Dissertation

Identification of Mosquito-Specific Viruses and Their Role in Virus-Virus-Host Interactions

submitted by

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Date of Disputation: 06. October 2023

Freie und Hansestadt Hamburg - May 20, 2023

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Abstract

The "virosphere" refers to the entire realm of viruses that exist on Earth. It includes all types of viruses, but up until recently, research has been mainly focused on those that directly affect humans and other animal or plant species of economic relevance.Yet, the virosphere is estimated to be incredibly vast, with millions of different viral species, and it is believed that many more viruses are yet to be discovered.

The study of the virosphere is crucial for understanding the diversity, evolution, and ecology of viruses and their interactions with their hosts. It is also important for identifying potential threats to human and animal health, such as emerging infectious diseases, and for developing strategies to prevent and control viral infections.

One part of the virosphere are arboviruses, or arthropod-borne viruses, a group of viruses that are transmitted by arthropods such as mosquitoes, ticks, and sandflies. Arboviral diseases, including dengue fever, chikungunya, Zika fever, West Nile fever, and yellow fever, have a significant impact on global public health. The emergence and re-emergence of arboviral diseases have become a major concern worldwide due to their increasing incidence and geographic spread.

While discovery and description of arboviruses primarily emanated from 'classical' virological research, the discovery of new virus species, and even whole families, is now often due to the proliferation of advanced sequencing technologies and bioinformatics tools. Another part of the virosphere, that is of some relevance to arboviruses, are insect- or mosquito-specific viruses. These are a group of viruses that have co-evolved with their insect hosts, particularly mosquitoes, and do not infect vertebrates.

These viruses have recently received increasing attention due to their potential as a tool for controlling mosquito-borne diseases. Research on insect- or mosquito-specific viruses is essential to understand their biology, evolution, and ecology as well as characterizing their potential to reduce the transmission of mosquito-borne diseases.

The presented study is a combined approach, using both sequencing and bioinformatical analysis as well as 'classical' virological and molecular techniques, to investigate the relationships of mosquito-specific viruses, arboviruses and the mosquito immune-system. Next-generation sequencing techniques were used to identify viral species from field samples. Small RNA sequencing was used to further analyse the interactions of the discovered viruses with the mosquito antiviral RNA interference machinery. Further, experiments were performed to analyse the influence of key effector proteins, Dicer and Argonaute, on the replication of these viruses. Finally, experiments on the interactions of mosquito-specific viruses with arboviruses, focused on viral replication and regulation of the host RNA interference system, were performed. In conclusion, the aggregated data expands our knowledge on the complex virus-virus-host interactions and supplements further approaches in studying mosquito-specific viruses as tools for arbovirus transmission control.

Kurzdarstellung

Die "Virosphäre" bezeichnet die Gesamtheit der Viren, die auf der Erde existieren. Sie umfasst alle Arten von Viren, jedoch lag bis vor kurzem der Fokus der Forschung hauptsächlich auf jenen, die sich direkt auf Menschen und andere wirtschaftlich relevante Tier- oder Pflanzenarten auswirken. Die Virosphäre wird jedoch auf Millionen verschiedener Virusspezies geschätzt.

Die Erforschung der Virosphäre ist entscheidend, um die Vielfalt, Evolution und Ökologie von Viren und ihrer Interaktionen mit ihren Wirtsorganismen zu verstehen. Ebenfalls relevant ist es potenzielle Bedrohungen für die menschliche und tierische Gesundheit, wie aufkommende Infektionskrankheiten, zu identifizieren und Strategien zur Prävention und Kontrolle viraler Infektionen zu entwickeln.

Ein Teil der Virosphäre sind die Arboviren oder arthropodenübertragenen Viren, eine Gruppe von Viren, die von Arthropoden wie Stechmücken, Zecken und Sandfliegen übertragen werden. Arbovirale Erkrankungen wie Dengue-Fieber, Chikungunya, Zika-Fieber, West-Nil-Fieber und Gelbfieber haben einen erheblichen Einfluss auf die globale öffentliche Gesundheit. Auftreten und Wiederkehr arboviraler Krankheiten sind aufgrund ihrer zunehmenden Inzidenz und geografischen Ausbreitung weltweit von Relevanz.

