmid EasyPure Column was placed into a 2 ml collection tube and supernatant from the lysate decanted into the column, centrifuged for 30 s at 1,000–2,000 x g. To wash and dry the silica membrane, 450 µl of Buffer AQ was added to the column, centrifuged for 1 min at full speed (> 12,000 x g). Finally, the DNA was eluted by placing the Nucleospin Plasmid EasyPure Column into a 1.5 ml microcentrifuge tube and 50 µl Buffer AE added, incubated for 1 min at room temperature and later centrifuged for 1 min at full speed (> 12,000 x g). The plasmid DNA was finally measured using NanoDrop Spectrophotometer.

2.2.19 Production of double-stranded RNA

Synthesis of double-stranded RNA (dsRNA) using Megascipt RNAi kit (Thermo Fischer Scientific) is a reverse transcription reaction using the T7 RNA polymerase in combination with PCR products harboring the T7 promoter sequences at the 5´and 3´ends. Gene specific primers flanked by T7 RNA polymerase promoter sequences were used to amplify special gene specific regions using KOD DNA polymerase PCR (section 2.2.9.2) and validated through Sanger sequencing. To perform the PCR; RNA isolated using QIAamp Viral RNA Mini Kit, cDNA synthesised using MMLV with oligo dT primer. The PCR was optimized using 53 ^{o}C annealing temperature, 10 s extension, and within 35 cycles. The PCR product for the target sections are cloned into a plasmid vector (pJet) using CloneJET PCR Cloning Kit (section 2.2.17) and sequenced. Another PCR is performed to make more linear DNA copies of the target/specific region. The PCR products were used for in vitro transcription reaction using RNAi Megascript kit following the manufacturer's instructions.

2.2.20 Transfection of Aag2 cells for double-stranded RNA knockdowns

Double-stranded RNA (dsRNA) produced against specific genes were used to knockdown expression of corresponding genes via transfection. Using 24 well plate, $1.8x10^5$ Aag2 cells were seeded per well in duplicates, 24 h before transfection. 200ng of dsRNA (For example, Ago1, 2, 3, Piwi4, 5, 6, or LacZ/eGFP as a negative control) per well were

transfected in duplicates at 24 h post-seeding with DharmaFECT 2 (Dharmacon) following manufacturer's instruction. 24 h post-transfection, the cells were infected with MeSV at the indicated MOI. 48 h post-infection, the cells from the duplicate wells were mixed and harvested for RNA extraction using TRIzol (Carlbad). Finally the concentration of the RNA was measured using using NanoDrop Spectrophotometer and later used for individual experiments.

2.3 Experiments

2.3.1 Establishment of Mesonivirus qRT-PCR

2.3.1.1 Mesonivirus qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) monitors the amplification of a targeted DNA molecule during the PCR in a real time. This is in contrast to the end-point analysis of a conventional PCR. The classification of detection can either be specific using fluorescent reporter probe method or non-specific using DNA-binding dyes such as SYBR Green.

In this study, SYBR Green detection was used. The fluorescent SYBR Green I enables the analysis of many different targets without having to use target-specific labelled probes. Using a HotStarTaq DNA Polymerase, the reaction mixture was conveniently set up at room-temperature without fearing that the polymerase activity would start prematurely. Threshold cycle (CT) and Melting curve analysis were generated for the interpretation of the results. The advantage of the detection method using SYBR Green, is the ability to filter out non-specific products using the specific melting temperature (Tm) of the products. CT values are used to obtain information about the concentration of a target in a sample via standard curve.

2.3.1.2 The standard curve

The standard curve method was used to determine the concentration of the target in a sample. To set up the standard curve for the MeSV qRT-PCR, a standard DNA template was produced and quantified. Using the standard template, serial dilution curves were prepared, which were analyzed using the MeSV qRT-PCR protocol (Table 2.16). Based on the analysis, 3 standard dilutions were chosen to make up the in-run standard curve.

To prepare the DNA template, viral RNA was isolated using QIAamp Viral RNA Mini Kit (section 2.2.7) from MeSV stock and cDNA synthesized using oligo dT primers (section 2.2.8). Afterwards, gel PCR was performed using KOD DNA polymerase (section 2.2.9.2). For this PCR, the specific primers (E-433, E-434), 50 ^{o}C annealing temperature, and 10 s extension time were used. The primers were designed closed to the 3' end of

the MeSV genome (part of the virus where the qRT-PCR primers bind) and were able to produce a product with a size of 459 bp. After the PCR, the products were checked for the correct size under UV light, excised, and DNA extracted using Macherey-Nagel NucleoSpin Gel and PCR Cleaned-up (section 2.2.10). Samples were submitted for Sanger sequencing at LGC Genomics (Berlin) to confirm the sequence.

The concentration of the DNA was determined using DNA copy number calculator via an online tool by Thermo Fisher Scientific (https://www.thermofisher.com/de/de/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html; Date accessed: 24.01.2019). Based on the results, a DNA copy of number of 6.7 x 10^{10} DNA copies/µl was determined and diluted to a desired stock concentration 1.0 x 10^{10} copies/µl for the serial dilution.

The serial dilution was done from 10^{10} to 10^{0} and qRT-PCR performed in triplicate for each dilution using SYBR green RT-PCR Kit (Qiagen). The results were analyzed and the concentrations 10^{6}, 10^{5}, and 10^{4} were chosen to calculate and produce in-run standard curve.

Table 2. 16: MeSV qRT-PCR protocol. (A) PCR master mix per a reaction and (B) PCR conditions.

QuantiTect SYBR Green RT-PCR kit (Qiagen) was utilized. Primer pair (E-439, E-440) was used and summarized in Table 2.7. The volume of master mix reaction and sample per well were 9 µl and 1 µl respectively (10 µl total volume/reaction). The acquisition modes at 72 °C/30 s cycling and 95 °C/1 s melting curve were set at single and continuous respectively.

A

Component	Stock concentration	Volume/Reaction [µl]
Nuclease free water	N/A	2.9
2X QuantiTect Master Mix	2x	5
Forward primer	10 pmol/µl	0.5
Reverse primer	10 pmol/µl	0.5
QuantiTect RT Mix	N/A	0.1
RNA template	N/A	1
Total mix		10

B

PCR step	Temperature [°C]	Time	# Cycles
Reverse Transcription (RT)	50	30 min	
Initial activation/denaturation	95	15 s	1
Denaturation	94	15 s	45
Annealing	52	30 s	
Elongation/Extension (single step)	72	30 s	
Melting curve	95	5 s	1
	50	15 s	
	95	1 s	
Cooling/End	40	30 s	1

2.3.2 Mesonivirus interactions with arboviruses

2.3.2.1 Growth characteristics of Mesonivirus in different cell lines

To characterize MeSV infection, growth kinetics in different mosquito-derived cells as well as immunofluorescence assays were performed. Cell lines derived from different mosquitoes (*Culex tarsalis*-derived Ct cells, *Culex quinquefasciatus*-derived Hsu cells, *Aedes albopictus*-derived C6/36 cells, and *Aedes aegypti*-derived Aag2 cells) were used for the viral growth kinetics (section 2.2.15). Immunostaining was done to detect the presence of MeSV in C6/36 cell lines using a monoclonal antibody detecting dsRNA and Alexa Fluor 488 conjugated anti-mouse IgG as secondary antibody as well as DAPI to stain the nucleus (section 2.2.14). Additionally, the immunostaining was repeated for MeSV in Hsu cells at different MOIs to understand infection efficiency.

2.3.2.2 Growth characteristics of Usutu virus in different cell lines

To characterize USUV infection, growth kinetics in different mammalian and mosquito-derived cells as well as immunofluorescence assays were performed. Cell lines derived from different mosquitoes (*Culex tarsalis*-derived Ct cells, *Culex quinquefasciatus*-derived Hsu cells, and *Aedes albopictus*-derived C6/36 cells) and Vero ATTC cells (from African Green Monkey kidney) were used for the viral growth kinetics (section 2.2.15). Immunostaining was done to detect the presence of USUV in Vero ATTC cell lines using monoclonal antibody to detect NS1 (arbovirus-flavivirus specific) and Alexa Fluor 488 conjugated anti-mouse IgG as secondary antibody as well as DAPI to stain the nucleus

(section 2.2.14). Additionally, RT-PCR was done for USUV confirmation in the various viral growth kinetics at 72 hpi using GoTaq DNA polymerase (see section 2.2.8.1). To perform the PCR; RNA isolated using QIAamp Viral RNA Mini Kit, cDNA synthesized using MMLV with random hexamers primer. Following, USUV F8 and USUV R8 primers were used during the GoTaq PCR protocol. The PCR was optimized using 53 ^{o}C annealing temperature, 30 s extension, and 35 cycles.

2.3.2.3 Cell lines persistently infected with Mesonivirus

The effects of MeSV on arboviruses were investigated i) when MeSV was persistently infecting the mosquito cells, ii) when mosquito cells were coinfected with MeSV and an arbovirus. Here, a persistent infection is characterized by the presence of infectious virus in specific cells after several passages. In this study, *Culex quinquefasciatus*-derived Hsu and *Culex tarsalis*-derived Ct cells persistently infected with MeSV were created. To do this, 1x10^{6} cells were seeded with 4 ml/well medium in a 6 well plate. After 24 h, the cells were inoculated with MeSV at MOI of 0.1. Cells were checked regularly and passaged as needed. Finally, the cell pellets and supernatants were tested for MeSV after several passages (at least five passages) via RT-PCR (Superscript III one Step RT-PCR, KOD DNA polymerase PCR, or GoTaq DNA polymerase PCR). After inconsistency in the detection of MeSV in the MeSV persistent Hsu and Ct cells, the presence of MeSV was also determined by $TCID_{50}$ on C6/36 cells and rechecked some of them with RT-PCR.

2.3.2.4 Effect of Mesonivirus on Usutu virus replication

To investigate the effect of mosquito-specific MeSV infection on the arboviral USUV infection, Ct cells were either (i) coinfected with MeSV and USUV or (ii) MeSV persistently infected Ct cells, sequentially infected with USUV.

During the first set-up, coinfection of MeSV and USUV, MOIs 0.1 and 10 were used respectively. Singly infected Ct cells with USUV (MO1 = 10) were used as negative control. During the second set-up, a persistent infection of Ct cell line with MeSV (MOI = 0.1) was created (section 2.3.2.3). After at least five passages, the MeSV persistent Ct cells were sequentially infected with USUV (MO1 = 10). Also, singly infected Ct cells with USUV (MO1 = 10) were used as negative control. For either experiment, the procedure for viral growth kinetics was followed (section 2.2.15). The supernatants were collected for USUV titration via $TCID_{50}$ on Vero ATTC cells at 0, 6, 10, 24, 48 and 72 hpi for three independent experiments in triplicate.

2.3.2.5 Effect of Mesonivirus on Semliki Forest virus and Bunyamwera virus replication

To determine the effect of MeSV on Semliki forest virus (SFV, *Togaviridae*) and Bunyamwera virus (BUNV, *Peribunyaviridae*) infection in *Culex* cells, Hsu cells were either (i) coinfected with MeSV and SFV-FLuc/BUNV-NLuc or (ii) MeSV persistently infected Hsu cells, sequentially infected with SFV-FLuc/BUNV-NLuc.

During the experiment with SFV-FLuc, the first set-up, coinfection of MeSV and SFV-FLuc, MOIs 0.1 and 10 were used respectively. Singly infected Hsu cells with SFV-FLuc (MO1 = 10) were used as negative control. The second set-up, a persistent infection of Hsu cell line with MeSV (MOI = 0.1) was created (section 2.3.2.3). After at least five passages, the MeSV persistent Hsu cells were sequentially infected with SFV-FLuc (MO1 = 10). Also, singly infected Hsu cells with SFV-FLuc (MO1 = 10) were used as negative control. For either experimental set-up, Hsu cells at each time points (6, 18, 24, 48, and 72 hpi) were lysed with passive lysis buffer and Firefly luciferase activity was measured using Luciferase assay kit and GloMax luminometer (section 2.2.13). Three independent experiments in triplicate were performed. RT-PCR was done to confirm the presence of MeSV in the growth kinetics at 72 hpi using GoTaq DNA polymerase (section 2.2.9.1). Therefore, RNA was isolated with TRIzol using cell pellet and cDNA synthesized using MMLV with random hexamers primer. The PCR was optimized using 55 ^{o}C annealing temperature, 1 min extension, 35 cycles, and MeSV Pan primers (E-384, E-385).

The same experimental set-ups above were used for the BUNV experiment with the following exceptions. MOI of 0.1 was used for both BUNV-NLuc and MeSV in the set-ups (i) and (ii) above. Additionally, the time points (18, 24, 48, and 72 hpi) were used.

2.3.3 Mesonivirus interactions with the mosquito host

2.3.3.1 Small RNA next generation sequencing profiling

To investigate the production of virus-specific small RNA in mosquito-derived cells against MeSV. Aag2 and Ct cells were infected with MeSV (MOI 0.1) for 24 h. RNA was isolated using TRIzol reagent (Carlsbad) according to manufacturer's protocol. RNA was also isolated from *Culex quinquefasciatus* mosquitoes injected with MeSV at 5 days post infection using Trizol LS (Carlsbad) and glycogen as carrier. After verification of a successful or negative MeSV infection by RT-PCR (GoTaq DNA polymerase PCR using cDNA templates synthesized by MMLV with random hexamers), at least 1 µg of total RNA was sent for small RNA sequencing using Illumina based system at BGI (BGISEQ-500) as previously described (Franzke et al. 2018). Small RNAs were mapped and analyzed to the genome and antigenome of MeSV as previously described (Franzke et al. 2018). Moreover, de novo mapping and contig assembly was performed as previously

described (Franzke et al. 2018) to identify virus-specific small RNAs corresponding to other viruses possibly present in the used cells.

2.3.3.2 Double stranded RNA-based silencing in *Aedes aegypti*-derived cells

The first experiment using dsRNA-based silencing approach was to investigate the antiviral effect of RNAi pathway proteins. To silence known transcripts of the *Aedes aegypti* RNAi pathway, sequence specific dsRNA molecules were used. Firstly, dsRNAs were produced against key proteins of the different RNAi pathway transcripts (miRNA: Ago1; siRNA: Ago2; piRNA: Ago3, Piwi4-6) in Aag2 cell lines (section 2.2.19). Afterwards, transfection of these dsRNAs was done to target the key transcipts (section 2.2.20) using LacZ as internal control and DharmaFECT 2 as transfecting agent. Following, MeSV (MOI 0.1) was used to infect the cells after 24 h post-transfection. RNA was isolated at 48 h post infection using TRIzol and cDNA synthesized using using MMLV with oligo dT primer. RNA levels of key RNAi protein transcripts and MeSV were determined via SYBR green qPCR (section 2.2.12) and MeSV qRT-PCR (section 2.3.1) respectively. The qPCR and qRT-PCR used cDNA and RNA as input respectively. The PCRs were performed using *Aedes aegytpi* S7 housekeeper transcript and LacZ-specific dsRNA transfected cells as negative control treatment. Delta-delta CT method was used to calculate the relative quantity of RNA levels in the transcripts for three independent experiments in duplicate.

The second experiment, induced silencing of MeSV was also investigated to determine if MeSV can theoretically be targeted by the RNAi pathway. Firstly, specific primers against the 5’ end, the middle, and the 3’ end of the MeSV genome were designed using Primer3Plus software. Specific care was taken that the produced dsRNA should have the same/ similar length for all three target regions. These specific primers flanked by T7 RNA polymerase promoter sequences were used to amplify specific MeSV regions validated through Sanger sequencing. Afterwards, dsRNAs were produced as described in section 2.2.19 and transfection of Aag2 cells with these transcripts were done to target the 5’ end, the middle, or the 3’ end, respectively of the MeSV genome as described in section 2.2.20. RNA was extracted after the transfection and used as template for qRT-PCR for MeSV, internal LacZ control, and endogenous housekeeper (S7, *Aedes aegypti*).The PCR was performed using LightCycler 480 (Roche) for three independent experiments in triplicate. Relative quantity for the respective dsRNA transcripts was calculated using delta-delta CT method as described in section 2.2.12.

2.3.3.3 Viral suppression of siRNA response by Mesonivirus in Aag2 cells

The assay was designed to show if MeSV was actively interfering with the siRNA pathway. RNAi suppressor assays were performed in Aag2 cells. $1.8x10^5$ Aag2 cells per well were seeded in a 24 well plate. After 24 h, the seeded cells were infected with MeSV (MOI = 1) and incubated 24 h at 28 °C. The cells were then cotransfected with 60 ng pIZ-FLuc (Ongus et al. 2006; Schnettler et al. 2008) and 10 ng of pAcIE1-RLuc (Ongus et al. 2006) (FLuc and RLuc expression plasmids respectively), and 1 ng dsRNA targeting FLuc or a control dsRNA targeting eGFP using 2 µl/well DharmaFECT 2 (Dharmacon). Experiments were repeated but instead of dsRNA, 0.1 ng siRNA targeting FLuc or control siRNA targeting HygB using DharmaFECT 2 were used. At 24 h post-transfection, cells were lysed in passive lysis buffer and FLuc and RLuc expression was determined using Dual luciferase assay kit (Promega) and GloMax luminometer (Promega) for luminescence detection. Relative luciferase activity (FLuc/RLuc) was normalized to control dsRNA (eGFP) or siRNA (HygB) cells. Experiments were performed in three independent experiments in triplicate.

2.3.3.4 Microinjection of *Culex* mosquitoes with Mesonivirus

This was a method deployed to introduce MeSV into *Culex* mosquitoes (*Culex quinquefasciatus* and *Culex pipiens molestus* mosquitoes) to determine if MeSV can infect and replicate in *Culex* mosquitoes. Nanoject (Drummond Scientific) was used for the intrathoracic inoculation of MeSV. This procedure was performed at the BSL3 insectary by Drs Mayke Leggewie and Mine Altinli of the BNITM. To do this, the Nanoject was first assembled and set/calibrated to the desired amount of virus to be injected. Mechanical aspirator was used to retrieve experimental mosquitoes from the cage and anesthetized using CO_2 and afterwards injected under a Stereomicroscope (Motic). A total of 30 mosquitoes were injected at each time point (3, 7, and 14 dpi). For this experiment, the Nanoject was calibrated to inject 1380 PFU of MeSV per single injection in 13.80 nl volume. The injected mosquitoes were fed with 8 % Fructose ad lib and kept at the BSL3 insectary. The optimal climatic conditions observed in rearing and maintaining these mosquitoes were 27-28 °C, 75-80 % humidity; 12 h day/night intervals. The survivors at each time point were collected and homogenized in 500 µl GMEM medium without supplement. Samples then heat inactivated at 60 °C for 2 h; following transfer to the BSL2 laboratory for further analyses (performed by me). Afterwards, they were kept at -80 °C until RNA extraction using TRIzol LS. RNA was used as the PCR template for the qRT-PCR in detecting MeSV (section 2.3.1).

2.3.4 Identification and characterization of mosquito-specific viruses in cell lines

This investigates the presence and kinetics of persistently infected MSVs in Aag2, Hsu and Ct cells.

Virus discovery was done using the small RNA deep sequencing data for the MeSV infected Aag2 and Ct cells as previously described (Franzke et al. 2018) (assistance from Dr Sreenu Vattipally, CVR, Glasgow, UK). In short, small RNAs were used for contig assembly, followed by de novo mapping. If at least 70 % of the viral genome was covered by the small RNAs, the hit was seen as real and follow up verification by RT-PCR was performed. To achieve this, de novo mapping and contig assembly was performed to identify virus-specific small RNAs corresponding to other viruses possibly present in the MeSV infected cells (Aag2, Ct cells).

The Ct and Hsu cells of the MeSV growth kinetic experiments (time point 72 hpi) were screened by RT-PCR FOR for the identified MSVs; Phasi Charoen-like virus (PCLV), Calbertado virus (CALBOV), Culex narnavirus (CxNV1), Flock house virus (FHV), and Cell fusing agent virus (CFAV) using virus specific primers. To do this, RNA was first extracted from the cell supernatants using QIAamp Viral RNA Mini Kit (section 2.2.7) and cDNA synthesized using MMLV with random hexamers primer. Afterwards, RT-PCR was performed using GoTaq DNA polymerase (section 2.2.9.1). The PCR condition was optimized with 55 ^{o}C annealing temperature, 1 min extension, and 35 cycles. Finally, the resulting PCR product was visualized using agarose gel electrophoresis.

MSVs overtime were also detected via qRT-PCR at the different time points (0, 24, 48, and 72 hpi) using the MeSV kinetic samples from Ct and Hsu cells. Specific primers for the above viruses and adapted MeSV qRT-PCR protocol (Table 2.16) were used to monitor the growth kinetics of these viruses. To do this, the extracted RNA above was used as the template for the PCR together with noninfected respective cell lines as negative controls.

2.3.5 Prevalence and phylogeny of Mesonivirus 8345

The prevalence of mesoniviruses and phylogeny of the MeSV 8345 strain were investigated to further characterize the virus. SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase (ThermoScientific) was used to screen field caught mosquitoes in Germany from the Arbovirology group of the BNITM to determine the prevalence of mesoniviruses (section 2.2.9.3). DNA samples were used as templates with the pan MeSV primers (E-384, E-385).

Bioinformatics analysis of the genome of MeSV 8345, regarding organization and Open Reading Frames (ORF), were investigated using Geneious Prime software (Biomatters, Inc.) by Dr Daniel Cadar of the BNITM. To investigate the phylogenetic relationship of

the new MeSV 8345 strain used in the study, Maximum Likelihood (ML) phylogenetic tree was constructed based on the protein sequence alignment of Open Reading Frame 1ab (ORF1ab) domain for MeSV 8345 and other mesoniviruses. This ORF1ab enclosed the conserved protein domains 3CLpro-RdRp-HEL1. The ORF1ab protein sequences derived from online database via National Center for Biotechnology Information (NCBI) were realigned using MAFFT 7 online version (Katoh et al. 2019). Since protein sequences were used, ProtTest version 3.4.2 was used to check for the best model with AIC/BIC and designated as LG+G. The tree was run on SeaView (Gouy et al. 2010) with 1000 bootstrap using only LG model. The constructed tree was rooted using Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) as outgroup which belongs to the same virus order *Nidovirales* as MeSV.

2.4 Statistical analyses

Data generated were analyzed using the statistical package GraphPad Prism (Country: USA, California, San Diego; Company: GraphPad Software Incorporated; Version: 7.00; Year: 2016; Founder: Dr. Harvey Motulsky). Microsoft Office tool (Excel) was also used for data cleaning and verification.

Group data table was created for each time point to analyze the growth kinetics. Using a column analyses, a t-test was chosen with unpaired experimental design. Mean and standard error of the mean were shown on the graphs and $p<0.05$ was considered significant. Each replicate for the three independent experiments (n=3) in triplicate was used separately in the descriptive statistics and inferences. The growth kinetics were further analyzed using linear regression to better understand the effect of MeSV on arbovirus replication based on the slopes and intercepts. Using the regression model, $p<0.05$ concludes that the difference between the slopes are significant. Hence comparing coinfections with single infections using a linear regression with $p<0.05$ was considered significant.

Data from dsRNA knockdown and luciferase assay experiments were also analyzed via unpaired t-test. The knockdown qRT-PCR data were analyzed using delta-delta CT approach ($RQ = 2^{-\Delta\Delta CT}$) (Livak and Schmittgen 2001). Thus, RQ is the relative quantification and CT is the cycle threshold of the knockdown transcripts. Regarding the dsRNA knockdown experiments, the results were normalized against the endogenous housekeeping control as well as the internal control. However, only internal controls were used in the luciferase assay experiments. Also, each replicate for the three independent experiments (n=3) in triplicate/duplicate was used separately in the descriptive statistics and inferences.

3 Results

3.1 Mesonivirus interactions with arboviruses

This study aims to unravel the interactions of MeSV with arboviruses. To best understand these interactions, the first thing to know is whether MeSV and the target arboviruses can grow well in cell lines derived from different mosquitoes or the susceptibility of cell lines to MeSV or arbovirus infection. This was done to see which cell line can help the virus replicates well and can be used for the onward infection experiments. The infection experiments of MeSV with the target arboviruses will then reveal the effect MeSV has on the replication of the target arboviruses. Arboviruses belonging to a selection of virus families were chosen, namely Usutu virus (USUV; Flavivirus), Bunyamwera virus (BUNV; Orthobunyavirus) and Semliki Forest virus (SFV; Alphavirus), and their interaction with MeSV was investigated in vitro. The chosen viruses covered the main arbovirus families and this could help to understand the interference of MeSV with arboviruses in a broader view. The changes in arboviral replication were determined via luciferase reporter assay or virus titration ($TCID_{50}$). These investigations conclude that the effect of MeSV on arbovirus replication is variable for the different arboviruses.

3.1.1 Growth characteristics of Mesonivirus in different cell lines

To characterize MeSV infection, immunofluorescence assays (IFAs) as well as growth kinetics in different mosquito-derived cells were performed.

Aedes albopictus-derived C6/36 cells were infected with MeSV (MO1 = 10) and detected at 4 dpi using dsRNA (primary antibody), anti-mouse alexa 488 (secondary antibody), and stained with DAPI (Fig. 3.1.A). The cells staining positive for double-stranded RNA (dsRNA) is indicative of RNA virus replication (intermediate virus replication stage). MeSV can infect C6/36 cells efficiently and the staining works. The IFA experiment was repeated using Hsu cells to estimate the number of infected cells (infection efficiency) at different MOIs (10, 1, 0.1, 0.01) (Fig. 3.1.B), (supplementary material Fig. 6.1). The results showed that infecting Hsu cells with MO1 10, 1, 0.1, 0.01 reported 7.8 % (20/255), 4.1 % (11/267), 4.0 % (9/224), and 1.3 % (3/224) infection efficiencies respectively. Theoretically, MOI 1 and 10 were expected to give 100 % efficiency but this was not shown experimentally. The reason could be that not all cells were susceptible for MeSV infection as Hsu cell is a polyclonal cell line. In summary, infecting cells with less viral particles per cell reduces the percentage of infected cells (Fig. 3.1.B), (supplementary material Fig. 6.1).

After successful detection of MeSV by IFA, cumulative growth curves were performed with MeSV in cell lines derived from different mosquitoes; *Culex tarsalis*-derived Ct cells, *Culex quinquefasciatus*-derived Hsu cells, *Aedes albopictus*-derived C6/36 cells, and *Aedes aegypti*-derived Aag2 cells (Fig. 3.1.C.D). These cell lines were chosen as they are known to be either susceptible to the main arbovirus families or from a mosquito species that was known to be infected with mesoniviruses. Selected cell lines were infected with MeSV (MOI = 0.1) and supernatants were collected at 0, 6, 10, 24, 48, 72 hours of post infection (hpi), follow by a $TCID_{50}$ (Fig. 3.1.D). MeSV replicates in all the cell lines used. Cytopathic effect (CPE) was also observed in all cell lines at the initial infection compared to mock infected cells as early as 24 hpi (Fig. 3.1.C).

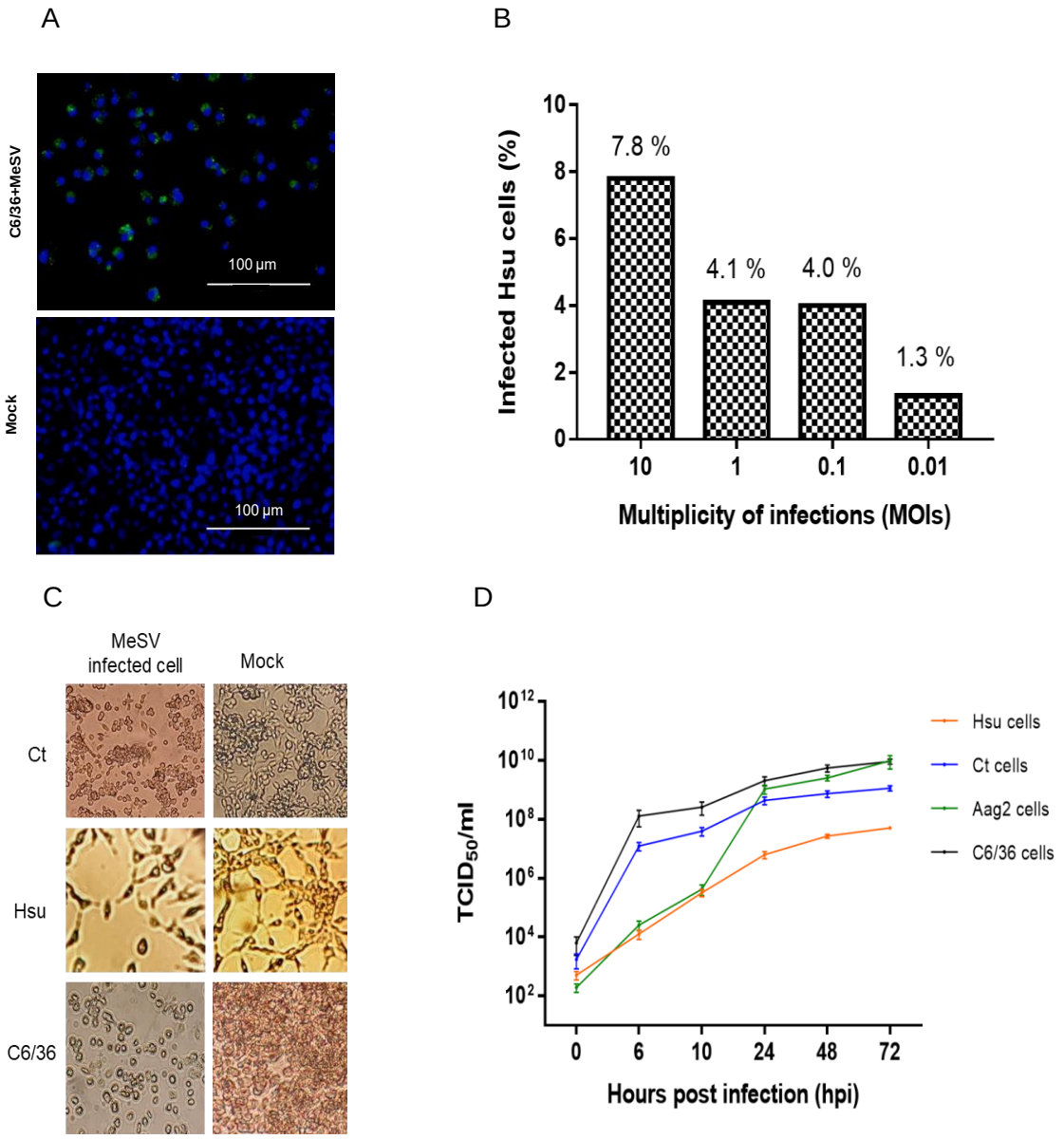

Fig. 3. 1: Growth characteristics of MeSV in different mosquito-derived cells.

Aedes albopictus-derived C6/36 cells were infected with MeSV at MOI 10. MeSV detected at 4 dpi using a monoclonal antibody detecting dsRNA and an Alexa 488 conjugated anti-mouse secondary antibody (green) as well as DAPI to stain the nucleus (blue). The mock cells were also stained with the respective antibodies. Scale bars are 100 µm **[A]**. The immunostaining was repeated in *Culex quinquefasciatus*-derived Hsu cells at differnt MOIs (10, 1, 0.1, 0.01). Percentage infection efficiency was calculated based on the total number of cells and the infected cells **[B].** Image of respective inoculated cell lines at 24 hpi using light microscopy (magnification = x400) **[C].** Supernatants were collected at different time points (0, 6, 10, 24, 48, 72 hpi) from the different MeSV (MO1 = 0.1) infected cells. MeSV detected by a $TCID_{50}$/ml on *Aedes albopictus*-derived C6/36 cells. Mean values of three independent experiments performed in triplicate are shown with SEM. *Culex tarsalis*-derived Ct cells (blue), *Culex quinquefasciatus*-derived Hsu cells (red), *Aedes albopictus*-derived C6/36 cells (black), and *Aedes aegypti*-derived Aag2 cells (green) **[D]**.

3.1.2 Growth characteristics of Usutu virus in different cell lines

To characterize USUV infection in more detail, IFAs and growth kinetics in different mammalian and mosquito-derived cells were performed. To establish the cell line to be used for USUV $TCID_{50}$, IFA was used to verify USUV replication in Vero ATTC cells. To do this, Vero ATTC cells and *Aedes albopictus*-derived C6/36 cells were infected with USUV and detected at 4 dpi using monoclonal antibodies NS1 (Fig. 3.2.A) and dsRNA (Fig. 3.2.B), anti-mouse alexa 488 (secondary antibody), and stained with DAPI. USUV was detected via IFA in Vero ATTC and C6/36 cells. Both NS1 flavivirus specific and dsRNA antibodies were used for USUV detection in Vero ATTC and C6/36 cells respectively, with NS1 showing a higher fluorescence. The results show that USUV is able to infect C6/36 and Vero ATTC cells efficiently and the infection can be detected successfully by different methods (IFA and $TCID_{50}$). The choice of detection method for follow up experiments, depends on the research questions.

To determine the growth kinetics, *Culex tarsalis*-derived Ct cells, *Culex quinquefasciatus*-derived Hsu cells, and *Aedes albopictus*-derived C6/36 cells were infected with USUV (MOI = 10), supernatants collected at 0, 6, 10, 24, 48 and 72 hpi and titer was determined by $TCID_{50}$. C6/36 cells and Ct cells showed a constant increase in USUV production. Although, titers were similar until 24 hpi, C6/36 cells produced 2 log more infectious virus at 48 and 72 hpi. Surprisingly, no significant increase of USUV was observed in Hsu cells and a strong decline after 24 hpi resulting in no infectious USUV particles at 72hpi (Fig. 3.2.D). To confirm this, RT-PCR using the cell supernatants of the USUV growth kinetics at 72 hpi were positive for USUV in Ct and C6/36 cells but not Hsu cells confirming the absence of USUV at 72 hpi (Fig. 3.2.E).

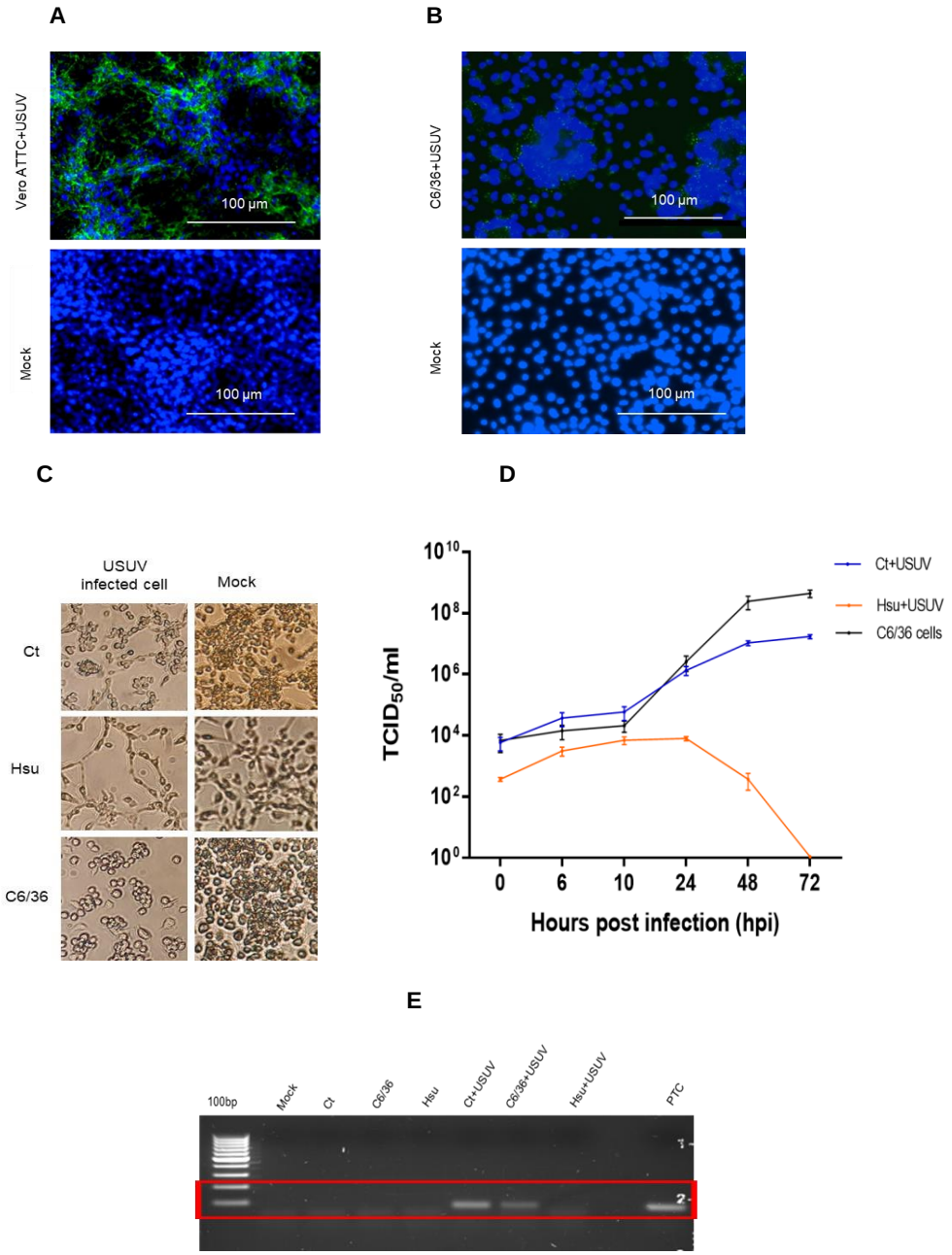

Fig. 3. 2: Growth characteristics of USUV in different derived cell lines

Vero ATTC cells (from African Green Monkey kidney) and *Aedes albopictus*-derived C6/36 cells were infected with USUV at MOI 10. USUV detected at 4 dpi using a monoclonal antibodies detecting NS1 **[A]** and dsRNA **[B],** and an Alexa 488 conjugated anti-mouse secondary antibody (green) as well as DAPI to stain the nucleus (blue). The mock cells were also stained with the respective antibodies. Scale bars are 100 µm **[A, B].** Image of respective inoculated cell lines at 24 hpi using microscopy (magnification = x400) **[C].** Supernatants were collected at different time points (0, 6, 10, 24, 48, 72 hpi) and USUV detected by a $TCID_{50}$/ml on Vero ATTC cells. Mean values of three independent experiments performed in triplicate are shown with SEM. *Culex tarsalis*-derived Ct cells (blue), *Culex quinquefasciatus*-derived Hsu cells (brown), and *Aedes albopictus*-derived C6/36 cells (black) **[D].** A gel PCR for USUV confirmation in the cell supernatants of the various kinetics at 72 hpi; Mock = water as negative control, PTC = positive control (virus stock) **[E]**.

3.1.3 Creation of Mesonivirus persistent infection in cell lines

Persistent infection is characterized by the presence of infectious virus particles in specific cells after several passages. The effect of MeSV on the replication of arboviruses were investigated using persistently infected cell lines. In this study, *Culex quinquefasciatus*-derived Hsu and *Culex tarsalis*-derived Ct cells persistently infected with MeSV (MOI = 0.1) were created. MeSV was confirmed in Hsu and Ct cells persistently infected with MeSV (Fig. 3.3.A.B). During the creation of the persistent infection, CPE was noticed after 24 hpi and the cells start to get better after at least 72 hpi. After creating the persistent infection, the presence of MeSV was verified after at least five passages. MeSV was detected in persistently infected Hsu and Ct cells via Superscript III one Step RT-PCR and KOD DNA polymerase PCR respectively (Fig. 3.3.A.B). Regarding the Superscript III one Step RT-PCR, RNA was extracted using TRIzol from MeSV persistent Hsu (Hsu+MeSV) cell pellets, and MeSV PAN primers were used (section 2.2.9.3), (Fig. 3.3.A). The KOD PCR was achieved through the following steps: RNA extracted from from MeSV persistent Ct (Ct+MeSV) cell pellets, cDNA synthesis using MMLV with random hexamers, and the generic protocol optimized using 50 °C annealing temperature with 15 s extension (section 2.2.9.2).

During the investigations, it was revealed that MeSV could not be detected at certain times with the persistently infected Hsu and Ct cells although CPEs were confirmed with $TCID_{50}$ (supplemenatry material Fig. 6.3). This could be due to possible mutation, enzymes or chemicals from the infected cell lines, or the presence of other persisting mosquito-specific viruses interfering with MeSV. However, the laboratory is still investigating the loss of susceptibility of the cell lines to MeSV infection.