Während die Entdeckung und Beschreibung von Arboviren hauptsächlich aus der 'klassischen' virologischen Forschung stammt, ist die Entdeckung neuer Virusarten und sogar ganzer Familien heute oft auf die Verbreitung von fortschrittlichen Sequenzierungstechnologien und bioinformatischen Anwendungen zurückzuführen. Ein weiterer Teil der Virosphäre, der für Arboviren von einiger Relevanz ist, sind insektenspezifische oder stechmückenspezifische Viren. Dies ist eine Gruppe von Viren, die sich mit ihren Insektenwirten, insbesondere Stechmücken, mitentwickelt hat und keine Wirbeltiere infiziert.

Diese Viren haben aufgrund ihres Potenzials zur Kontrolle von Stechmücken übertragenen Krankheiten in letzter Zeit zunehmende Aufmerksamkeit erhalten. Die Erforschung von stechmückenspezifische Viren ist entscheidend, um ihre Biologie, Evolution und Ökologie zu verstehen sowie ihr Potenzial zur Reduzierung der Übertragung von durch Stechmücken übertragenen Krankheiten zu charakterisieren.

Die vorliegende Studie ist ein kombinierter Ansatz, der sowohl Sequenzierung und bioinformatische Analysen als auch 'klassische' virologische und molekulare Techniken nutzt, um die Beziehungen von stechmückenspezifischen Viren, Arboviren und dem Stechmücken-Immunsystem zu untersuchen. Next-Generation-Sequenzierungstechniken wurden verwendet, um virale Spezies aus Feldproben zu identifizieren. Die kleine RNA-Sequenzierung wurde verwendet, um die Interaktionen der entdeckten Viren mit der antiviralen RNA-Interferenzmaschinerie der Stechmücken weiter zu analysieren. Es wurden weiterhin Experimente durchgeführt, um den Einfluss von Schlüsseleffektorproteinen, Dicer und Argonaute, auf die Replikation dieser Viren zu analysieren. Schließlich wurden Experimente zur Untersuchung der Interaktionen von stechmückenspezifischen Viren mit Arboviren durchgeführt, die sich

auf die virale Replikation und Regulation des RNA-Interferenzsystems des Wirts konzentrierten. Zusammenfassend erweitern die aggregierten Daten unser Wissen über komplexe Virus-Virus-Wirt-Interaktionen und ergänzen weitere Ansätze zur Untersuchung von mücken-spezifischen Viren als Werkzeug zur Kontrolle der Arbovirusübertragung.

Table 1: Table of Abbreviations

Abbreviation	Full Form
A10	adenosine at the 10th position
Ae.	Aedes
Ago1	Argonaut-1
Ago2	Argonaut-2
ASALV	Agua Salud alphavirus
bp	base pairs
CALBOV	Calbertado virus
CPE	cytopathic effect
CT	cycle of threshold
CT-EI	CT cells persistantly infected with EILV
CT-NI	CT cells persistantly infected with NIEV
Cx.	Culex
DaesV	Daeseongdong virus
Dcr1	Dicer-1
DENV	Dengue virus
DeziV	Dezidougou virus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	desoxyribonucleic acid
dpi	days post infection
ds	double stranded
EILV	Eilat virus
exo	exogenous
FCS	fetal calf serum
FHV	Flock House virus
FRC	Fructose solution
GFP	green fluorescent protein
hpi	hours post infection
ICTV	International Committee on Taxonomy of Viruses
ISV	insect-specific virus
kb	kilo base pairs
L15	Leibovitz L-15 medium
MesoV	Mesonivirus
mi	micro
MOI	multiplicity of infection
MSV	mosquito-specific virus
NegV	Negevirus
NGS	Next-generation sequencing
NIEV	Niénokoué virus
ns	non-significant
nsP	non-structural protein
nt	nucleotide
NVD1	Nelorpivirus dungfly 1
ORFs	open reading frames
p. mem. prot.	putative membrane protein
P/S	penicillin & streptomycin
PBS	Phosphate Buffered Saline
PCLV	Phasi-Charoen-like virus
PCR	polymerase chain reaction
PFU	plaque forming units
pi	Piwi-interacting
PIWI	P-element Induced WImpy testis
pre	precursor
pri	primary
RNA	ribonucleic acid
SDS	Sodium dodecyl sulfate
SFV	Semliki Forest virus

si	short-interfering
sP	structural protein
sRNA	small RNA
ss	double stranded
TCID50	50% tissue culture infectious dosis
Tm	50% melting temparature
U1	uracil at the 1st position
USUV	Usutu Virus
UTR	untranslated region
WNV	West Nile Virus
YFV	yellow fever virus
YicV	Yichang virus

1 Introduction

1.1 Virosphere

Currently the International Committee on Taxonomy of Viruses (ICTV) lists 10.434 virus species, representing a thirty-six fold increase since their first report in 1971. Only very recently has the Serratus project utilized high-throughput computational methods to identify over 10^5 putative virus species in already existing public databases, only within the realm of *Ribovirida* [Edgar et al., 2022].