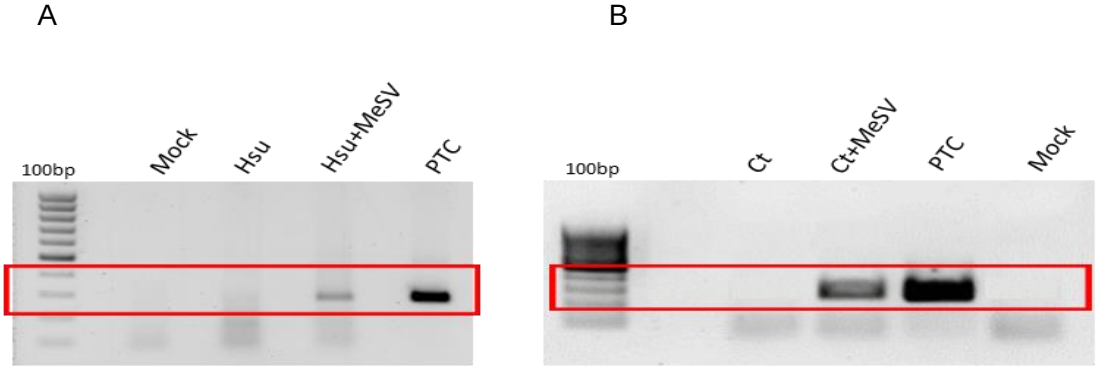

Fig. 3. 3: Cell lines persistently infected with Mesonivirus

Culex quinquefasciatus-derived Hsu cells were infected with MeSV (Hsu+MeSV), MOI = 0.1. RNA from persistently infected cells was isolated using TRIzol and presence of MeSV verified after five passages using Superscript III one Step RT-PCR **[A]**. *Culex tarsalis*-derived Ct cells were infected with MeSV (Ct+MeSV), MOI = 0.1. RNA from persistently infected cells was isolated using TRIzol, cDNA isolated using MMLV with random hexamers, and presence of MeSV verified after five passages using KOD DNA polymerase RT-PCR **[B]**. Mock = water as negative control, PTC = positive control (virus stock), Hsu = noninfcted Hsu cell, Ct = noninfected Ct cell.

3.1.4 Effect of Mesonivirus on Usutu virus replication

To investigate the effect of the mosquito-specific MeSV infection on the arboviral USUV infection in *Culex* cells, Ct cell were either (i) coinfected with MeSV (MOI = 0.1) and USUV at high MOI (10) (Ct+MeSV+USUV) or (ii) first infected with MeSV (MOI = 0.1) and after establishment of a persistent infection (Section 3.1.3), sequentially infected with USUV (MOI = 10) (Ct+pMeSV+USUV). Singly infected Ct cells with USUV (MOI = 10) (Ct+USUV) were used as control. Growth kinetics of USUV was determined in the supernatants at 0, 6, 10, 24, 48, and 72 h post infection (hpi) via a $TCID_{50}$ on Vero ATTC cells.

For all conditions, USUV concentration steadily increased from 24 hpi until 72 hpi (last time point) (Fig. 3.4). In case of acute coinfections, less MeSV effect was observed for all time points except at 48 and 72 hpi with a significant difference ($p<0.05$) (Fig. 3.4.A). Similar results were observed for USUV infection in MeSV persistently infected Ct cells, but this time increased USUV concentration was only significant at 24 and 48 hpi for the Ct+pMeSV+USUV ($p<0.0001$) (Fig. 3.4.B).

USUV growth kinetics in Ct+pMeSV+USUV were compared to growth kinetics in Ct+USUV using linear regression and were significantly different ($p=0.0032$) (supplementary material Fig. 6.2.A). Similar, USUV growth kinetics in Ct+MeSV+USUV were compared to growth kinetics in Ct+USUV using linear regression and were also significantly different ($p=0.002$) (supplemenatry material Fig. 6.2.B).

Taken together, the acute infection of USUV was reduced by MeSV in both co- and persistent infection. The $TCID_{50}$/ml values are documented in the supplementary material (Table 6.1.A.B).

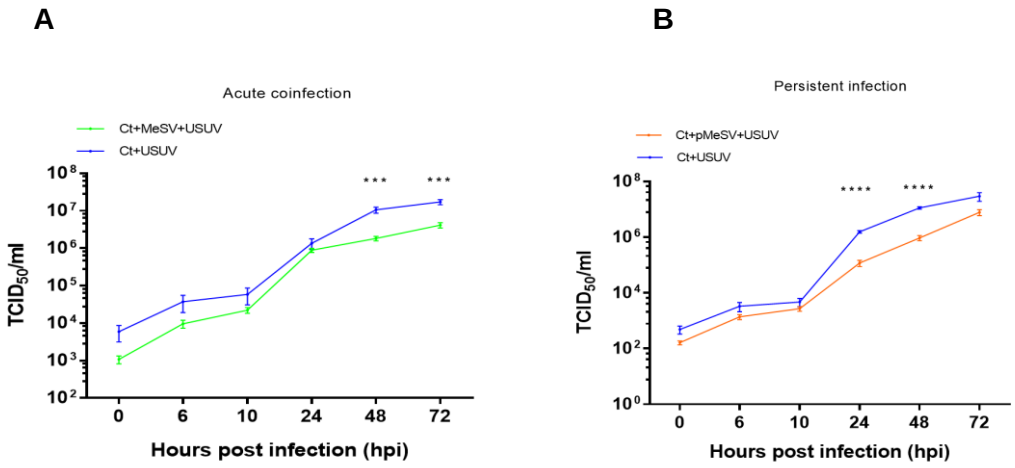

Fig. 3. 4: Growth kinetics of USUV in Mesonivirus infected *Culex* cells

Culex tarsalis-derived Ct cells were infected singly with USUV (Ct+USUV), coinfected with MeSV and USUV (Ct+MeSV+USUV); MOI 10 = USUV, MOI 0.1 = MeSV **[A]**: or first MeSV persistently infected followed by USUV infection (Ct+pMeSV+USUV); MOI 10 = USUV, MOI 0.1 = MeSV **[B]**. Supernatants were collected for USUV titration via $TCID_{50}$ at 0, 6, 10, 24, 48 and 72 hpi for three independent experiments in triplicate. Mean values with SEM are shown. Statistics via unpaired t-test (*p<0.05: ****=p<0.0001, ***p=0.0002).

3.1.5 Effect of Mesonivirus on Semliki Forest virus replication

To determine if the inhibitory effect of MeSV is USUV specific or can be broadened to another arbovirus families, growth kinetics were performed with the alphavirus, SFV. *Culex quinquefasciatus*-derived Hsu cells were used together with a Firefly luciferase reporter expressing SFV, where Firefly luciferase is expressed in the non-structural protein cassette and a representative of virus replication and production. Hsu cells were either single infected with SFV-FLuc (MOI = 10) (Hsu+SFV-FLuc), coinfected with MeSV (MOI = 0.1) and SFV-FLuc (MOI = 10) (Hsu+MeSV+SFV-FLuc) or MeSV (MOI = 0.1) persistently infected Hsu cells were sequentially infected with SFV-FLuc (MOI = 10) (Hsu+pMeSV+SFV-FLuc). Cells were lysed at different time points (coinfection: 6, 24, 48, and 72 hpi; persistent infection: 6, 18, 24, 48, and 72 hpi) and Firefly luciferase determined.

For all situations, luciferase expression by SFV increased after 6 hpi and steadily decreased from 24 hpi until 72 hpi (Fig. 3.5). The difference between the coinfection (Hsu+MeSV+SFV-FLuc) compared to the single (Hsu+SFV-FLuc) infection at all time points were not significant (p>0.05) (Fig. 3.5.A). Similar results were observed for SFV-Fluc infection in MeSV persistently infected Hsu cells, but this time luciferase expression of SFV was only significantly different compared to single infected cells at 72 hpi (p<0.05) (Fig. 3.5.B). To confirm the presence of MeSV in the growth kinetics, RT-PCR was performed using cell pellets of the acute coinfection at 72 hpi. MeSV specific bands were detected for all samples (Fig. 3.5.C).

Taken together, MeSV seems to have no effect on the replication of the target arbovirus, SFV. The values for the relative luciferase activities are documented in the supplementary material (Table 6.2.A.B).

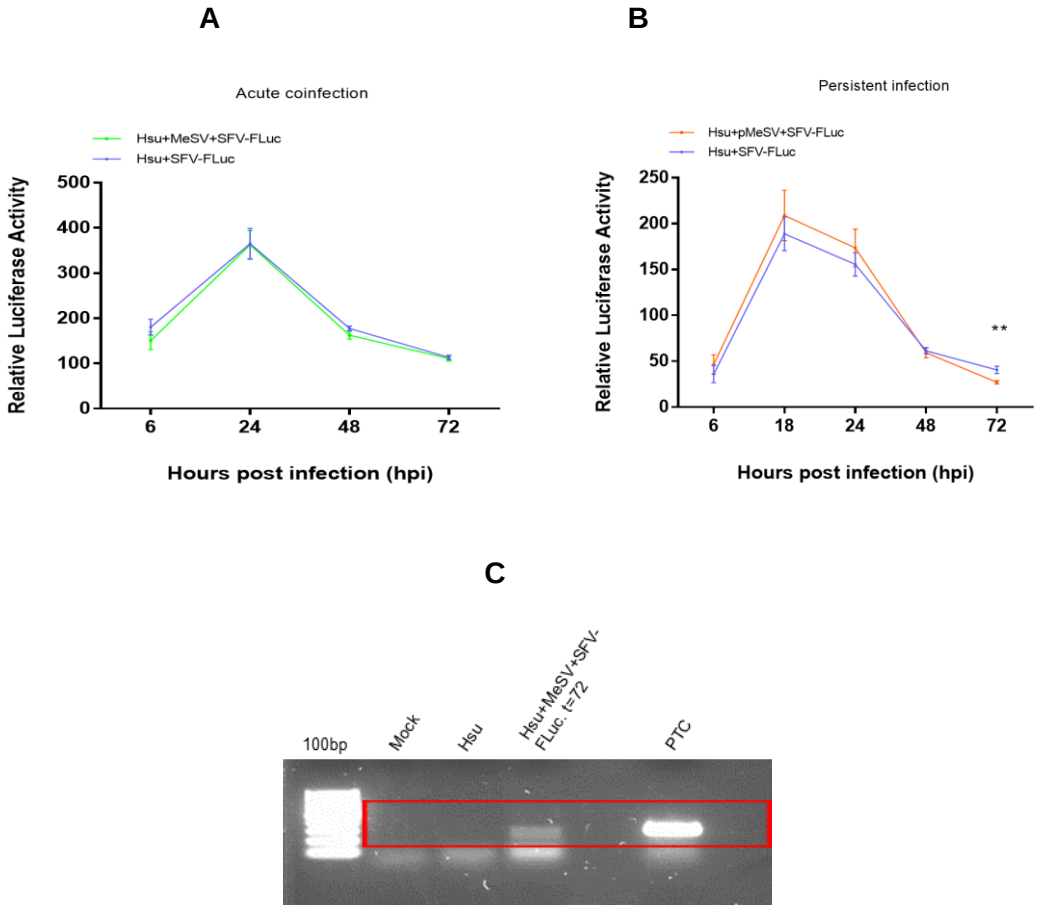

Fig. 3. 5: Growth kinetics of Semliki Forest virus in Mesonivirus infected *Culex* cells

Culex quinquefasciatus-derived Hsu cells were singly infected with SFV-FLuc (Hsu+SFV-FLuc), coinfected with MeSV and SFV-FLuc (Hsu+MeSV+SFV-FLuc); MOI 10 = SFV-FLuc, MOI 0.1 = MeSV **[A]**: or first MeSV persistently infected followed by SFV-FLuc (Hsu+pMeSV+SFV-FLuc); MOI 10 = SFV-FLuc, MOI 0.1 = MeSV **[B]**. Cells were lysed at 6, 18, 24, 48, 72 hpi, and Luciferase activity measured for three independent experiments in triplicate using a reporter luciferase assay. Mean values with SEM are shown. Statistics via unpaired t-test (*p<0.05: **p=0.0071). A gel PCR was performed for MeSV confirmation using cell pellets of the acute coinfection at 72 hpi; Mock = water as negative control, PTC = positive control (virus stock) **[C].**

3.1.6 Effect of Mesonivirus on Bunyamwera virus replication

To broaden the investigation regarding the effect of MeSV on arbovirus infections, another arbovirus family was included (*Peribunyaviridae*). Therefore, growth kinetics were performed with the orthobunyavirus, BUNV. To investigate the possible effect of MeSV on BUNV, a similar approach for the SFV experiment was adapted. *Culex quinquefasciatus*-derived Hsu cells were used together with a Nano luciferase reporter expressing

BUNV, where Nano luciferase is expressed in the non-structural protein cassette which is also involved in virus replication and production. Hsu cells were either single infected with BUNV-NLuc (MOI = 0.1) (Hsu+BUNV-NLuc), coinfected with MeSV (MOI = 0.1) and BUNV-NLuc (MOI = 0.1) (Hsu+MeSV+BUNV-NLuc) or MeSV (MOI = 0.1) persistently infected Hsu cells were sequentially infected with BUNV-NLuc (MOI = 0.1) (Hsu+pMeSV+BUNV-NLuc). Cells were lysed at different time points (coinfection: 24, 48, and 72 hpi; persistent infection: 18, 24, 48, and 72 hpi) and Nano luciferase determined.

In the acute infections, luciferase expression by BUNV increased until 48 hpi in both the single and coinfected cells with no significant difference between the two treatments (Fig. 3.6). In contrast, in the persistent infection assay, luciferase expression increased steadily until 48 hpi for both the single and persistently infected cells. Interestingly, luciferase expression further increased (from 48 to 72 hpi) in the case of the single infection and in contrast to the persistent infection where luciferase expression stayed steady from 48 to 72 hpi (Fig. 3.6.B). This results in a significant difference of luciferase expression between single and persistent infection at 48 and 72 hpi (Fig. 3.6.B). The reason for variable luciferase expression for the single infection until 48 hpi in both the acute and persistent infection could be due to difference in susceptibility of cells after a certain passage (age) as both experimental set-ups were performed on different occasions (Fig. 3.6.A.B). To confirm the presence of MeSV in the growth kinetics, RT-PCR was performed using cell pellets of the acute coinfection at 72 hpi. MeSV specific bands were detected for all samples (Fig. 3.6.C).

To conclude, MeSV seems to have variable effect on the replication of the BUNV. The values for the relative luciferase activities are documented in the supplementary material (Table 6.3.A.B).

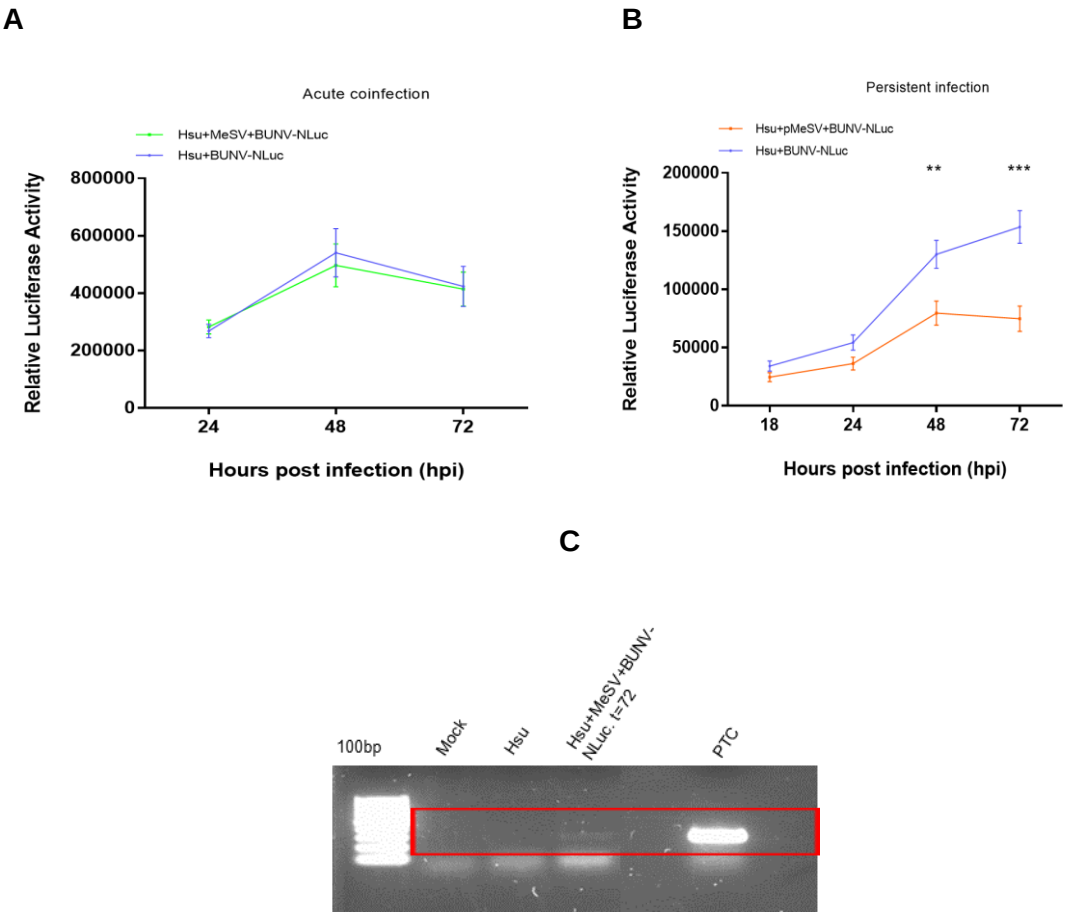

Fig. 3. 6: Growth kinetics of Bunyamwera virus in Mesonivirus infected *Culex* cells

Culex quinquefasciatus-derived Hsu cells singly infected with BUNV-NLuc (Hsu+BUNV-NLuc), coinfected with MeSV and BUNV-NLuc (Hsu+MeSV+BUNV-NLuc); MOI 0.1= BUNV-NLuc, MOI 0.1= MeSV **[A]**: or first MeSV persistently followed by BUNV-NLuc (Hsu+pMeSV+BUNV-NLuc); MOI 0.1= BUNV-NLuc, MOI 0.1= MeSV **[B]**. Cells were lysed at 18, 24, 48, 72 hpi, and Luciferase activity measured for three independent experiments in triplicate using a reporter luciferase assay. Mean values with SEM are shown. Statistics via unpaired t-test (*p<0.05: **p=0.0058, ***p=0.0004). A gel PCR for MeSV confirmation in the growth kinetics using cell pellets of the acute coinfection at 72 hpi; Mock = water as negative control, PTC = positive control (virus stock) **[C]**.

3.2 Mesonivirus interactions with the mosquito host

The investigation of the interactions of MeSV with the mosquito host was started with in vitro studies as these are easier to perform than in vivo experiments and are very good to understand the interactions. Little is known regarding the interactions of MSVs with the mosquito hosts, especially the antiviral response and its effect on virus infection. Therefore, the interactions of MeSV with antiviral RNAi response was investigated. The interaction of MeSV with the RNAi response involved different investigations illustrating the induction of RNAi response, the ability to exogenously target MeSV by RNAi, impact

of silencing key RNAi proteins, and inhibition of RNAi response. The study revealed production of MeSV specific small RNAs in *Aedes* and *Culex* cells with hot spots of small RNAs at the 5' end of the MeSV genome. It was shown that the siRNA pathway was able to target MeSV successfully in case of exogenous induction. Finally, MeSV was able to suppress the siRNA pathway.

3.2.1 In vitro interactions of Mesonivirus with the mosquito host

3.2.1.1 Interactions of the mosquito RNAi response and Mesonivirus

RNAi is believed to be the main antiviral response in mosquitoes against viruses; however little is known about its involvement with MSVs. Key findings to proof the antiviral response of the RNAi pathway against a virus would be: (i) production of virus specific small RNAs, (ii) increase of virus infection upon knockdown of key RNAi proteins, and sometimes (iii) expression of a viral RNAi suppressor.