Identification and description of viruses species have been all but unbiased [Harvey and Holmes, 2022]. The vast majority of known viruses are associated with only two host phyla, the Chordata and Arthopoda (fig. 1 A). Within the Chordata, virus species of interest are those that either infect humans directly, serve as a reservoir for human pathogenic viruses, or infect economically relevant species. Identification and description of virus species, that are associated with these vertebrate hosts, still vastly outnumber our knowledge on the virosphere of invertebrate organisms (fig. 1 B).

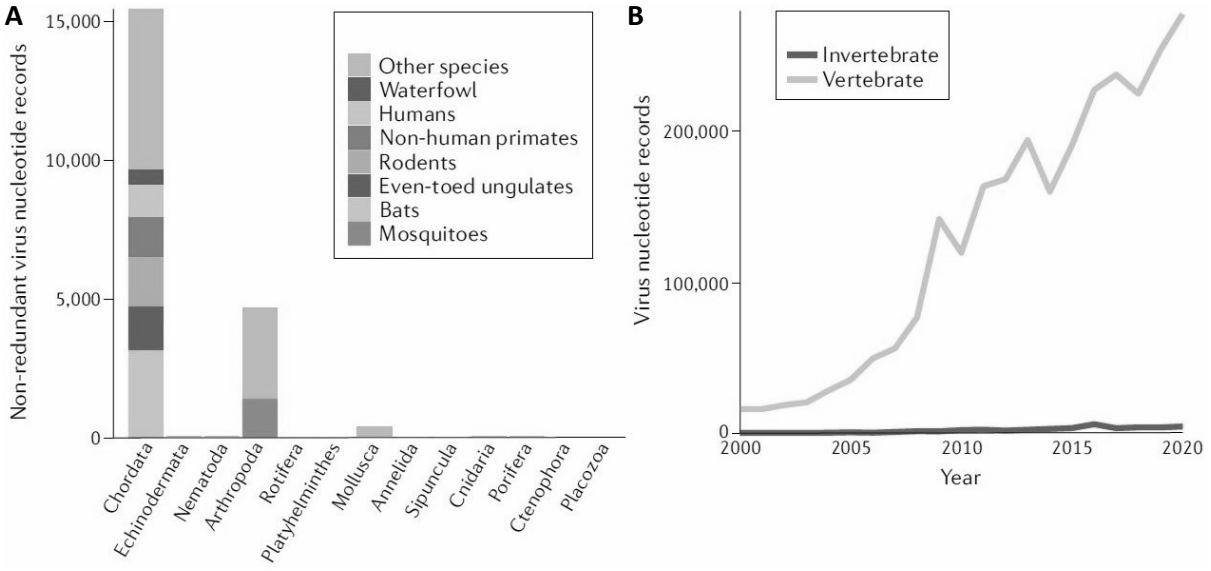


Figure 1: **Virome Sequencing by Phylum.** (A) Cumulative amount of unique viral sequences in GenBank per animal phyla. (B) Number of vertebrate and invertebrate associated viruses entries in GenBank per year. [Harvey and Holmes, 2022]

With the *Arthropoda*, the invertebrates hosts the phylum with far the greatest diversity of species among all animals. And with this variety of host species also comes a great diversity of virus species. Within the *Arthopoda*, a taxon of mostly hematophagous animals, the *Culicidae*, also commonly referred to as mosquitoes, are included. Adult mosquitoes primarily rely on nectar, honeydew or phytophagy as their source of nutrients [Peach and Gries, 2020]. But in order to produce eggs, most female mosquitoes, as well as other arthropods, such as ticks (*Ixodida*), biting midges (*Ceratopogonidae*) and

sand flies (*Phlebotominae*), require the uptake of a blood meal from a vertebrate. This ecto-parasitic behavior of mosquitoes, and the other arthropods mentioned, gave rise to an unique ecological niche, in which viruses evolved that can infect both invertebrate and vertebrate species. These are the so-called arthropod-borne viruses, commonly referred to as arboviruses.

1.2 Vectors

Arbovirus is a non-taxonomic term, relating to a group of species that belong to diverse virus families, mainly with RNA genomes. While some arboviruses have been a burden to humans for a long time, and new species are constantly (re-)emerging, following their introduction into pristine host-vector systems. For the maintenance of arbovirus infections, out of the 113 recognized *Culicidaen* genera, two are of outstanding interest due to their major functions as vectors for arboviruses relevant to humans, *Aedes* and *Culex* [Harbach, 2013].

1.2.1 Vector Ecology

The narrow corridor of vector-virus pairing is a result of the complex interplay of multiple external biotic and abiotic as well as internal factors, such as immune functions and co-infections [Viglietta et al., 2021]. Only in a fine orchestration of these factors, it is possible for a mosquito species to become a competent vector for a given arbovirus [Hardy, 1983]. Alongside these factors, both *Aedes* and *Culex* mosquitoes exhibit a broad global distribution as well as ample anthropophagic host patterns, explaining their outstanding relevance for arbovirus related research efforts [Kraemer et al., 2015, Shocket et al., 2020, Farajollahi et al., 2011, Cebrián-Camisón et al., 2020, Fikrig and Harrington, 2021, Pruszynski et al., 2020]. Species of the *Culex* genra, which are in the focus of the presented work, can be found on every continent, except Antarctica, within a wide range of climates (fig. 2 A, [Shocket et al., 2020]). With their primary blood meal hosts being birds, but still being sufficiently opportunistic to feed on humans and other mammals, they present as capable bridge vectors, connecting enzootic arbovirus transmission cycles between avian hosts with surrounding mammalian populations (fig. 2 B, [Farajollahi et al., 2011]).

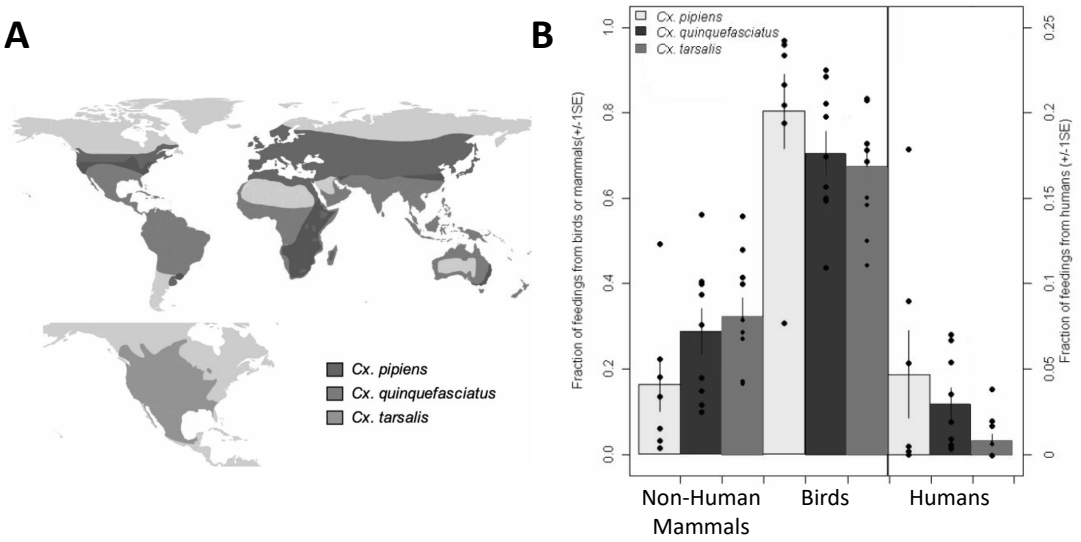


Figure 2: ***Culex* spp. distribution and feeding-patterns.** (A) Schematic representation of the geographic occurrence of *Culex pipiens* (gray), *Culex quinquefasciatus* (red) and *Culex tarsalis* (blue). (B) Bar graph showing the fractions of blood meals originated from non-human mammals and birds (left) and humans (right) found in *Cx. pipiens* (yellow), *Cx. quinquefasciatus* (red), and *Cx. tarsalis* (blue) [Shocket et al., 2020, Farajollahi et al., 2011, Darsie, 1981] [modified].

1.2.2 Infection of the Vector

In general, the vector mosquito acquires an arbovirus during a blood meal on a sufficiently viremic host. To close the transmission cycle, the virus has to end up in the mosquito's saliva and infect a vertebrate host by subsequent blood meals. As mentioned above, this vector competence is a multi-factorial process, of which here a short overview of the intrinsic, organismic functions involved are exhibited (fig. 3). First, there are mechanical barriers like the peritrophic membrane and the epithelial basal lamina of the mid gut. These structures are, solely with their physical properties, able to prevent infection and dissemination of arboviruses at different points [Houk et al., 1979, Girard et al., 2005]. Second, there are biological barriers and obstructions. Most arboviruses have to perform multiple infection cycles inside the mosquito, namely in the mid gut epithelium, internal organs and the salivary glands. At all points in this process, the virus encounters the hosts immune system as well as other co-infecting microorganisms and viruses, with the potential to interfere with the final arbovirus transmission [Jupatanakul et al., 2014, Blair and Olson, 2014, Vasilakis and Tesh, 2015]. These will be discussed in greater detail in the chapters 1.5 Virus-Host Interactions and 1.6 Virus-Virus Interactions.