3.2.1.1.1 Small RNA deep sequencing profiling

To investigate the production of MeSV specific small RNAs in mosquito cells, small RNA next generation sequencing was performed. To do this, Aag2 and Ct cells were infected with MeSV (MOI = 0.1) and RNA isolated with TRIzol at 24 hpi. Successful MeSV infection was verified by RT-PCR (Fig. 3.7). Following, small RNAs were sequenced using next generation sequencing at BGI and analyzed by mapping the small RNAs to both the MeSV genome and the antigenome (Fig. 3.8). Results were similar for both Aag2 (Fig. 3.8; Right panel) and Ct (Fig. 3.8; Left panel) cells. However, total clean reads in MeSV-infected Aag2 cells were more than MeSV-infected Ct cells and this was similar to the number of 21 nt reads in Aag2 and Ct cells. The percentage of 21 nt siRNA reads which aligned to the MeSV genome in Aag2 cells was 0.2 % which was more than 0.05 % in Ct cells (supplementary material Table 6.4.A). This is possibly representing a higher infection in Aag2 compared to Ct cells (Fig. 3.7; Fig. 3.1D). For both cells, MeSV-derived small RNAs were predominantly 21 nt in length and map to similar extent to the genome and antigenome, thereby showing typical features of a Dcr-2-based siRNA production pathway with a dsRNA-based inducer molecule (i.e. dsRNA replication intermediates) (Fig. 3.8.A). However, MeSV has shown a unique distribution of the siRNAs as the small RNA profile revealed a concentration (hot spots) of small RNAs at the 5' end of the MeSV genome unlike other viruses where normally the distribution was along the whole genome/ antigenome (Morazzani et al. 2012). The specific characteristics due to piRNA ping-pong production are; A10 bias for sense small RNAs, U1 bias for antisense small RNAs, as well as 10 nts complementarity at the 5' ends between the sense and antisense small RNAs (Miesen, Joosten, et al. 2016). The results revealed only a small number of

MeSV-specific small RNAs which were found in the size of piRNAs (24-30 nts) and they did not show the ping-pong specific production signature (Fig. 3.8.C.D). Taken together, this data suggests that an antiviral RNAi response in Aag2 and Ct cells is induced following MeSV infection and produces (exclusively) vsiRNAs targeting viral sequences mostly at the 5' end of the genome. MeSV-specific small RNAs in the size of piRNAs were identified to a low extent, however, they did not exhibit the specific ping-pong characteristics and follow up experiments would be needed to determine if they are real piRNAs.

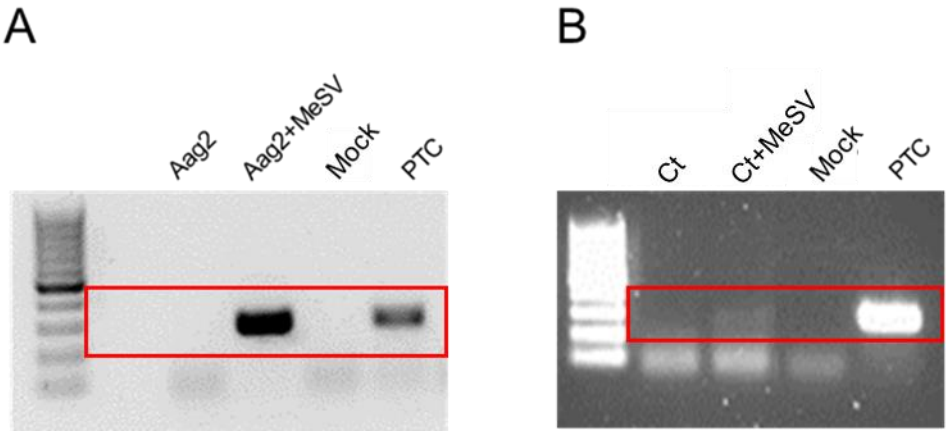

Fig. 3. 7: Mesonvirus detection in *Aedes* and *Culex* cells

Aedes aegypti-derived Aag2 **[A]** and *Culex tarsalis*-derived Ct **[B]** cells were infected with MeSV (MOI = 0.1). RNA was isolated from the MeSV infected Aag2 (Aag2+MeSV) and Ct (Ct+MeSV) cells using TRIzol after 24 hpi, cDNA synthesized using MMLV with random hexamers. The presence of MeSV was verified using GoTaq DNA polymerase RT-PCR using cDNA templates. Mock = Water as negative control; PTC = MeSV as positive test control. Aag2 and CT were non-MeSV infected cells.

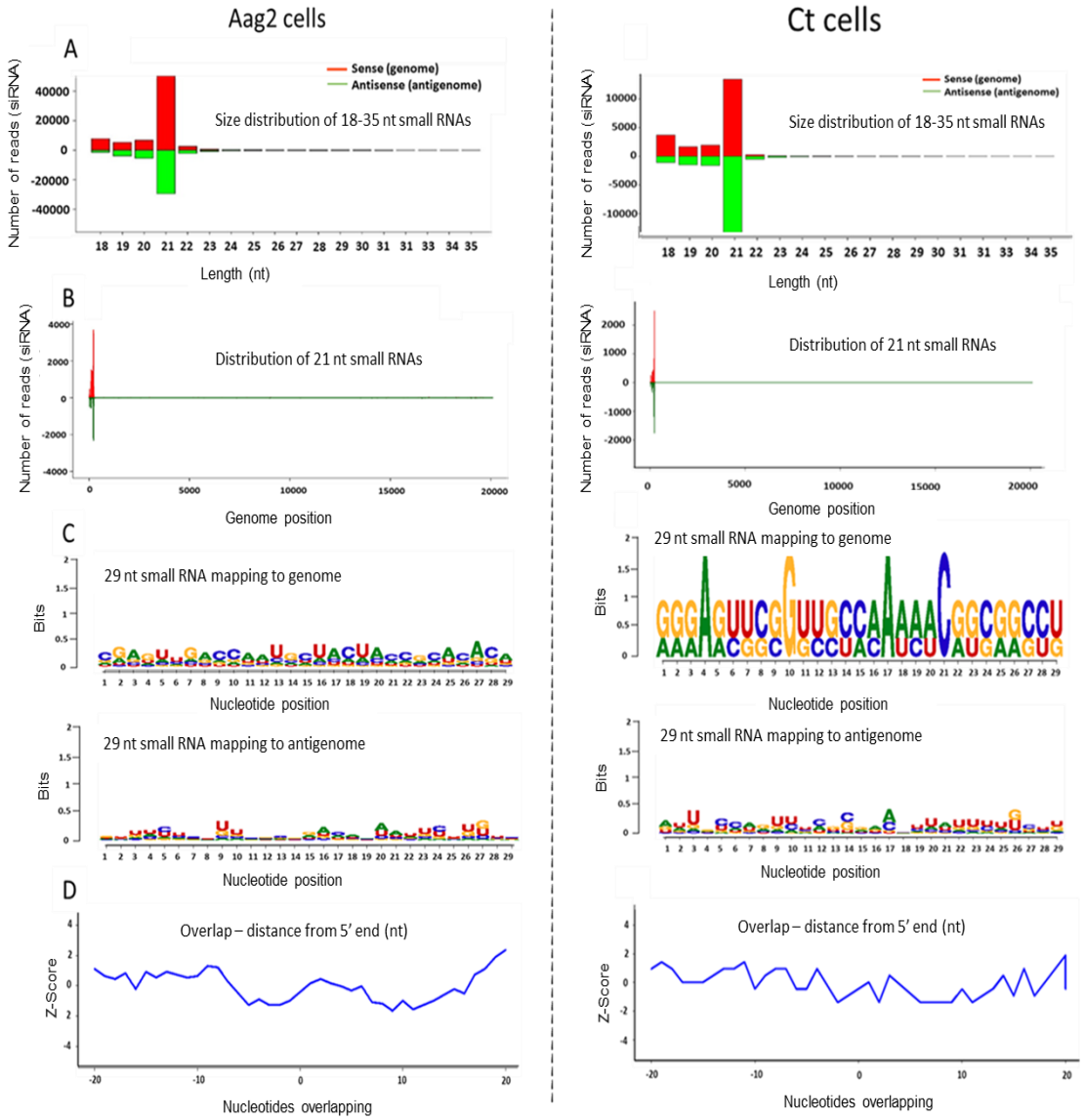

Fig. 3. 8: Characteristics of Mesonivirus-derived small RNAs in *Aedes* and *Culex* cells.

Aedes aegypti-derived Aag2 (Left panel) and *Culex tarsalis*-derived Ct (Right panel) cells were infected with MeSV (MOI = 0.1). After 24 hpi, RNA was isolated using TRIzol and followed by small RNA sequencing. **[A]** Size distribution 18-35 nt of small RNAs from MeSV infected cells mapped to MeSV genome (+, red) and antigenome (-, green). **[B]** Genome distribution of 21 nt small RNAs that map to the genome of MeSV (red) and antigenome (green). Frequency of small RNAs (y-axis) mapped to corresponding nucleotide location (x-axis). **[C]** Relative nucleotide frequency at 29 nt long small RNAs mapping to MeSV genome and anti-genome. **[D]** Probability of overlap of the sense (MeSV genome) and antisense (MeSV antigenome) small RNAs (length of 25-29 nt). The results shown are representative of two independent experiments.

3.2.1.1.2 Knockdown of antiviral RNA interference proteins in Aag2 cells

The production of MeSV-specific siRNAs in infected Aag2 cells, shows the induction of the siRNA pathway upon infection. However, it is not yet known if the produced MeSV-specific siRNAs are able to target MeSV and thereby act antiviral. Besides, no information is present about a possible antiviral or proviral activity of the other RNAi pathways, like miRNA or piRNA. Therefore, to investigate this functionality, key proteins of the different RNAi pathways (miRNA: Ago1; siRNA: Ago2; piRNA: Ago3, Piwi4-6) were silenced by dsRNA-transfection, followed by MeSV infection. Successful silencing of at least 50% was achieved for all chosen transcripts (Fig. 3.9.A). MeSV infection increased in cells silenced for Piwi4, 5 and 6 with the strongest effect in Piwi4 silenced cells; however, due to diverge amounts of increase, especially for Piwi4 silenced cells, the observed effect was not significant. No effect on MeSV infection compared with control cells were observed for cells silenced for Ago1 nor Ago2 (Fig. 3.9.B). Taken together, although MeSV induces the production of virus-specific siRNAs, it seems that the siRNA pathway is unable to target MeSV successfully as knockdown of the siRNA pathway key protein Ago2 has no effect on MeSV infection. A possibility could be that MeSV interferes with the “antiviral” siRNA response either by directly expressing an RNA silencing suppressor or by indirect interaction, like decoy or hiding strategies. On the other hand, knockdown of Piwi proteins of the piRNA pathway results in an insignificant increase of MeSV infection, and no significant MeSV-specific piRNAs were produced (Fig. 3.9).

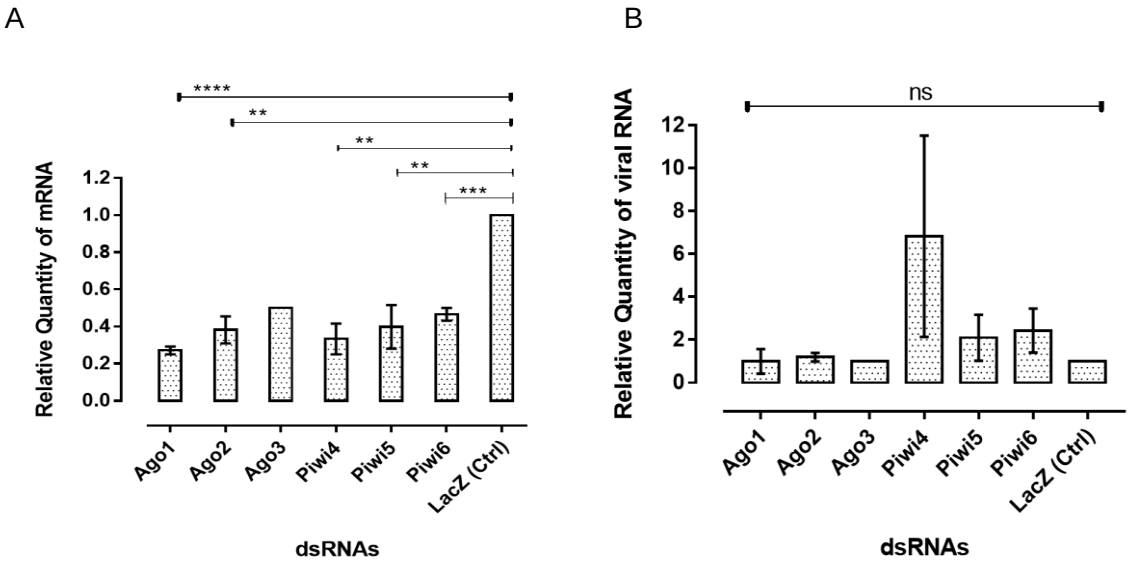

Fig. 3. 9: Antiviral activity of different RNAi pathways in *Aedes* cells against Mesonivirus infection.

Aedes aegypti-derived cell line (Aag2) were transfected with dsRNA specifically targeting key RNAi protein transcripts (Ago1-3, Piwi4-5) or LacZ control. After 24 h post transfection, the cells were infected with MeSV (MOI=0.1). RNA was isolated at 48 h post infection and RNA levels of key RNAi protein transcripts (**A**) and MeSV (**B**) determined with the deltadelta Ct method usinig *Aedes aegytpi* S7 as housekeeper and LacZ-specific dsRNA transfected cells as control treatment. Mean values of three independent experiments in duplo are shown with SEM. Statistics via unpaired t-test (*P<0.05: **=0.0010, 0.0013, 0.0068, ***=0.0001, ****<0.0001).

3.2.1.1.3 Induced silencing of Mesonivirus

Previous research on SFV in mosquito cells has shown that genome areas with siRNA hot spots (high production of siRNAs) are not well accessible by the RNAi response in contrast to cold spots (low production of siRNAs) (Siu et al. 2011). This was seen as a viral decoy strategy to ensure successful viral infection even in the presence of the antiviral RNAi response. Since MeSV-specific small RNAs were positioned only at the 5' end of the MeSV genome, a similar decoy strategy might be suggested for MeSV. To verify this, the ability to exogenously silence MeSV in Aag2 cells using different synthetic dsRNA inducer molecules was investigated. Therefore, sequence specific dsRNA (with a similar length) was produced from the 5' end, the Middle, and the 3' end of the MeSV genome. Following, the ability of these different dsRNAs to silence MeSV infection in Aag2 cells was determined. MeSV specific dsRNA (5` end, middle or 3` end) or control dsRNA (LacZ specific) was transfected 24 h prior to MeSV infection (MOI = 0.1), followed by RNA isolation and qRT-PCR for MeSV at 48 hpi. Biologically active siRNAs targeting

the MeSV genome should mediate the degradation of viral RNA and thus reduce the relative quantity of viral RNA. The results showed that targeting MeSV-specific dsRNAs to the 5' end, middle and 3' end of MeSV genome lead to a significant reduction in MeSV ($p<0.0001$) (Fig. 3.10). Thus, the overall ability of hot spot dsRNA mimics at the 5' end and cold spot dsRNA mimics at the middle and 3' end is significant to inhibit MeSV replication. To conclude, the hot spots at the 5' end are theoretically accessible by the RNAi response similar than the cold spots at the middle and 3´ end. There was therefore no decoy strategy by MeSV and the logical follow up experiment would be to check if MeSV instead was expressing an RNAi suppressor molecule.

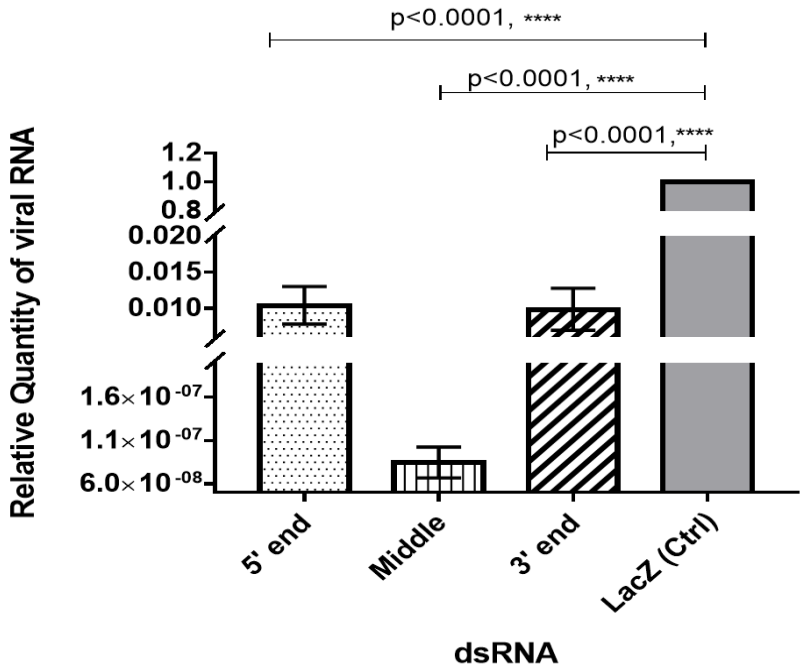

Fig. 3. 10: Effect of Mesonivirus-specific dsRNA silencing on MeSV replication.

Aedes aegypti-derived Aag2 cells were transfected with MeSV-specific dsRNA against the 5' end, Middle, and 3' end and subsequent infection with MeSV (MOI=0.1). After 48 hpi, changes in MeSV replication were measured using qRT-PCR for three independent experiments in triplicate. Values were normalized by the housekeeping gene ribosomal protein S7 and relative quantity of viral RNA was calculated using deltadelta CT method with cells transfected with dsLacZ as control group. Mean values with SEM are shown. Statistics via unpaired t-test (*p<0.05: **** = p<0.0001).

3.2.1.1.4 Viral suppression of siRNA response by Mesonivirus in *Aedes aegypti* cells

Instead of decoy strategies, viruses often encode proteins or molecules that directly interact and inhibit the antiviral RNAi response; so called viral RNAi suppressors (VSRs). To determine whether MeSV encodes a VSR, a luciferase reporter based assay was

performed in Aag2 cells. Therefore, MeSV or mock infected cells were used and constructs expressing Firefly luciferase (FFLuc) and Renilla luciferase (RLuc, as internal control) were co-transfected together with either siRNAs (Firefly luciferase specific, siFFLuc, or Hygromycin B specific, siHygB, as control) or dsRNAs (Firefly luciferase specific, dsFFLuc, or eGFP specific, dseGFP, as control). Luciferase activity was measured to assess the ability of the virus to prevent either dsRNA-mediated knockdown or siRNA-mediated knockdown of the target mRNA (Firefly luciferase) in Aag2 cells.

During virus infection viral siRNAs (vsiRNAs) are produced from long dsRNAs by Dcr-2-mediated cleavage, thus it is important to control whether MeSV could affect dsRNA processing into functional siRNAs. The luciferase reporter assay using dsRNA as inducer molecule, therefore assessed the ability of MeSV to interfere with Dcr-2-dependent silencing. The knockdown showed at least 50% successful silencing efficiency in control cells. The presence of MeSV in the cells resulted in an increase of luciferase compared to mock infected cells, although not significant. In other words, the silencing efficiency of FFLuc-specific dsRNAs was reduced in the presence of MeSV (Fig. 3.11.A).

The siRNA-based mediated knockdown experimental set-up, on the other hand assessed the ability of MeSV to interfere with the silencing response downstream of Dcr-2 activity. Again, at least 50% successful silencing efficiency was achieved in control cells. Increased levels of FFLuc were observed in MeSV infected cells transfected with siFFLuc compared to mock infected cells. Hence, the presence of MeSV significantly affects the efficiency of siRNA-based silencing of FFluc in Aag2 cells Fig. 3.11.B).

Taken together, MeSV interferes with siRNA-mediated silencing in Aag2 cells and shows a similar trend for dsRNA-mediated silencing; supporting the presence of a RNAi suppressor molecule encoded by MeSV. MeSV's suppression was not at the dsRNA, Dcr-2, and the siRNA production sites. Likely, it could be from or after the RISC complex formation with regards to the exogenous siRNA pathway.