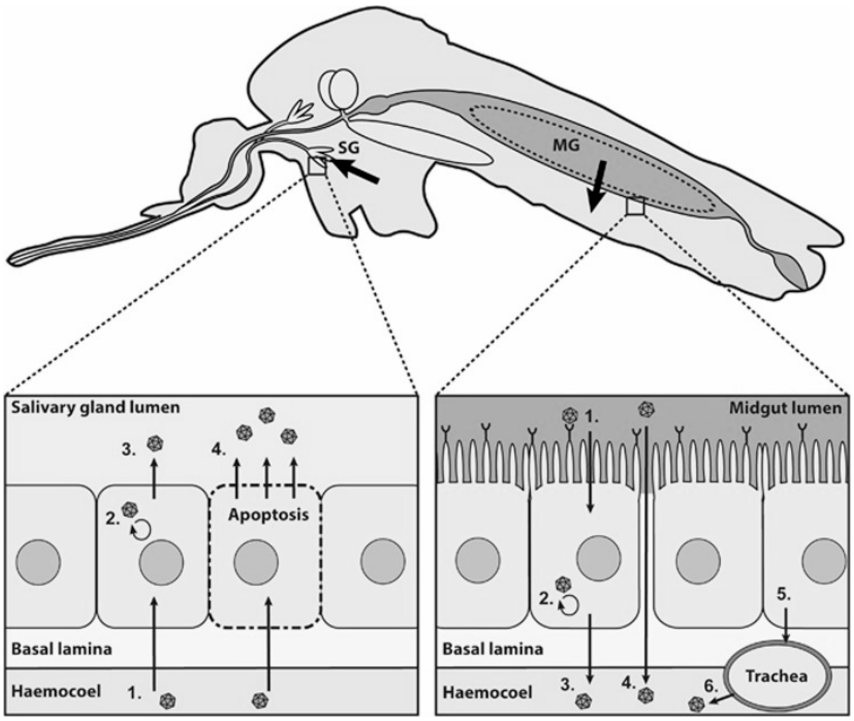


Figure 3: **Schematic overview of an Arbovirus infection of a Mosquito.** The infectious blood meal becomes digested in the mosquitoes mid gut, from where virus particles have to pass through the mid gut epithelium and its basal lamina into the hemocoel. This process is either achieved by a proper infection of the mid gut cells (right 1.-3.) or by bypassing the epithelium (right 4.). Beside direct dissemination into the hemocoel, virus particles can also be disseminated by the tracheal system (right 5.). Virus particles that reached the salivary glands can pass through their basal lamina and infect the glands epithelial cells (left 1.-2). Virus particles are released into the saliva by either budding or apoptotic processes (right 3.-4.). [Vogels et al., 2017]

1.3 Arboviruses

1.3.1 *Flaviviridae*

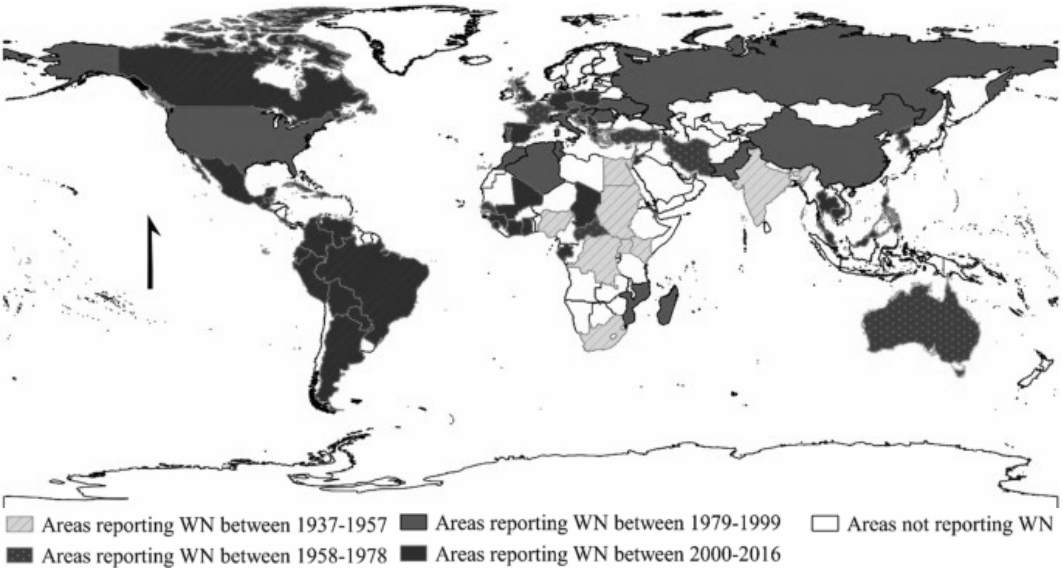
The term arbovirus, the field of virology and tropical medicine are historically intricately connected with what today is classified as the family of *Flaviviridae*. The eponymous yellow fever virus (YFV), causing the correspondent yellow fever disease, was the first virus species for which the direct connection between a virus, disease and mosquitoes was proposed in 1881, experimentally proven in 1900 and was finally successfully isolated in 1928 [Finlay, 1881, Reed et al., 1900, Stokes et al., 1928]. Also, the second human pathogenic virus to be described in the early 1900's was an arthropod-borne flavivirus, the causative agent of dengue fever, Dengue virus (DENV) [Ashburn et al., 1907]. As both YFV and DENV were responsible for recurring disease outbreaks in human populations, screening efforts for both of these viruses led to the identification of another arbo-flavivirus. This time it was not named after an associated human disease, but rather the location it was first found. In the serum from a monkey in Uganda's Zika Forest, the Zika virus (ZIKV)

was identified, later also found in mosquitoes, and described as the causative agent of disease in humans [Dick et al., 1952, Smithburn, 1952]. Since then, it has had minor outbreaks in Africa and Asia, until it caused a recent epidemic in South America, primarily Brazil, in 2015/2016 [Kindhauser et al., 2016].

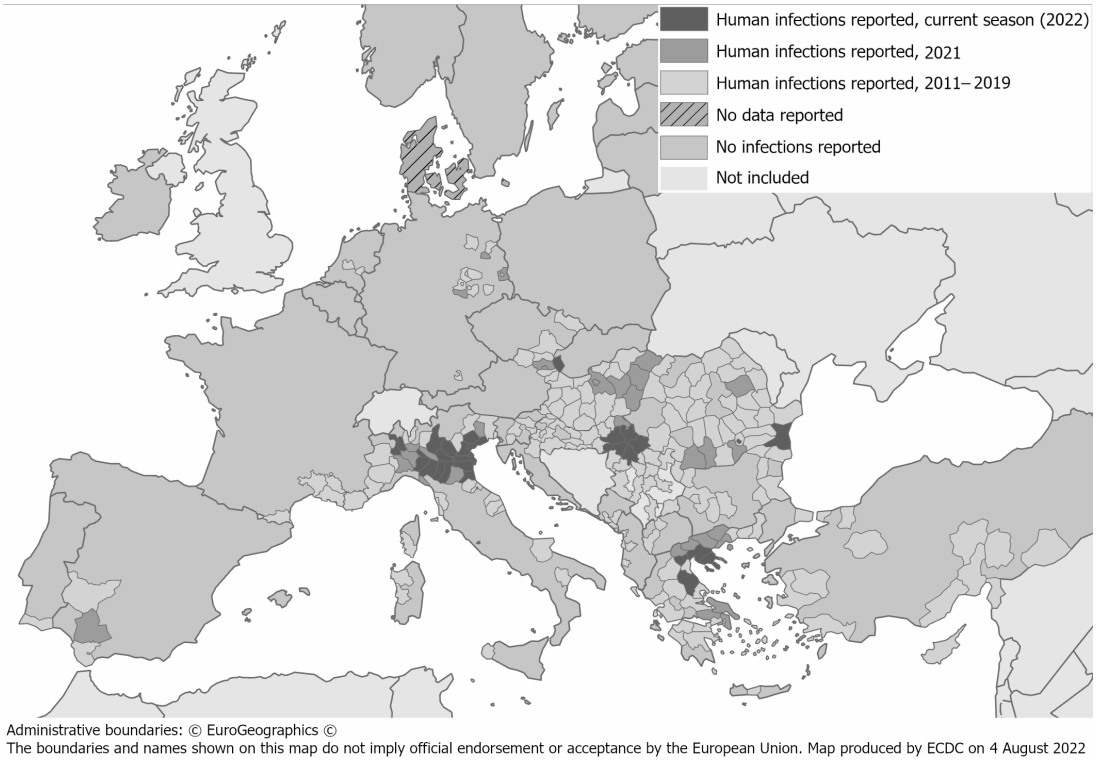
A common characteristic of YFV, DENV and ZIKV, besides belonging to the same taxon, is that their primary vector organisms are mosquitoes of the genus *Aedes* with *Ae. aegypti* being the most important one. But, as mentioned earlier, mosquitoes belonging to the genus of *Culex* are also known to be relevant vectors for arboviruses. One of the most pertinent Culex-borne flaviviruses to mention is the Japanese Encephalitis virus (JEV). Its disease was also already described as early as the 1870's and was first isolated in 1935 [Solomon et al., 2000]. Aside from JEV, two other viruses have most recently sparked research interests as emerging and co-circulating viruses, primarily in Europe; West Nile Virus (WNV) and Usutu Virus (USUV) [Nikolay, 2015, Zannoli and Sambri, 2019, Vilibic-Cavlek et al., 2019].