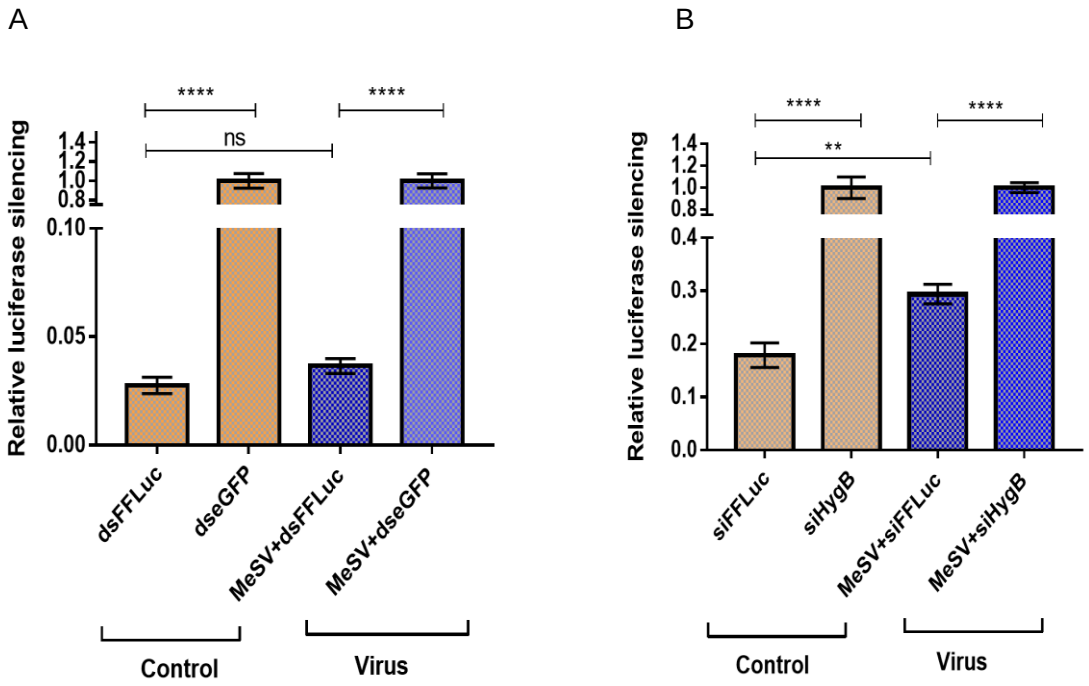

Fig. 3. 11: Mesonivirus suppression of siRNA silencing response in *Aedes aegypti* cells

Aedes aegypti-derived Aag2 cells were inoculated with MeSV (MOI=1). 24 h post infection, the cells were co-transfected with FFLuc and Rluc expressing reporter plasmids (pIZ-Fluc and pAcIE1-Rluc as internal control), dsRNAs targeting FFLuc (dsFFLuc) or dseGFP (control) **[A]**, or alternatively siRNAs against FFLuc (siFFLuc) or Hygromycin B (siHygB) as control **[B]**. Cells were lysed at 24 h post transfection and relative luciferase (FFluc/ Rluc) normalized to control cells (dseGFP or siHygB, respectively) was determined. The mean values with standard error are shown for three independent experiments conducted in triplicate. Mean values with SEM are shown. Statistics via student unpaired t-test (*p<0.05: **=p<0.0026, 0.0013; **** = p<0.0001); ns = No significant difference.

3.2.2 In vivo interaction of Mesonivirus with the mosquito host

After extensive research on the MeSV interaction with the mosquito host in vitro, in vivo experiments were performed to determine the ability to transfer the in vitro results to whole mosquitoes. This includes infection kinetics in *Culex* mosquitoes and small RNA sequencing profiling of MeSV-infected mosquitoes.

3.2.2.1 Microinjections of *Culex* mosquitoes with Mesonivirus

Culex quinquefasciatus and *Culex pipiens molestus* mosquitoes were used for the in vivo investigation. MeSV is known to infect a broad range of mosquitoes including *Culex* mosquitoes. To investigate if this is true for our isolate, MeSV infection was initiated in

Cx.quinquefasciatus and *Cx. pipiens molestus* mosquitoes by intrathoracic microinjection, followed by MeSV detection. Approximately 10^3 PFU of MeSV was injected into each mosquito, survivors at each time point (3, 7, 14 dpi) were homogenized, and RNA extracted using TRIzol LS. Total, 30 mosquitoes were injected for each time point. RNA was used as the PCR template for MeSV detection via qRT-PCR using primer labels 439 and 440.

For MeSV injectecd *Cx. quinquefasciatus* mosquitoes, 5 were positive out of 28 survivals (17.86 % infection rate) at 3 dpi. At 7 dpi, 3 positive MeSV mosquitoes were recorded out of 30 (10 % infection rate). Lastly, Out of 22 mosquitoes, MeSV was detected in 5 (22.73 % infection rate) at 14 dpi. The infection rate declined after 3 dpi and increased again after 7 dpi (Fig. 3.12).

Regarding MeSV injected *Cx. pipiens molestus* mosquitoes, 13 were positive out of 30 survivals (43.33 % infection rate) at 3 dpi. At 7 dpi, 16 positive MeSV mosquitoes were recorded out of 24 (66.67 % infection rate). Lastly, Out of 21 mosquitoes, MeSV was detected in 1 (4.76 % infection rate) at 14 dpi (Fig. 3.12). The infection rate increased after 3dpi and declined after 7dpi in contrast to *Cx. quinquefasciatus* mosquitoes. Infection rate is rather low for both mosquito species at 14 dpi. Therefore, it is doubtful if interaction experiments could easily be performed with arboviruses in vivo. Although, the infection rate of *Cx. pipiens molestus* at 7 dpi could be sufficient for onward experiment, a vector control strategy needs the virus to keep a high infection rate at later time points. Usually, 100 % infection rates were recorded after injection with other tested viruses (Abbo et al. 2020; Romo et al. 2018).

Taken together, the results showed that *Cx. pipiens molestus* mosquitoes had high infection rate compared to *Cx. quinquefasciatus* mosquito counterparts. Therefore, *Cx. pipiens molestus* looks promising for onward USUV coinfection experiments (Fig. 3.12).

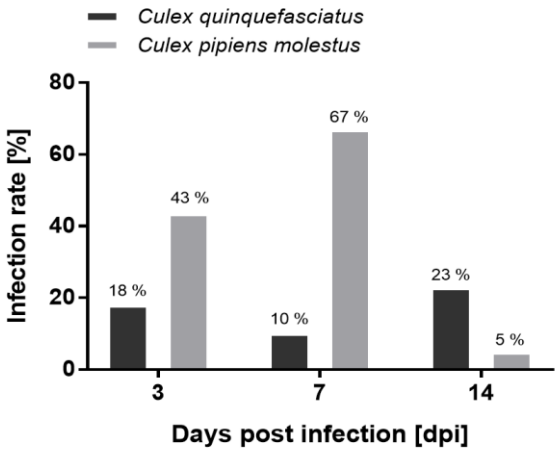

Fig. 3. 12: Infection rate of Mesonivirus in *Culex* mosquitoes

Culex quinquefasciatus and *Culex pipiens molestus* mosquitoes were inoculated with MeSV. Surving mosquitoes at each time point (3, 7, and 14 dpi) were harvested, homogenized, and RNA extracted using TRIzol LS. SYBR Green qRT-PCR was performed to detect MeSV at the various time points. Infection rates at various time points indicated above respective bars.

3.2.2.2 Small RNA sequencing of *Culex quinquefasciatus* mosquitoes

Having characterized MeSV-derived small RNAs in vitro, it important to also understand the production of small RNAs in vivo using related mosquito vector. Literature revealed that, results from in vitro experiments can be replicated to produce equivalent results in vivo. Therefore, I hypothesized that MeSV microinjected *Culex quinquefasciatus* mosquitoes could also produce high concentration (hot spot) of small RNAs at the 5' end and the unique distribution of small RNAs of the MeSV genome as found in mosquito-derived cells. *Cx. quinquefasciatus* mosquitoes were chosen because they were the ones available for microinjection at the time and known vector for MeSV. To investigate this, MeSV confirmed *Cx. quinquefasciatus* mosquitoes at 5 dpi via qRT-PCR were chosen. Two *Cx. quinquefasciatus* mosquitoes were pooled and homogenized, RNA was isolated using TRIzol LS and sent for small RNA deep sequencing.

The amount of MeSV-specific small RNAs in the size of 21 nts in the infected mosquitoes is rather low, making up <1 % of all clean small RNA reads (supplementary material Table 6.4.B). Small RNAs in the size of siRNAs (21 nts) were elicited at the sense and antisense orientations of the MeSV genome but greater at the sense orientation (Fig. 3.13.A). Mapping of the 21 nts MeSV-specific small RNAs showed that they distribute along the whole genome/antigenome of MeSV (Fig. 3.13.B, Left panel) unlike the results in cells. Taking the results together, MeSV-specific small RNAs (siRNA) were produced in the MeSV infected *Cx. quinquefasciatus* mosquitoes (Fig. 3.13), but showing a different mapping to infected cells.

To understand the infection rate results (Fig. 3.12) in more detail, small RNA sequencing was also performed from MeSV injected *Cx. quinquefasciatus* with a negative MeSV detection by RT-PCR. It could either be that the injected virus is unable to infect the mosquito at all or is cleared by the mosquito after initial replication. In case of initial replication, MeSV-specific siRNAs mapping to the genome and antigenome would be expected. The injected, MeSV-negative *Cx. quinquefasciatus* mosquitoes produced insignificant amount of siRNAs (21 nts), but other small RNAs (mapping to the genome and antigenome) were generated in the size of 18-20 nts at the 5' end (Fig. 3.13.A, Right panel). This results suggest that MeSV is unable to replicate in this injected mosquitoes or only to a very low level. The reason for the presence of smaller RNAs from the genome and antigenome is not known at this point. Follow up experiments would be needed to determine if these could represent the leader small RNAs of the subgenomic RNAs of MeSV.

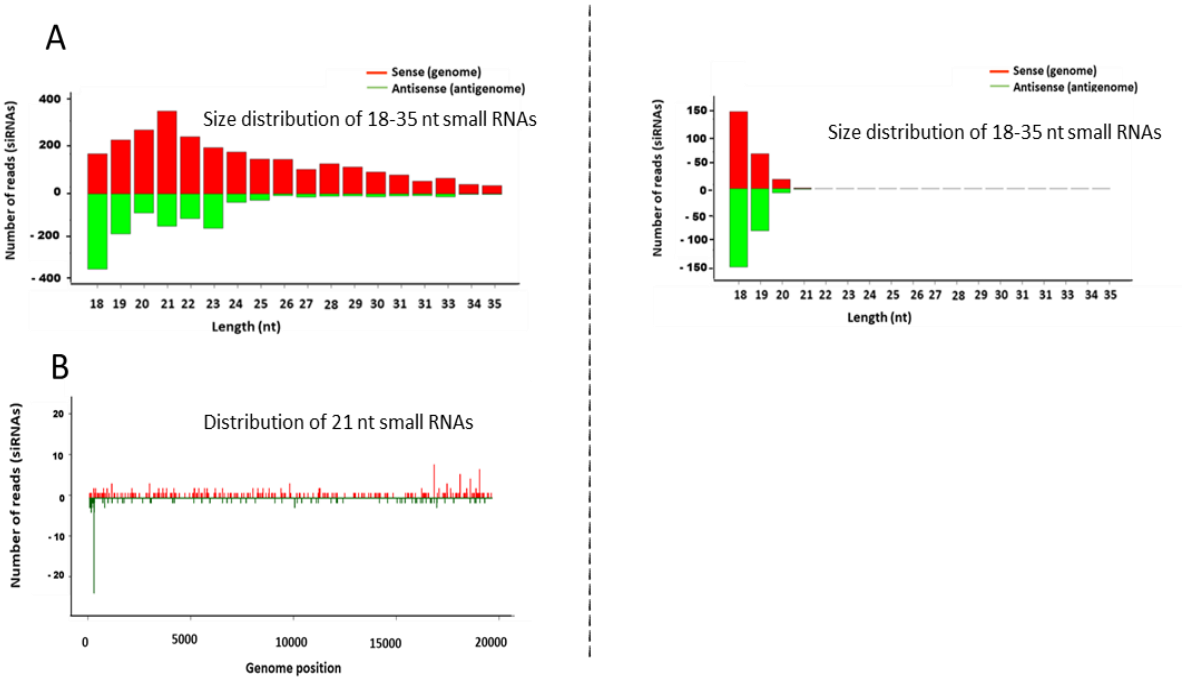

Fig. 3. 13: Characteristics of Mesonivirus-derived small RNAs in *Culex quinquefasciatus* mosquitoes.

RNA was isolated from *Culex quinquefasciatus* mosquitoes injected with MeSV, either MeSV positive (Left Panel) or negative (Right Panel), followed by small RNA sequencing. **[A]** Size distribution 18-35 nt of small RNAs from MeSV infected mosquitoes mapped to the to MeSV genome (+, red) and antigenome (-, green). **[B]** Genome distribution of 21 nt small RNAs that map to the genome of MeSV (red) and antigenome (green). Frequency of small RNAs (y-axis) mapped to corresponding nucleotide location (x-axis).

3.3 Identification of other mosquito-specific viruses

During my laboratory investigations, I have observed that I could not detect MeSV in Hsu and Ct cells at certain times, although CPEs were confirmed with $TCID_{50}$. A possible explanation could be that other persistently infecting MSVs present in the cells could interfere with MeSV and/or change their characteristics (for example: resulting in CPE). Little was known regarding persistent MSV infections in Ct cells at the beginning of my thesis project. In contrast, MSVs persistently infecting Aag2 cells (CFAV, PCLV) were already well characterized. However, cross-contamination of MSVs in cell culture have been previously reported (Geisler and Jarvis 2018). Therefore, small RNA sequencing results of Aag2 and Ct cells infected with MeSV (Section 3.2.1.1.1) were also used to identify and verify MSV infections in these cell lines. Moreover, MSV infection in Hsu and Ct cells were reported by others during the course of my project (Göertz, Miesen, et al. 2019; Rückert et al. 2019).

3.3.1 Unbiased identification of mosquito-specific viruses by small RNA sequencing

Virus specific small RNAs normally map along most of the genome and antigenome in insects and therefore they can be used for contig assembly and virus discovery (Franzke et al. 2018). Using the previously described bioinformatics pipline, small RNAs from MeSV infected Aag2 and Ct cells were used for unbiased virus discovery (Franzke et al. 2018). The assembly of small RNA reads revealed the presence of other MSVs in MeSV acutely infected Aag2 and Ct cells. Viruses were identified in case the small RNAs covered at least 70 % of the viral genome/ antigenome (Franzke et al. 2018). Therefore, the following viruses were identified: Phasi Charoen-like virus (PCLV), Calbertado virus (CALBOV), Culex Narnavirus 1 (CxNV1), and Cell fusing agent virus (CFAV). CFAV and PCLV were identified in Aag2 cells. In Ct cells CxNV1, CALBOV, and PCLV were identified. The identified viruses belong to the following families: *Flaviviridae*, *Narnaviridae*, and *Phenuviridae* (Table 3.1). The identified MSVs in Aag2 cells correspond with previous findings showing persistent infections of PCLV and CFAV in Aag2 cells (Franzke et al. 2018; Schnettler et al. 2016). During the course of the project, other groups (Göertz, Miesen, et al. 2019; Rückert et al. 2019) have reported the same persistent MSV infections in Ct cells and virus specific small RNA production of these viruses. This strongly supports the finding that the identified MSV infections are present in Ct cells and not due to cross-contaminations. Finally, the small RNA sequence data were confirmed with RT-PCR using supernatants of MeSV growth kinetics in these cell lines as illustrated below (Fi. 3.14A).

Table 3. 1: Mosquito-specific viruses identified by the assembly of small RNA reads.

BLAST-hit was retrieved for the listed viruses. The genomic size and percentage of nucleotide identity between the query and the subject sequence.

Virus	Family	Cell line	Accession #	% Identity
Cell fusing agent virus (CFAV)	*Flaviviridae*	Aag2	KU936054.1	91
Culex Narnavirus 1 (CxNV1)	*Narnaviridae*	Ct	MK628543.1	95
Calbertado virus (CALBOV)	*Flaviviridae*	Ct	KX669689.1	77
Phasi Charoen-like virus (PCLV)	*Phenuviridae*	Aag2	MH310079.1	98
		Ct	NC_038263.1	78

3.3.2 Identification of additional mosquito-specific viruses in Mesonivirus growth kinetics

This was done to confirm the presence of the known MSVs in the Ct and Hsu cell lines used in the MeSV growth kinetics and to determine their infection kinetics during an acute MeSV infection. The supernatants of the MeSV growth kinetics were analyzed for the presence of the known persistent infecting MSVs via RT-PCR and qRT-PCR with virus specific primers. The results revealed that Ct cells in culture and those used for the MeSV growth kinetics showed the presence of CALBOV, FHV, CxNV1, and PCLV. On the other hand, Hsu cells in culture and those used for the MeSV growth kinetics revealed the presence of Merida virus (Fig. 3.14.A.) as previously reported (Weger-Lucarelli et al. 2018). All these viruses were detected both in the MeSV kinetics and the relative noninfected control cells. An increase in the MSVs replication over time in the MeSV growth kinetics was demonstrated (Fig. 3.14.B). Non-MeSV infected Hsu and Ct cells did not show CPEs on *Aedes albopictus*-derived C6/36 cells (supplementary material Fig. 6.3). Therefore the persistent infected MSV seems not to produce CPE or their virus production was so low that we did not see the CPE. Taken together, the data suggest that any effects seen in the growth kinetics of MeSV were due to the presence of acutely infecting MeSV.

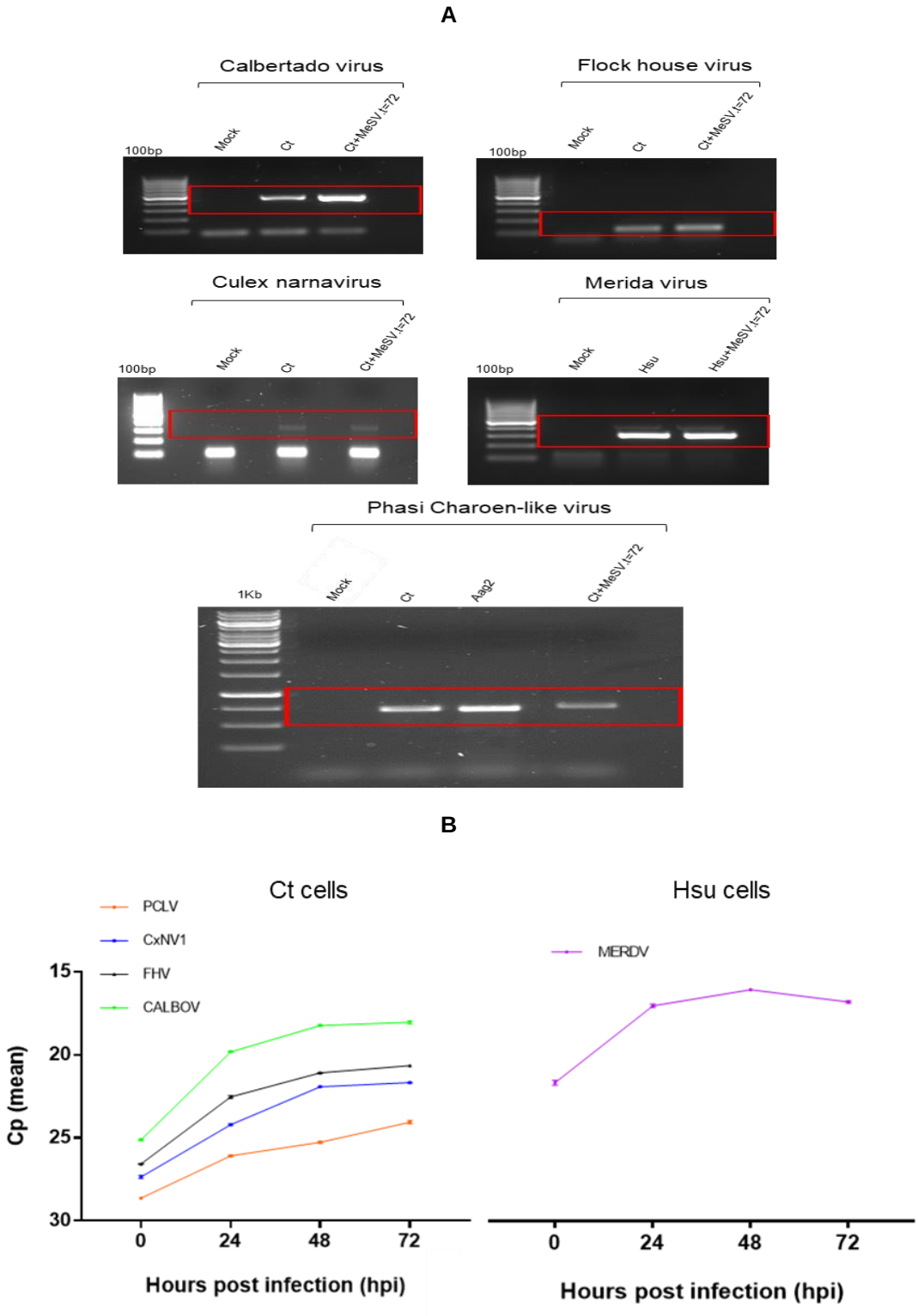

Fig. 3. 14: Detection of other mosquito-specific viruses in Mesonivirus growth kinetics in *Culex* cells

Supernantants of MeSV growth kinetics at 72 hpi in *Culex quinquefasciatus*-derived Hsu and *Culex tarsalis*-derived Ct cells were used. Apart from using MeSV growth kinetics in Hsu cells for MERDV detection, MeSV

growth kinetics in Ct cells were used for the detection of other viruses. RNA was extracted from the supernatants and used as template for the PCRs. MeSV detection performed via RT-PCR **[A]**. Mosquito-specific virus detection in MeSV growth kinetics at different time points (0, 24, 48, 72 hpi) via qRT-PCR for a single experiment in duplo **[B].** Mock = water as negative control, PCLV = Phasi Charoen-like virus, CxNV1 = Culex narnavirus, FHV = Flock house virus, CALBOV = Calbertado virus, MERDV = Merida virus. *Aedes aegypti*-derived Aag2 cells = positive control for PCLV.