West Nile Virus Like ZIKV, WNV was first identified in sub-saharan Africa, also in Uganda but in the West Nile Province as part of a YFV research project [Smithburn et al., 1940]. The first major WNV epidemic recorded occurred 1951 in the Kibbutz Ma'ayan Tzvi near Haifa, Israel [Bernkopf et al., 1953]. There, 41% (123 of 303 inhabitants) developed West Nile disease, mainly leading to fevers, exanthemata, severe headache, anorexia, vomiting and abdominal pain, causing mortality in 21% of patients older than 16 years and between 100% and 34% in younger individuals.

From this time onward, WNV outbreaks remained limited to the areas around the Mediterranean Sea and the African continent [Sejvar, 2003, Murgue et al., 2001] (fig. 4a). In 1996 WNV caused a disease outbreak in Romania, this time mostly causing encephalitis and meningitis with 393 hospitalizations and 17 fatalities [Campbell et al., 2001]. Three years later, in 1999 WNV was introduced to the Americas, leading to a remarkable outbreak, starting in the New York area, causing stupendous mortality among birds, infecting humans and continuously spreading throughout America [Steele et al., 2000, Nash et al., 2001, Komar et al., 2001, Ludwig et al., 2002].



a)



b)

Figure 4: **Spatio-temporal Distribution of WNV occurrences.** a) Representation of reported WNV infections on national level globally, divided into time intervals (color shaded areas) from 1937 to 2016. [Fall et al., 2019] [modified]. b) Regional representation of recorded WNV infections in human from 2011 to 2022 in the European Union and neighboring regions [ECDC, modified].

In 2004 an outbreak of WNV among birds was recorded in Hungary [Erdélyi et al., 2007]. Remarkably, this was the first observation of an outbreak caused by a different distinct genetic variant of WNV. Based on molecular phylogenies, WNV is sub-divided into several lineages, of which lineage 1 (WNV1) and 2 (WNV2) are considered the most

human-pathogenic variants [Pesko and Ebel, 2012, Pauli et al., 2013]. And prior to the 2004 outbreak, WNV2 has not been identified outside of Sub-Saharan Africa [Zeller and Schuffenecker, 2004]. In the following years, other Western, Central, Southern and Eastern European Regions also reported the occurrence of WNV2 [Camp and Nowotny, 2020] (fig. 4b). Between the years 2011 and 2015 WNV2 displaced WNV1 in Italy [Rizzo et al., 2016]. In 2018 a major WNV outbreak occurred, mainly in the Po Valley, Albania, Greece, Romania and Hungary, also caused by lineage 2 [Camp and Nowotny, 2020].

In Germany, no autochthonous WNV infections were recoreded prior to 2018 [Frank et al., 2022, Michel et al., 2019]. In that year, WNV2 was detected in birds and horses in Eastern Germany [Ziegler et al., 2019, Ziegler et al., 2020]. From there on, infections with WNV are occuring every transmission season in humans, birds and horses [Pietsch et al., 2020, TSIS, 2023].

Usutu Virus Two years after the WNV outbreak in New York, a similarly alarming event of morbidity among birds occurred in Austria, affecting primarily black birds (*Turdus merula*) around Vienna [Weissenböck et al., 2002]. Immunohistochemical stainings suggested the presence of WNV antigen in brain and kidney samples of affected animals. But subsequent *in situ* hybridization and PCR testing for WNV were negative. But the researches were able to amplify an infectious agent from tissue samples in cell culture, and analyzed it with a universal flavivirus PCR, identifying it as Usutu Virus (USUV).