3.4 Prevalence and phylogeny of Mesonivirus 8345

The MeSV strain used in the study was isolated from *Coquilletidia richiardii* mosquito at the Bernhard Nocht Institute for Tropical Medicine (BNITM). However, previous analysis by Dr. Börstler showed a high prevalence of mesoniviruses in mosquitoes collected in Germany (Börstler 2016). To determine if this is is generally seen in Germany or was specific to the previously investigated collection years, the prevalence of mesoniviruses in *Culex* mosquitoes collected in Germany and the phylogeny of MeSV 8345 were investigated. MeSV was detected in a few mosquito samples investigated and and the phylogeny revealed that the MeSV 8345 strain clustered with Yichang virus from China.

3.4.1 Prevalence of Mesoniviruses

MeSV has been identified in a variety of mosquito species all over the world. Between 2013 and 2015, Dr. Börstler during her PhD collected mosquitoes from Germany within the German arbovirus surveillance program and screened them for mesoniviruses. She collected a total of 234,869 mosquito specimens, screened 2,141 mosquito pools for different mosquito species, and 935 (43.7 %) different mosquito species were positive for MeSV (Börstler 2016). To follow up on this previous findings regarding the prevelance of MeSV in german mosquitoes, screening of a pool of 300 mosquito larvae collected in Germany in the years 2016 - 2018 were performed. Unfortunetly, only DNA samples were available for the above mentioned samples. As MeSV is an RNA virus it could be difficult to detect them in DNA samples however previous laboratory experience (Prof. Dr. Esther Schnettler personal communication, BNITM) has successfully detected RNA viruses as RNA contamination in DNA samples. Out of 300, 5 were positive for MeSV (1.7% prevalence) and were *Culex torrentium* (2) and *Culex pipiens pipiens* (3). Previously, 43.7% prevalence of MeSV was determined in mosquitoes collected from 2013 to 2015; using RNA as starting material (Börstler 2016). The low infection rate suggests a major difference in MeSV infection prevalence in mosquitoes between the two different time periods. However, other reasons are more likely, including the different numbers of samples analyzed, the sample type (DNA versus RNA), and thereby a possibly low amount of RNA in the DNA samples. It also has to be noticed that, it was not possible to

verify the MeSV sequence in the 5 positive samples, as sequencing the MeSV amplicons was unsuccessful due to the low DNA concentration.

3.4.2 Phylogeny of Mesonivirus 8345

Mesoniviridae is a recently established family comprising a group of positive-sense, single stranded RNA (+ssRNA) insect viruses (Lauber et al. 2012).The phylogeography of several mesoniviruses were previously investigated to understand their evolutionary relationships (Vasilakis et al. 2014). Hence, the relatedness of the new MeSV 8345 strain to other mesoniviruses was investigated via phylogeny.

The initial genomic sequence of MeSV 8345 was provided by Dr Daniel Cadar of the Arbovirology group at the BNITM. He also performed the identification of the open reading frames (ORFs) using Geneious Prime (Biomatters, Inc.) bioinformatics software. The entitre genome of MeSV 8345 comprises 20,102 nucleotides (nts). Six ORFs; ORF1a-ORF1b-ORF2b-ORF2a-ORF3b-ORF3a in increasing order of nts of the genome were detected. The sequence for ORF1a and ORF1b were approximately 500 – 8500 and 8500 – 16200 nt respectively. There is an overlap between ORF2a and ORF2b as well as ORF3a and ORF3b (Fig. 3.15.A).

To determine the phylogenetic relationships of MeSV 8345, a maximum likelihood (ML) phylogenetic tree was constructed based on the alignments of ORF1ab with the conserved domains such as 3C-like serine protease ($3CL^{pro}$), RNA-dependent RNA polymerase (RdRp), and Zinc-binding helicase (ZnHel) (Fig. 3.15.B). ORF1ab was previously used in studies as its domains are highly conserved for *Mesoniviridae* (Vasilakis et al. 2014; Zirkel et al. 2013). The analysis exhibited a highly similar topologies and clustering within previsously described nidoviruses in the *Mesoniviridae* family (Example: Kadiweu virus, Ofaie virus, Meno virus, and Nse virus) (Fig. 3.15). Karang sari virus and Bontang virus were isolated from the same mosquito species (*Culex vishnui*) and clustered together in the tree (Fig. 3.15). On the other hand, Houston virus, Odorna virus, and Cavally virus were isolated from *Aedes* mosquitoes; this was illustrated by the tree with the formation of a specific clade. Overall, mesoniviruses used in the generation of the phylogenetic tree were from different mosquito species including *Culex*, *Aedes*, *Coquillettidia*, *Mansonia*, and *Uranotaenia* mosquitoes. Interesting, MeSV 8345 and Yichang isolates formed a monophyletic clade, although isolated from different mosquito species and different geographical areas (Fig. 3.15).

Fig. 3. 15: Genomic characterization of Mesonivirus 8345.

[A] Schematic diagram of organization of MeSV 8345 genome. The genome diagram was generated using Geneious Prime (Biomatters, Inc.) showing the six ORFs (Cadar et al, unpublished). **[B]** Phylogenetic tree of Mesonivirus 8345 including other mesoniviruses isolated from different mosquito species. The tree was constructed with protein domains of ORF1ab: 3CLpro-RdRp-HEL1 using Maximum-likelihood method with 1000 bootstrap replicates. Realignment was done using MAFFT 7 online version and the tree run on SeaView. The constructed tree is rooted to Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2).

4 Discussion

Mosquito-specific viruses (MSVs) do not replicate in vertebrate cells but persist in mosquito populations and are highly prevalent in nature. Prior infection with these viruses can regulate the infection and transmission of pathogenic arboviruses (Hall-Mendelin et al. 2016). Mesoniviruses constitute the only mosquito-associated genus in the order *Nidovirales* and are found in mosquito populations globally (Diagne et al. 2020). The global distribution of mesoniviruses make it possible for mosquito vectors of public health importance to be coinfected with disease causing arboviruses. Recently, MeSV 8345 strain was isolated at the BNITM from a *Coquilletidia richiardii* mosquito pool, collected in Germany. However, little is known about the interaction of MeSV with the mosquito host or arboviruses. Previous experimental investigations involving MSV and arboviruses have shown that MSVs have the potential to reduce, increase, or have no effect on the replication of the target arbovirus (Agboli et al. 2019). MeSV 8345 strain used in this study showed approximately 90 % identity to YCV (*Mesoniviridae*) isolated in China. During the thesis project, YCV was recently reported to significantly inhibit proliferation of DENV-2 and ZIKV, in cell culture, and reduce transmission rate of DENV-2 in *Aedes albopictus* mosquito (Ye et al. 2020). This is the only report showing the interference of mesoniviruses with arboviruses and was just published at the end of the thesis project.

Recent studies have shown that arbovirus replication and spread in mosquitoes is not inactively tolerated but prompts host reponses to control these pathogens (Donald et al. 2012). Antiviral RNAi is a typical example of these responses that is initiated in mosquitoes. RNAi is a sequence-specific knockdown response, which is divided into different pathways depending on the small RNA molecules (miRNA, siRNA, piRNA) involved (Kean et al. 2015; Leggewie and Schnettler 2018). Although, the exogenous siRNA pathway establishes an effective antiviral response in mosquitoes, vpiRNA has been involved in the response to arboviruses in *Aedes* mosquitoes (Rückert et al. 2019). There is no available information regarding induction of RNAi upon MeSV infection in vitro and the effect of this response on the vector competence of the mosquito. Therefore, understanding the interaction of MeSV with the mosquito host to induce RNAi is needed to add to the increasing knowledge regarding MSVs.

This study seeks to understand the tripartite interactions between the mosquito host, the mosquito-specific MeSV, and arboviruses. The interaction determines the effect of MeSV on replication of arboviruses and antiviral RNAi in the mosquito host. The experimental data were generated using mosquito-derived cell lines. To futher understand this newly isolated MeSV, a brief phylogeographical relationship with other mesoniviruses and its prevalence was also investigated and discussed in this study.

4.1 Mesonivirus interactions with coinfecting viruses

Generally, MSVs are known for their inability to replicate in mammalian (vertebrate) cells (Agboli et al. 2019; Wang et al. 2017). This is true for MeSV, as it was previously reported by Dr. Börstler in her PhD thesis, to be unable to replicate in vertebrate cell lines such as monkey (Vero E6) cells (Börstler 2016). Therefore, I did not investigate this in my experiments. Some MSVs have been reported to be very specific and only replicate in some mosquito-derived cells. For example, Parramatta River virus (PaRV) replicates only in *Aedes*-derived cells (McLean et al. 2015). It is not known if this is true for MeSV and previous work only shown the replication of MeSV in C6/36 and Aag2 cells (Börstler 2016). If there is no host restriction, C6/36 cells have the highest chance for MSV replication as they are defective in an important antiviral response, Dcr-2 of RNAi (Brackney et al. 2010). It is not surprising that MeSV replicated in C6/36 as it was the cell line used for the virus isolation. To check if MeSV is also able to infect *Culex*-derived cells, growth kinetics were performed in several cell lines.

Chosen mosquito-derived cell lines (*Aedes albopictus*-derived C6/36 cells, *Aedes aegypti*-derived Aag2 cells, *Culex tarsalis*-derived Ct cells, and *Culex quinquefasciatus*-derived Hsu cells) were used to monitor the replicative potential and the growth kinetics of MeSV at different time points. MeSV was able to grow and replicate in all the four different cell types (C6/36, Aag2, Ct, and Hsu). This supports the fact that mesoniviruses have a wide host range, and an expansive geographical distribution (Vasilakis et al. 2014). To the best of my knowledge, this is the first report showing the susceptibility of *Culex*-derived cells like Hsu and Ct cells to MeSV infection.

Virus growth variability, replicative potential, and CPE depends on the passage history of the virus, country of isolation, tissue tropism, and magnitude of viral replication (Blitvich and Firth 2015). MeSV caused CPEs in all the mosquito-derived cell lines especially in C6/36 cells where morphological changes were more noticeable during the initial infection. However, the extent of the CPE and the titer were independent of the mosquito-derived cell line. When the cell lines became persistently infected with MeSV, CPE was not noticeable anymore.

Replication potential of viruses are necessary to determine model cell lines for experimental designs to investigate MSV and arbovirus interactions. It was observed that *A. albopictus*-derived C6/36 cells had a higher growth support for MeSV and *Culex quinquefasciatus*-derived Hsu cells the least. This growth variability could be due to following reasons or a combination of them: (i) different cell tropism, (ii) different species tropism, and (iii) difference in antiviral response. To investigate the interaction of MeSV with arboviruses, specifically USUV, a *Culex*-derived cell line was chosen as USUV is known to be transmitted by *Culex* mosquitoes. The choice of *Culex* cells was confirmed recently where German *Culex pipiens* biotype *molestus* and *Culex torrentium* were found to be vector-competent for USUV (Holicki et al. 2020). This becomes a good choice as both

MeSV and USUV strains were isolated in Germany. Also, the MeSV 8345 strain from Germany is close to YCV which is also a mesonivirus isolated from *Culex* mosquitoes in China (Wang et al. 2017), revealed by alignment of the sequence at the NCBI database.

Granting that, *Culex* mosquitoes are good vectors for USUV, successful infection in *Culex*-derived cells had to be verified as in vitro and in vivo experiments cannot always be transferred. This is supported by the lack of successful USUV replication in Hsu cells in contrast to Ct and *Aedes*-derived cells. USUV could not replicate in Hsu cells, although initial infection was possible and this could be due to several reasons. For example: additional MSV infection, cell type and therefore receptors and host factors. Thus, Hsu cells were derived from the ovary of *Culex quinquefasciatus* mosquito (Hsu, Mao, and Cross 1970b) and known to contain an actively replicating persisting MERDV (Weger-Lucarelli et al. 2018). It is possible that the presence of MERDV inhibits or lowered the replication of USUV in Hsu cells. Taken together, MeSV generated high and low titers in C6/36 and Hsu cells respectively; this is similar for USUV.

Reproducible and consistent titer detection is critical for virus research. However, it is not achievable sometimes due to unknown factors. In the past, this was a regular problem for arboviruses of the flavivirus genus, including DENV and USUV (personal communication, Prof. Dr. Esther Schnettler, BNITIM, Germany; Prof. Dr. Gorben Pijlman, WUR, Netherlands). Initially, USUV 490 strain was used but later produced inconsistency in CPE via $TCID_{50}$ on Vero ATTC cells. In contrast, USUV 491 strain produced a consistent CPE in Vero ATTC cells. The reason for the inconsistency in CPE is unknown as both strains were isolated from Black bird (*Turdus merula*).

Infection experiments conducted to understand the effect of MeSV on the replication of different arboviruses in *Culex*-derived cells showed differences depending on the investigated arbovirus. Overall, either an inhibitory effect or no effect was observed. In case of SFV, no strong interference was observed, which could also be linked to the low infection kinetics of SFV in the used Hsu cells. The replication of SFV declined afer 24 hpi represented by a strong decrease in luciferase. Thus, Hsu cell does not seem to support the growth of SFV overtime. This suggests that Hsu cells are able to efficiently target and possibly clear SFV infections even in the absence of MeSV. In contrast, BUNV infection was successful overtime in Hsu cells and reduced effects by MeSV were only observed in case of persistent MeSV infections. This is in contrast to previous reports regarding MSV interference with arboviruses. Most studies so far have been performed by coinfections or sequential infections with arbovirus shortly after the MSV infection. Recent results investigating the effects of persistent PCLV infection in *Aedes aegypti*-derived cells on arbovirus infection showed no effects (Fredericks et al. 2019). Similar to the current finding, PCLV infection of CFAV-positive *Aedes albopictus* cells inhibited the growth of LACV, an orthobunyavirus (Schultz et al. 2018). However, it is not known which

of the two persistent viruses (PCLV or CFAV) are responsible for this interference. Interesting, USUV infection was reduced during co- and persistent infection of MeSV (at least significant at certain time points).

Until now, most studies investigating interference of MSVs with arbovirus infections focus on MSV and arbovirus belonging to the same virus family. Morevover, most studies have been focused on arboviruses belonging to the flaviviruses, like DENV, ZIKV, and WNV and the corresponding mosquito-specific flaviviruses. Mosquito-specific flaviviruses such as NHUV, CxFV, CFAV, and PCV have been investigated (Baidaliuk et al. 2019; Bolling et al. 2012a; Hobson-Peters et al. 2013; Romo et al. 2018). NHUV resulted in a significant reduction in WNV, SLEV, and JEV production (Kenney et al. 2014). Contrary, CFAV was able to increase the replication of DENV (Zhang et al. 2017). Taken together, inhibitory effects have often been observed regarding interference of MSV with arbovirus of the same virus family but not in all cases. In contrast, interaction and inhibition between viruses of different virus families were observed in the current study. The possible mechanisms of suppression could be due to a triggered innate immune response of the mosquito or competition for indispensable cellular factors. Although, MeSV inhibits the replication of USUV, it is only possible at certain time points. However, linear regression analyses yielded significant differences for the infection experimental set-ups with MeSV and USUV. This conforms to Bolling and colleagues' study where linear regression analysis involving WNV and CxFV showed a significant difference (Bolling et al. 2012b).

It would be important to verify the observed inhibitory effects of MeSV on arbovirus infection during mosquito infections in the future. So far, studies have shown that results from in vitro experiments are comparable to in vivo set-ups but this is not always the case (Agboli et al. 2019). For example NHUV respectively inhibits the replication of ZIKV in *Aedes aegypti* mosquitoes (Romo et al. 2018) and WNV in *Cx. quinquefasciatus* mosquitoes (Goenaga et al. 2015; Kenney et al. 2014). Also, CxFV infection in *Culex pipiens* mosquitoes did not inihibit the transmission potential of RVFV (Talavera et al. 2018). As mentioned earlier, the transmission rate of DENV-2 in *Ae. albopictus* mosquito was reduced by YCV (Ye et al. 2020). The only report so far involving mesoniviruses.

Taking the results together, it is revealing that the effect of MSV on replication of arboviruses is variable depending on the infecting MSV, the cell line or mosquito species, and the virus family. If the MSV and the target arbovirus belong to the same family or different families, the effect is often variable. Regarding mosquito infection experiments, the infection route and the mosquito species are factors that could determine the effect of MSVs on arbovirus replication. The use of MSVs as biological agents to control arbovirus infection is promising but more studies are needed in this regard.

4.2 Mesonivirus interactions with the mosquito host

MSVs have to interact closely with their mosquito hosts to be able to infect and cause persistent infection. This happens at different interfaces and an example is the antiviral RNAi response that the mosquito host mounts after encountering an MSV. RNAi is a biological process in which RNA molecules inhibit gene expression or translation, by neutralizing targeted RNA molecules.

Different factors need to be investigated if RNAi acts antiviral against MeSV in mosquitoes. Production of viral specific small RNAs prove the ability of the RNAi path-way to recognize MeSV infection. Moreover, the characteristics of the small RNAs can give information about which RNAi pathway might act antiviral. Small RNA sequencing of MeSV infected *Aedes aegypti*-derived Aag2 and *Culex tarsalis*-derived Ct Cells showed that MeSV-derived small RNAs were produced in both cell lines. The predominantly 21 nt MeSV-derived vsiRNAs mapped to the genome and antigenome showed siRNA production pathway with a dsRNA-inducer molecule (dsRNA replication intermediates). However, MeSV-specific small RNAs have a unique distribution as the small RNA profile revealed the majority of small RNAs produced at the 5' end of the MeSV genome/antigenome. In contrast, MeSV-injected *Culex quinquesfasciatus* mosquitoes with acute infection showed MeSV-derived small RNAs distributed along the whole length of the MeSV genome. Such distribution of vsiRNA across the genome and antigenome in different mosquito species (and derived cells) with a variety of arboviruses and some MSVs have been previously reported. In addition, it has been reported that the siRNA production looks totally different between virus infection in cells versus whole mosquitoes (Brackney et al. 2010, 2009; Hess et al. 2011; Morazzani et al. 2012; Myles et al. 2008; Myles, Morazzani, and Adelman 2009; Scott et al. 2010; Siu et al. 2011). Taken together, the strong bias for 21 nts production and distribution is very unique for MeSV.

Only a small number of MeSV-specific small RNAs were found in the size of piRNAs (24-30 nts) and none showed the ping-pong specific production signature (U1, A10 biases and overlap of sense and antisense piRNAs at 10 nts) in the cell lines (Aag2 and Ct) and *Cx. quinquefasciatus* mosquitoes. Although, vpiRNAs have been reported for a variety of viruses (arbovirus and MSVs) in mosquitoes and derived cells, recent findings suggest that their production is dependent on the combination of virus and mosquito species. Some virus families are better piRNA producers (*Bunyavirales*, alphaviruses) than others (e.g. flaviviruses) (Leggewie and Schnettler 2018; Liu et al. 2019; Varjak, Leggewie, et al. 2018). Many detailed analyses have shown significant differences in vpiRNA characteristics (some for example not showing all the ping-pong production characteristics). Flavivirus-specific piRNAs for example, in *Aedes aegypti* cells vpiRNAs of DENV and ZIKV can only be mapped to a few foci on the genome and antigenome and biases for A10 or U1 was lacking (Miesen, Ivens, et al. 2016; Scott et al. 2010; Varjak, Donald, et

al. 2017). As a few MeSV-specific piRNAs were identified in *Cx. quinquefasciatu*s mosquitoes without the ping-pong signature in the current study, more research is needed to characterize the vpiRNAs.

Antiviral activity has not yet been reported for vsiRNAs detected against MSVs (Lee et al. 2017; Schnettler et al. 2016; Scott et al. 2010). As production of virus-specific small RNAs only suggests an antiviral activity of the corresponding RNAi pathway, silencing experiments are needed. It is generally assumed that the siRNA pathway is the major antiviral response in mosquitoes, supported by the findings that Ago2 knockdown results in high viral increase for nearly all tested viruses so far (Liu et al. 2019). Interestingly, no significant increase of MeSV infection was observed in case of Ago2 knockdown, although MeSV-specific siRNAs were produced. Such lack of antiviral activity by Ago2 has until now only been reported for ZIKV (Varjak, Donald, et al. 2017), here, Dcr-2 activity is sufficient for the antiviral response. This opens the question, if the produced MeSV-specific siRNAs is not biological functional or maybe the dicing of MeSV RNA by Dcr-2 into vsiRNAs is already sufficient as antiviral response. Reasons why Ago2 silencing shows no increase in MeSV replication could be: (i) MeSV produces a strong RNAi suppressor and therefore does not care about vsiRNA or Ago2 activity, (ii) Produced siRNAs are not needed as Dcr-2 cutting is antiviral enough, (iii) Silencing efficiency is not enough and the resisting Ago2 is sufficient for the antiviral activity, (iii) Produced siRNAs are not biologically active/ incorporated in Ago2, and (iv) Produced siRNAs cannot target MeSV RNA (because the RNA is hidden or coated with a protein).

RNA-based decoy strategies have been reported for several arboviruses in mosquitoes, ensuring successful viral replication in the presence of the antiviral siRNA pathway. In case of SFV, genome areas with siRNA hot spots were shown not to be well accessible by the RNAi response unlike the cold spots (Siu et al. 2011). However, this is not the case for MeSV as dsRNA targeting the 5´end genome for siRNA hot spot were as efficient in targeting MeSV as dsRNA targeting the middle or 3´end of the MeSV genome.

Another possibility to ensure successful viral infection in mosquitoes would be the presence of an RNAi suppressor molecule. Arboviruses and MSVs are known to encode proteins that interfere with the antiviral RNAi response. Suppressor assay experiment for siRNA was done to investigate if MeSV produces RNAi suppressor and therefore does not care about siRNA or Ago2 activity. Fascinating, it came out that MeSV encodes a suppressor that interferes on the level of siRNAs. Similar to the current finding of MeSV, CYV (*Birnaviridae*) was previously shown to encode a suppressor protein VP3. This protein binds long dsRNA to prevent cleavage by Dcr-2 and binding of siRNA prevents incorporation into RISC (van Cleef et al. 2014). Taking the previous reports together, the main suppression activity by viruses so far is dsRNA or Dcr-2 binding. However, the suppressor for MeSV in the current study is unknown and yet to be investigated.

Contrary to the effect of Ago2 silencing, predominant MeSV infection increase was determined for Piwi4 (not significant due to high variations in the independent repeats). Further, the silencing effect of other components of the piRNA pathway proteins, Piwi5 and Piwi6, showed a trend of increase for MeSV (not significant). This effect of silencing of the piRNA proteins is surprising as no ping-pong-specific MeSV piRNA could be detected upon infection. However, even for viruses that produced piRNAs, no antiviral activity of Piwi5 and Piwi6 have been reported yet (Dietrich, Jansen, et al. 2017; Dietrich, Shi, et al. 2017; Varjak, Dietrich, et al. 2018). In contrast, for all tested viruses so far, Piwi4 was antiviral (Liu et al. 2019). However, the current study did not yield a significant effect of Piwi4. Additionally, Piwi4 could not yet be directly linked to vpiRNA production, but rather intermingles with proteins of the piRNA and siRNA pathway (Varjak, Maringer, et al. 2017).

Another pathway of the RNAi is the miRNA which was also investigated via Ago1 knockdown with a characteristic 21-22 nt length. The knockdown did not show antiviral activity. In contrast, reports revealed that knockdown of Ago1 yielded antiviral and proviral activity depending on the virus (Dietrich, Shi, et al. 2017). In contrast to arboviruses, little information is available on the effects on miRNA expression during MSV infections in mosquitoes. Similar to the current study, knockdown of Ago1 resulted in decreased virus infection for mosquito-specific flavivirus Parammatta River virus (Personal communication, Prof. Dr. Esther Schnettler, BNITM). Differential miRNA expression has also been reported upon blood feeding itself in addition to changes in miRNA expression upon virus infection. It is not known yet if these miRNA changes influence the initial virus infection (Fu, Dimopoulos, and Zhu 2017).

Taken together, MeSV interacts with the RNAi response in mosquitoes; however, differences to commonly found results (antiviral activity of Ago2, siRNA distribution) have been observed. Additional experiments are needed to determine if these differences are MeSV specific, MeSV-mosquito species specific or can be broadened to other mesoniviruses. Finally, to the best of my knowledge, very limited information is available about MSVs with regards to understanding the functionality of key RNAi proteins in the mosquito host. Therefore, it is worth mentioning that the current study is adding knowledge to bridge the gap.

4.2.1 Microinjection of *Culex* mosquitoes with Mesonivirus

Microinjection of *Culex pipiens molestus* mosquitoes resulted in a higher infection rate than that of *Culex quinquefasciatus* mosquitoes, albeit both rates were lower than expected as microinjection of viruses normally results in 100 % infection rate (Abbo et al. 2020; Göertz, van Bree, et al. 2019; Romo et al. 2018). In contrast, the newly identified Dianke (*Mesoniviridae*) showed high infection rates if infected by blood meal (Gaye et al. 2020). Blood meal feeding of MSVs is strongly artificial as MSVs are known to be

incapable of infecting and replicating in vertebrates. Moreover, blood feeding results in a variety of changes in the midgut, which can either support or inhibit virus uptake. However, it is not expected that this would increase infection rates for MeSV as it has been commonly shown in the past that microinjection gives higher infection rates compared to virus feeding, even in the case of arboviruses (Kent et al. 2010b; Kumar and Puttaraju 2012; Romo et al. 2018). Upon injection, the virus can directly start to replicate and disseminate and do not need to pass the midgut barrier, which is an important and known barrier strongly restricting virus infections (Danet et al. 2019; Franz et al. 2015). In this regard, *Cx. quinquefasciatus* mosquito is probably not a possible host for MeSV. In contrast, a recent study identified the highest prevalence of YCV (closely linked to MeSV) in *Cx. quinquefasciatus* (prevalence rate of 8.9 %) (Wang et al. 2017). Additional research is needed to determine if these differences are due to the different viruses (MeSV versus YCV) or the chosen infection route.

4.3 Identification of other mosquito-specific viruses

Studies have shown that laboratory mosquito cell lines and mosquito colonies can be persistently infected with MSVs without any CPE or affecting cell growth, but possible interference with experimental set-ups. Earlier reports proved that small RNAs can be used to identify persistent viral infection in mosquito-derived cells and mosquitoes (Franzke et al. 2018; Wu et al. 2010). Using this approach, several MSVs were identified in the used cell lines, some previously reported or recently reported during the course of the thesis by others (Phasi Charoen-like virus, PCLV; Cell Fusing Agent virus, CFAV; Flock House virus, FHV; Calbertado virus, CALBOV; and Culex Narnavirus 1, CxNV1) (Göertz, Miesen, et al. 2019; Maringer et al. 2017; Rückert et al. 2019; Scott et al. 2010). PCLV and CFAV were found in the Aag2 cells while CALBOV, CxNV1, and PCLV were identified in Ct cells. The small RNA sequencing results were confirmed with RT-PCR, proving actively replicating persistent infection of theses viruses. Interestingly, there are additional studies of invertebrate cell culture systems depicting persistent infection with MSVs or insect-specific viruses (Bell-Sakyi and Attoui 2016; Greninger 2018; Webster et al. 2015; Wu et al. 2010).

Several studies involved the use of MSVs and some were done to see the effect of MSVs on the replication of arboviruses. Therefore, this knowledge is vital and should be considered when designing experiments and anlysing data from these immune-competent mosquito cell lines which seem to harbour MSVs. Follow up experiments are needed to determine possible interference of the present MSVs in the used cell lines regarding MeSV and arbovirus infections.

4.4 Prevalence and phylogeny of Mesonivirus 8345

Mosquito larvae were collected in Germany during the years 2016-2018 and were taxonomically justified to be *Culex* mosquitoes by the Arbovirology group of the BNITM. The prevalence of mesoniviruses in these samples was 1.7 % (5 out of 300 DNA samples). This is lower than expected as previous research (Dr. Jessica Börstler PhD thesis, UHH,BNITM) showed a 43.7% prevalence of MeSV from adult female mosquitoes (935 out of 2,141 mosquito pools, RNA samples) collected during the years 2013-2015 (Börstler 2016). Wang and colleagues reported that the prevalence of Yichang virus (*Mesonivridae*) in adult female mosquitoes caught in 2014 in China (Hubei) was 16.5 % (35 out of 212 pools, RNA samples) (Wang et al. 2017). This highlights the fact that the prevalence of MeSV infection in mosquitoes can vary. In Wang and colleagues' report, the virus was mostly detected in *Culex* mosquitoes especially *Cx. quinquefasciatus* (prevalence rate of 8.96 %) (Wang et al. 2017) which conforms to the current study but with a low prevalence rate. The difference in the prevalence rates could be due to the sample type and the total number of samples used in the various studies. As little is known regarding the transmission of MeSV, investigating larvae versus adults can have a strong effect on the prevalence in case the virus is mostly transmitted horizontally between adult mosquitoes. Taking the results together with previous reports in relation to the mosquito host, it is worth to know that MeSV was identified in several different species of mosquitoes (For example; *Coquilletidia sp*, *Ochlerotatus sp*, *Anopheles sp*, *Aedes sp*, and *Culex sp*).

The ORF1ab phylogenetic tree indicated that MeSV 8345 strain had a close relationship with the Yichang virus (Wang et al. 2017). The ORF1ab used comprised 3CLpro, spike protein, and RdRp domains which are conserved in the *Mesoniviridae* family. Interestingly, Yichang virus and MeSV 8345 were isolated from different mosquitoes; namely, *Culex* sp and *Coquilletidia richiardii* respectively. However, the phylogenetic tree illustrates that the clustering of the mesoniviruses is independent of their mosquito hosts. Finally, it is difficult to conclude that the new MeSV 8345 strain constitutes a new species as there are outstanding species demarcation criteria which were previously employed (Lauber et al. 2012). Therefore, future work is needed to further characterize MeSV 8345 in an evolutionary perspective.

4.5 Conclusion

The focus of this work was to investigate the tripartite interaction between mosquito host, mosquito-specific MeSV, and arboviruses. A prevalence of 1.7 % was reported in field caught mosquitoes for mesoniviruses and the genome of MeSV 8345 strain was 20, 102 nucleotides with six ORFs which is similar to other members of the *Mesoniviridae* family. Furthermore, the phylogeny of the MeSV 8345 strain illustrates a clustering and similarity

with Yichang virus. The characterization of USUV and MeSV in cell lines showed that different mammalian and invertebrate cell lines were susceptible to USUV and MeSV infections. Interaction of MeSV with arboviruses caused an inhibition in the replication of USUV (*Flaviridae*) in both acute and persistent infections unlike SFV (*Togaviridae*). However, the replication of BUNV (*Peribunyaviridae*) was hindered by the presence of MeSV in the persistent infection unlike the acute infection. The significance of the MeSV effect on USUV replication was variable at various time points. However, regression analyses proved the overall significance of the infection experiment. Regarding the reporter viruses, BUNV replicates better than SFV at the presence of MeSV. Overall, the infection experiments discovered that the effect of MeSV is dependent on the family of the target arbovirus. This is the first report trying to understand the effect of MeSV on these main arbovirus families. However, several other studies reported effects of MSVs on arbovirus replication and different observations were made. In these previous studies, MSV either inhibits, increases, or has no effect on the replication of the target arbovirus.

The RNAi pathway is the major innate antiviral response mosquito vectors use to restrict arbovirus infections and the exogenous siRNA pathway constitutes the most efficient antiviral response. Antiviral RNAi response was induced upon MeSV infection in *Aedes aegypti*-derived Aag2 cell line, *Culex tarsalis*-derived Ct cell line, and *Culex quinquefasciatus* mosquitoes as shown via small RNA deep sequencing data. The induction produces vsiRNAs as the major small RNA molecules and a few small RNAs in the size of piRNAs (24-30 nt) without ping-pong amplification features. A hot spot of small RNAs were produced at the 5’ end of the MeSV genome, a unique distribution exhibited by MeSV as most other virus studied revealed distribution of small RNAs across the whole genome/ antigenome of the infecting virus. The study looks further to understand the functionality of the induced small RNAs in *Aedes aegypti*-derived cells via knockdown of key RNAi proteins. Interestingly, although MeSV-specific siRNAs were elicited via the small RNA profiling, knockdown of the key siRNA pathway, Ago2, had no effect on MeSV infection; suggesting the inability of the produced siRNAs to target MeSV effectively. Fascinating, it was found that MeSV was expressing an RNAi suppressor protein making it difficult for the siRNA pathway to target MeSV, although the siRNA pathway is generally seen as the most important antiviral response in mosquitoes. On the other hand, targeting different areas of the MeSV genome, by induced RNAi, using dsRNA molecules, was successful; proving the general ability of siRNAs to silence MeSV. This is the first study investigating the antiviral RNAi response in mosquitoes against a mesonivirus and only a handful of studies investigating the interaction of the antiviral RNAi response and MSVs in general. Besides, using the virus-produced small RNAs for virus discovery, persistent infections in *Culex tarsalis*-derived Ct cells and *Aedes aegypti*-derived Aag2 cells of several MSVs were discovered. The same MSVs were either previously (Aag2 cells) or over the course of the project reported by others (Ct cells), supporting the persistent infections in the cell lines.

Taken together, this study widens our understanding of the complex interactions of MSVs, the mosquito hosts, and arboviruses. Due to diverse interactions of MeSV and MSVs in general with arboviruses, they therefore have the potential to be used as disease control agents in vector populations. Indeed, more studies are needed for a better understanding of MSV or arthropod-restricted viruses' diversity and prevalence.

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6 Supplementary Material

6.1 Infection efficiency of Mesonivirus in Hsu cells

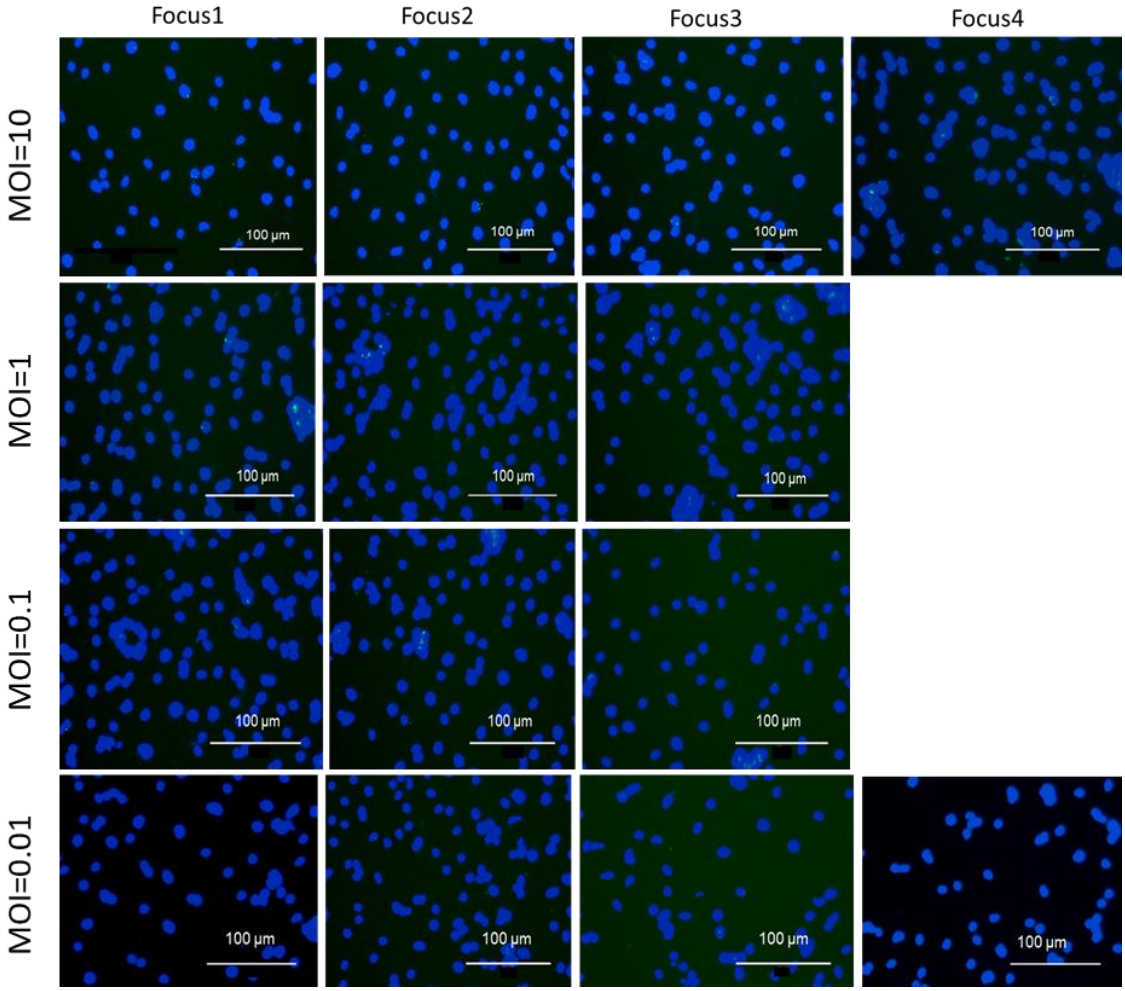


Fig. 6. 1: Estimation of infection efficiency of Mesonivirus in *Culex* cells

Culex quinquefasciatus-derived Hsu cells were infected with MeSV at differnt MOIs (10, 1, 0.1, 0.01). MeSV detected at 4 dpi using a monoclonal antibody detecting dsRNA and an Alexa 488 conjugated anti-mouse secondary antibody (green) as well as DAPI to stain the nucleus (blue). At each MOI, randomly selected sections of the well were focused. Percentage infection efficiency was calculated based on the total number of cells and the infected cells. Scale bars are 100 µm.

6.2 Interaction of Mesonivirus with Usutu virus

Table 6. 1: Growth kinetics of Mesonivirus persistent cells coinfected with USUV and acute coinfection of Mesonivirus with USUV using Ct cells.

Culex tarsalis-derived Ct cells were infected singly with USUV (Ct+USUV) in tandem with **[A]** MeSV persistently infected Ct cells (Ct+pMeSV+USUV) and **[B]** coinfection with MeSV and USUV (Ct+MeSV+USUV). MOI 10=USUV; MOI 0.1=MeSV. Supernatants were collected for USUV titration via $TCID_{50}$ at 0, 6, 10, 24, 48 and 72 hpi for three independent experiments in triplicate. Mean values with SEM are shown. Statistics via unpaired t-test; $p<0.05$ is significant.

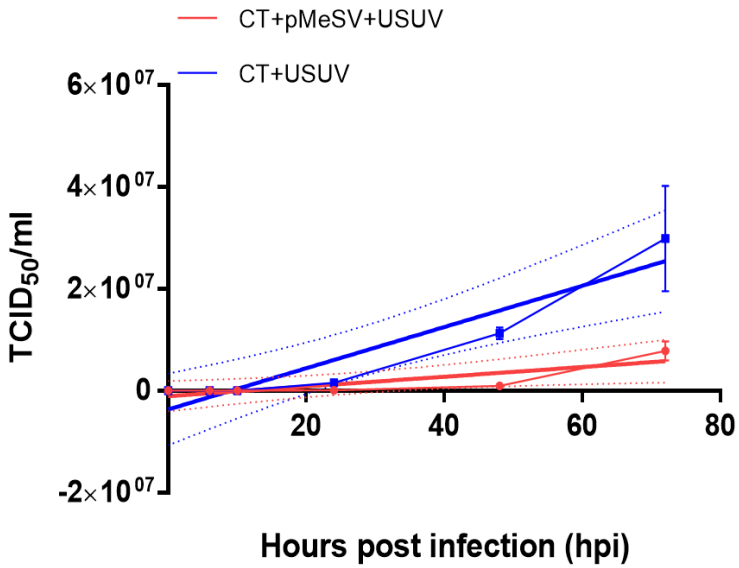
A

Hour post infection	$TCID_{50}$/ml (Mean ± SEM)		p-value
	Ct+pMeSV+USUV	Ct+USUV	
0	163.6 ± 26.51	488.4 ± 153.8	0.0538
6	1394 ± 281	3314 ± 1202	0.1395
10	2721 ± 532.2	4762 ± 1560	0.2335
24	118732 ± 30264	1562704 ± 149170	<0.0001 ****
48	954739 ± 206552	11322761 ± 1138712	<0.0001 ****
72	7837308 ± 1890528	29881322 ± 10349251	0.0524

B

Hour post infection	$TCID_{50}$/ml (Mean ± SEM)		p-value
	Ct+MeSV+USUV	Ct+USUV	
0	1073 ± 254	5921 ± 2785	0.1022
6	9617 ± 2364	37202 ± 17950	0.1471
10	22337 ± 3949	58764 ± 27961	0.2154
24	884887 ± 110444	1356194 ± 431110	0.3053
48	1824799 ± 245038	10640454 ± 1940602	0.0004 ***
72	4141700 ± 660928	17199652 ± 2695009	0.0002 ***

A



B

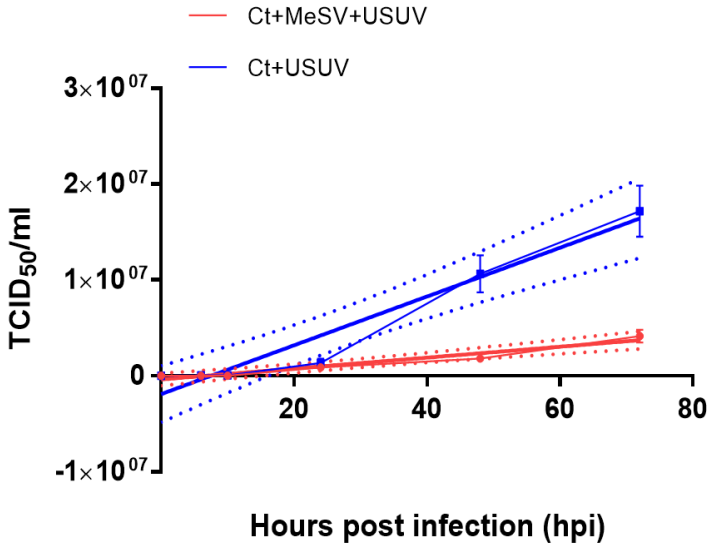


Fig. 6. 2: Linear regression analysis of Mesonivirus persistent cells coinfected with USUV and acute coinfection of Mesonivirus with USUV using Ct cells.

Culex tarsalis-derived Ct cells were infected singly with USUV (Ct+USUV) in tandem with MeSV persistently infected Ct cells (Ct+pMeSV+USUV) **[A]**, and coinfection with MeSV and USUV (Ct+MeSV+USUV) **[B]**. MOI 10=USUV; MOI 0.1=MeSV. Supernatants were collected for USUV titration via $TCID_{50}$ at 0, 6, 10, 24, 48 and 72 hpi for three independent experiments in triplicate. Linear regression model showing significance difference between Ct+pMeSV+USUV (p=0.0032) or Ct+MeSV+USUV (p=0.002) compared to Ct+USUV.

6.3 Interaction of Mesonivirus with Semliki Forest Virus

Table 6. 2: Growth kinetics of Mesonivirus persistent cells infected with SFV-FLuc and acute coinfection of Mesonivirus with SFV-FLuc using Hsu cells.

Culex quinquefasciatus-derived Hsu cells were singly infected with SFV-FLuc (Hsu+SFV-FLuc) in tandem with **[A]** MeSV persistent Hsu cells infected with SFV-FLuc (Hsu+pMeSV+SFV-FLuc) and **[B]** coinfection of Hsu cells with MeSV and SFV-FLuc (Hsu+MeSV+SFV-FLuc). MOI 10=SFV-FLuc; MOI 0.1=MeSV. Luciferase activity was measured at 6, 18, 24, 48 and 72 hpi for three independent experiments in triplicate using a reporter luciferase assay. Mean values with SEM are shown. Statistics via unpaired t-test; $p<0.05$ is significant.

A

Hours post infection	Relative Luciferase Activity (Mean ± SEM)		p-value
	Hsu+pMeSV+SFV-FLuc	Hsu+SFV-FLuc	
6	46.33 ± 10.74	35.89 ± 9.48	0.4764
18	208.9 ± 27.46	188.9 ± 18.46	0.5541
24	173.4 ± 20.43	155.4 ± 12.87	0.4669
48	59.33 ± 5.495	61.33 ± 3.403	0.7610
72	26.89 ± 1.933	40.56 ± 3.99	0.0071 **

B

Hours post infection	Relative Luciferase Activity (Mean ± SEM)		p-value
	Hsu+MeSV+SFV-FLuc	Hsu+SFV-FLuc	
6	150.6 ± 19.81	180.4 ± 17.7	0.2771
24	362.8 ± 31.7	365.2 ± 33.73	0.9585
48	162.8 ± 8.783	177.9 ± 5.106	0.1563
72	110.9 ± 5.445	113.4 ± 4.933	0.7325

6.4 Interaction of Mesonivirus with Bunyamwera virus

Table 6. 3: Growth kinetics of Mesonivirus persistent cells coinfected with BUNV-NLuc and acute coinfection of Mesonivirus with BUNV-NLuc using Hsu cells.

Culex quinquefasciatus-derived Hsu cells singly infected with BUNV-NLuc (Hsu+BUNV-NLuc) in tandem with **[A]** MeSV persistent Hsu cells infected with BUNV-NLuc (Hsu+pMeSV+BUNV-NLuc) and **[B]** coinfection of Hsu cells MeSV and BUNV-NLuc (Hsu+MeSV+BUNV-NLuc). MOI 0.1=BUNV-NLuc; MOI 0.1=MeSV. Luciferase activity was measured at 18, 24, 48 and 72 hpi for three independent experiments in triplicate using a reporter luciferase assay. Mean values with SEM are shown. Statistics via unpaired t-test; $p<0.05$ is significant.

A

Hours post infection	Relative Luciferase Activity (Mean ± SEM)		p-value
	Hsu+pMeSV+ BUNV-NLuc	Hsu+ BUNV-NLuc	
18	24547 ± 3860	34011 ± 4351	0.1232
24	36198 ± 5548	54211 ± 6572	0.0525
48	79589 ± 10325	130056 ± 12022	0.0058 **
72	74733 ± 10801	153533 ± 14063	0.0004 ***

B

Hours post infection	Relative Luciferase Activity (Mean ± SEM)		p-value
	Hsu+MeSV+ BUNV-NLuc	Hsu+ BUNV-NLuc	
24	282087 ± 24068	268319 ± 23309	0.6866
48	497102 ± 74858	541287 ± 84284	0.7003
72	414119 ± 59468	423556 ± 70393	0.9197

6.5 Reads aligning to Mesonivirus in small RNAs

Table 6. 4: Summary of reads aligning to mesonivirus in small RNAs from cell lines and mosquiotes

Aedes aegypti-derived Aag2 and *Culex tarsalis*-derived Ct cells were infected with MeSV (MOI = 0.1). After 24 hpi, RNA was isolated using TRIzol and followed by small RNA sequencing. The results shown are two independent experiments. First experiment (shown as first row in the table for each Aag2 and Ct cells) were used for the mappings **[A]**. RNA was isolated from *Culex quinquefasciatus* mosquitoes infected with MeSV at 5 dpi, followed by small RNA sequencing. PCR negative MeSV injected *Culex quinquefasciatus* mosquitoes were used as controls. The results shown are representative of one experiment **[B].**

A

Cell line	Total Clean reads[a]	MeSV reads (21nt)[b]	% MeSV reads (21 nt)[c]	MeSV reads (28 nt)[d]	% MeSV reads (28 nt)[e]
Aag2	27,381,110	50401	0.184072158	176	0.000642779
	27,179,670	43180	0.158868743	112	0.000412073
Ct	24,906,587	13424	0.053897389	4	1.606E-05
	22,576,201	19200	0.085045309	8	3.54355E-05

[a] Total clean reads for each sample
[b] total number of 21 nt reads in each sample
[c] Percent of total 21 nt sRNA reads which align to the respective viral genome
[d] total number of 28 nt reads in each sample
[e] Percent of total 28 nt sRNA reads which align to the respective viral genome

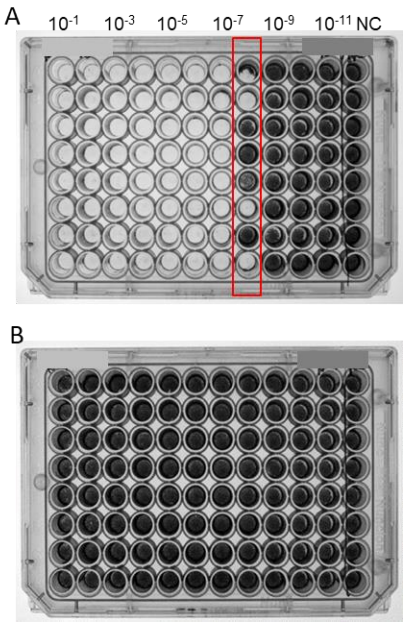
B

Culex quinquefasciatus mosquito	Total Clean reads[a]	MeSV reads (21 nt)[b]	% MeSV reads (21 nt)[c]	MeSV reads (28 nt)[d]	% MeSV reads (28 nt)[e]
MeSV Positive	27,523,440	374	0.001358842	136	0.000494124
MeSV Negative	27,994,751	1	3.5721E-06	0	0

[a] Total clean reads for each sample
[b] total number of 21 nt reads in each sample
[c] Percent of total 21 nt sRNA reads which align to the respective viral genome
[d] total number of 28 nt reads in each sample
[e] Percent of total 28 nt sRNA reads which align to the respective viral genome

6.6 Mesonivirus persistent infection in *Culex* cells validation

Culex tarsalis-derived Ct cells



Culex quinquefasciatus-derived Hsu cells

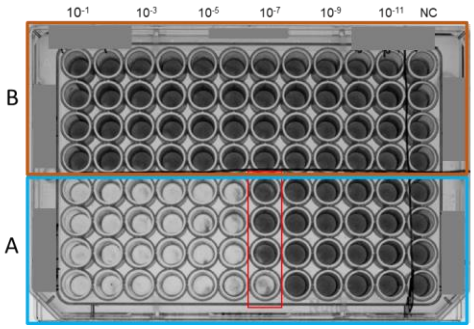


Fig. 6. 3: Proof of Mesonivirus replication in persistently infected *Culex* cells

Supernatants of MeSV persistently infected *Culex tarsalis*-derived Ct cells and *Culex quinquefasciatus*-derived Hsu cells were taken and frozen overnight at -80ºC together with noninfected corresponding cells. Left panel shows the Ct cells while the right panel displays the Hsu cells. Supernatants were collected for titration via $TCID_{50}$ on *Aedes albopictus*-derived C6/36 cells. **A** =MeSV persistent infected cell supernant, **B** =Naïve cell supernatant. The numbers show the dilution at each well and NC is the noninfected well. Plain wells show typical cytopathic effects (CPEs).

Abstract

Mesonivirus (MeSV) is a mosquito-specific virus, which has been found to infect mosquito populations worldwide, including also species that can be vectors to arboviruses of public health concern. This highlights the possibility for mosquitoes to be coinfected with MeSV and an arbovirus. However, it is not clear, if MeSV infection triggers the antiviral immune response of the mosquito host, namely the RNA interference (RNAi) pathways. Furthermore, it is unclear whether this response or any other interactions have an effect on potential coinfections with arboviruses, which can in turn influence vector competence of the mosquito. Therefore, investigating these interactions in vitro is a vital first step to exploring these possibilities.

Different arboviruses were chosen, namely Usutu virus (USUV; Flavivirus), Bunyamwera virus (BUNV; Orthobunyavirus) and Semliki Forest virus (SFV; Alphavirus), and their interactions with MeSV was investigated in vitro. Infection experiments were conducted on *Culex*-derived cells and changes in arboviral replication were determined via reporter assay or $TCID_{50}$. Small RNA deep sequencing was performed to investigate the possible presence of MeSV-specific small RNAs in acute infections. After detecting MeSV-specific small RNA molecules, their functionalities were elucidated by knockdown of key RNAi pathway proteins in an *Aedes aegypti* cell line and subsequent infection with MeSV. After infection, the changes in MeSV replication were measured using qRT-PCR. Suppressor assay was also used to understand the direct effect of MeSV on the antiviral RNAi pathway.

Interesting, the infection experiments suggest different effects of MeSV depending on the arboviruses. In the case of SFV, MeSV infection does not seem to have an effect on viral replication. However, BUNV and USUV replication seems to be suppressed at the presence of MeSV. MeSV-specific 21 nts siRNAs were produced with a unique hot spot at the 5’ end of the MeSV genome. Besides, antiviral RNAi activity of several RNAi mosquito proteins was recorded and the presence of an RNAi suppressor by MeSV was observed. Using a recent virus discovery pipeline based on small RNAs, several persistent viruses were identified in the used *Culex*-derived cells.

Taken together, the data suggest that the interaction between MeSV and arboviruses differs depending on the arbovirus. Antiviral RNAi pathway was stimulated by an acute MeSV infection. However, follow-up experiments are necessary to verify these observations and confirm a potential link between MeSV-triggered RNAi and effects on vector competence for arboviruses. The study widens our understanding of the complex interactions of mosquito-specific viruses, the mosquito hosts, and arboviruses.

Zusammenfassung

Das Mesonivirus (MeSV) ist ein mückenspezifisches Virus, das weltweit Stechmückenpopulationen infiziert. Darunter fallen auch Vektoren von Arboviren, die von Relevanz für die öffentliche Gesundheit sind. Es ist daher möglich, dass Stechmücken sowohl mit MeSV als auch mit einem Arbovirus infiziert sein können. Es ist jedoch nicht klar, ob eine MeSV-Infektion die antivirale Immunantwort des Mückenwirts, den RNA-Interferenz (RNAi)-Signalweg, auslöst. Weiterhin ist unklar, ob diese Reaktion oder andere Wechselwirkungen einen Einfluss auf mögliche Koinfektionen mit Arboviren haben, die wiederum die Vektorkompetenz der Mücke beeinflussen können. Die Untersuchungen dieser Wechselwirkungen in vitro sind daher ein wichtiger erster Schritt, um die Möglichkeit einer Beeinflussung der Vektorkompetenz für Arboviren durch MeSV zu erforschen.

Im Rahmen dieser Arbeit wurden folgende Arboviren im Zusammenhang mit einer MeSV Koinfektion in vitro untersucht: Usutu-Virus (USUV; Flavivirus), das Bunyamwera-Virus (BUNV; Orthobunyavirus) und das Semliki-Forest-Virus (SFV; Alphavirus). Die Koinfektionexperimente wurden in einer *Culex*-Zelllinie durchgeführt und Veränderungen der arboviralen Replikation wurden mittels Reporter-Assay oder TCID50 bestimmt.

Zudem wurde eine RNA-Sequenzierung durchgeführt, um das Vorhandensein von MeSV-spezifischen kleinen RNAs bei akuter Infektion zu überprüfen. Im Anschluss wurde die antivirale Funktionalität der identifizierten MeSV-spezifischen kleinen RNAs in einer *Aedes aegypti*-Zelllinie untersucht. Hierfür wurde die Menge an mRNA spezifischer RNAi-Schlüsselproteine gezielt runterreguliert und im Anschluss die MeSV Replikation mittel qRT-PCR gemessen. Abschließend wurde ein Suppressor-Assay durchgeführt, um die direkte Wirkung von MeSV auf den antiviralen RNAi-Signalweg zu verstehen.

Die Ergebnisse der Koinfektionsexperimente deuten darauf hin, dass die Auswirkungen einer Koinfektion mit MeSV stark abhängig von dem jeweiligen Arbovirus sind. Im Falle von SFV scheint die MeSV-Infektion keinen Einfluss auf die virale Replikation zu haben. Die Replikation von BUNV und USUV scheint jedoch in Anwesenheit von MeSV unterdrückt zu werden.

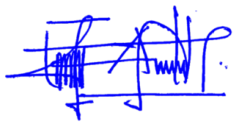
Weiterhin induziert eine akute MeSV die Produktion Virus-spezifischer siRNAs (21 Nukleotide lang), die sich an einem sogenannten Hotspot am 5‘-Ende des MeSV Genoms gruppieren. Außerdem wurde die antivirale Aktivität mehrerer verschiedener RNAi-Schlüsselproteine festgestellt und eine RNAi-Suppressor-Aktivität bei MeSV beobachtet. Schlussendlich wurde im Rahmen der RNA-Sequenzierung und unter

Verwendung einer neuen Pipeline für Virusidentifikation, die auf kleinen RNAs basiert, mehrere persistente Viren in den verwendeten *Culex*-Zellen identifiziert.

Zusammengenommen deuten die Daten darauf hin, dass sich die Interaktion zwischen MeSV und Arboviren je nach Arbovirus unterscheidet. Zudem scheint eine akute MeSV-Infektion den RNAi-Signalweg zu stimulieren. Es sind jedoch Folgeexperimente notwendig, um diese Beobachtungen zu verifizieren und eine mögliche Verbindung zwischen MeSV-stimulierten RNAi-Signalweg und Auswirkungen auf die Vektorkompetenz für Arboviren zu bestätigen. Diese Arbeit erweitert unser Verständnis für die komplexen Wechselwirkungen zwischen mückenspezifischen Viren, den Vektoren und Arboviren.

Declaration of oath

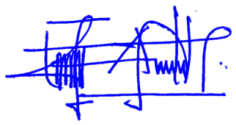
I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids.

Hamburg, 07.04.2021 

City, Date (Eric Agboli)

Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg, 07.04.2021 

Ort, Datum (Eric Agboli)

Publications

This section includes scientific communications related to the study.

Main Publication:

1. **Eric Agboli**, Mayke Leggewie, Mine Altinli, Esther Schnettler. **Mosquito-Specific Viruses-Transmission and Interaction**. Viruses. 2019;11:873. doi: 10.3390/v11090873.

Key conference abstract publications:

1. **Eric Agboli**, Mayke Leggewie, Stephanie Jansen, Anna Heitmann, Renke Lühken, Daniel Cadar, Egbert Tannich, Jonas Schmidt-Chanasit, Esther Schnettler. **Effect of Mesonivirus on arboviral infection in mosquito cells.** *International Symposium on Zoonoses Research, 2019. Abstract # N02. https://www.zoonosen.net/sites/default/files/redaktion/dateien/Program%20Zoonoses%202019.pdf*

2. **E. Agboli**, M. Leggewie, S.Jansen, E. Tannich, J. Schmidt-Chanasit, E.Schnettler. **Interaction of arboviral Usutu virus and insect-specific Mesonivirus and its effect on vector competence of mosquitoes.** *Junior Scientist Zoonoses Meeting, 2018. Poster # 1. https://www.openagrar.de/receive/openagrar_mods_00039461*

Key results in this dissertation will be published in the near future with these related titles:

1. Antiviral RNA interference response in mosquitoes to Mesonivirus infection.
2. Interactions of arboviruses with mosquito-specific Mesonivirus in cell lines.

Curriculum vitae

Lebenslauf entfällt aus datenschutzrechtlichen Gründen.

Curriculum vitae is not applicable for data protection reasons.

Acknowledgement

First of all, I would like to express my gratitude to Prof. Dr. Esther Schnettler, my supervisor for the acceptance to train me. I cannot forget the time I was looking for a supervisor just to work on mosquitoes and I was given a tight timeline. You came through at that particular stage of my life and I cannot thank you enough. My starting was like riding a bicycle, you kept encouraging me and very patient with me as the field was new. To me, you are such a humble, affable, talented scientist, and a mother I ever met. Year by year, I kept riding that bicycle to keep my balance in the field I desire. Here I am today putting the pieces together to create my story.

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My sustenance in Germany was fueled by the German Academic Exchange Service (DAAD) and also Friends of the BNITM. Your financial support was very helpful. My premier focal person, Caroline Wiegand, and the head of the section ST32 at the DAAD, Cay Etzold were indeed supportive at my initial challenge with the DAAD. I say, thank you to the management of the BNITM especially Prof. Dr. Egbert Tannich for the recommendation and support.

Finally, I want to thank my wife, Rosemary Esiawonam Atisu for sacrificing her Nursing job to come and stay with me in Germany to the completion of my studies. You shared most of my stories and I really appreciate your encouragement.

Dedication

I dedicate this work to the following personalities in my life:

- My Mother (**Adzotor Kpekpena**)
- My wife (**Rosemary Esiawonam Atisu**)
- My daughter (**Erica Wosiaminam Agboli**)
- My son who was born during my studies (**Caleb Woyram Agboli**)