First described in 1959 around Ndumu, South Africa, USUV presented as antigenically closely related to WNV and was likewise found in *Culex* mosquitoes [Williams et al., 1964]. And prior to the Austrian outbreak, USUV had only been isolated in Sub-Saharan Africa [Nikolay et al., 2011].

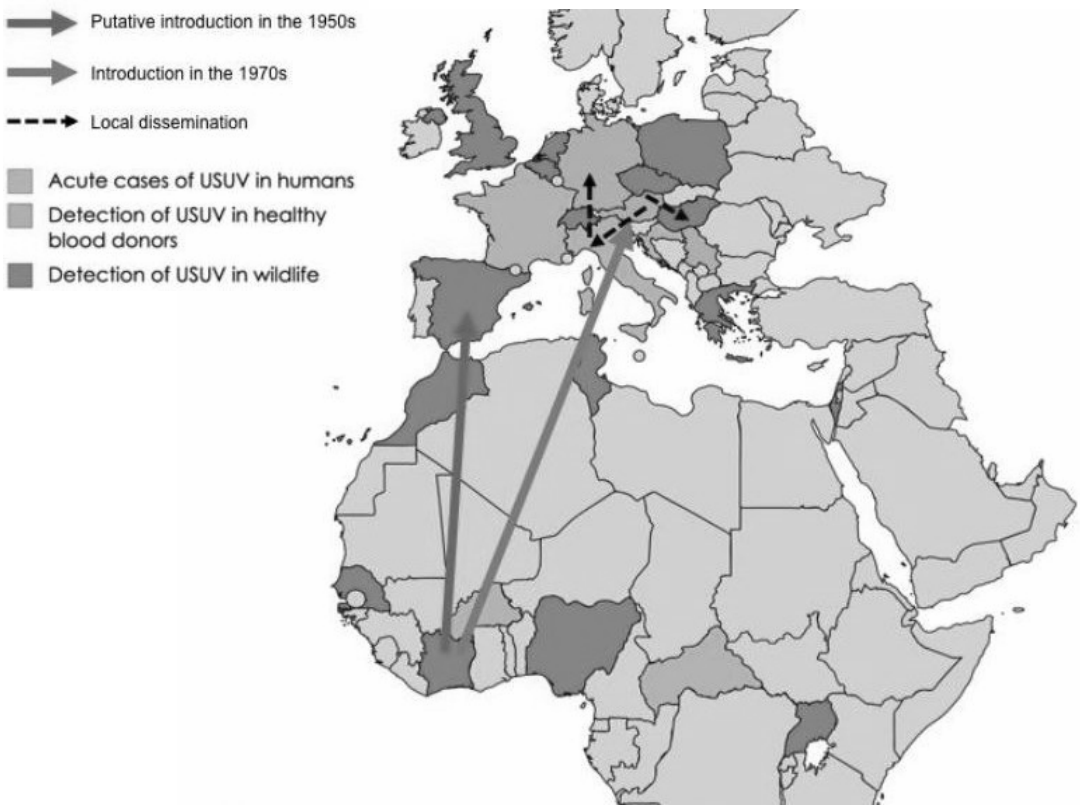


Figure 5: **Geographical distribution of USUV.** Representation of reported infections with USUV on a national level in Africa, West Asia and Europe (yellow, blue and green areas) and reconstruction of possible introduction (red and blue arrows) and local dissemination events (black arrows), based on molecular genetic characteristics of virus samples. [Roesch et al., 2019] after [Engel et al., 2016] [modified].

Though, back in 1998 a remarkable mortality among black birds with an unknown cause was noted in Tuscany, Italy. Unfortunately, the only report on that incident was published in a veterinary journal, only in Italian language [Mani et al., 1998]. But as some of the same researchers who reported the first European USUV outbreak in 2001 analyzed the preserved samples, and they found that the event in Italy was in fact also caused by USUV, preceding the possible beginning of its circulation in Europe by three years [Weissenböck et al., 2013].

Since then, USUV infections, often accompanied by bird mortality but few human cases, occurred in many European regions (a detailed review of USUV epidemiology was recently conducted by [Vilibic-Cavlek et al., 2020]). Current phylogeographic analysis suggests that the introduction of USUV to Europe happened even prior to the recorded events described above, sometime in the 1970s (fig. 5, [Engel et al., 2016]).

Ecology of WNV & USUV Both WNV and USUV are enzootic viruses that switch in their natural life cycles between ornithophagic mosquitoes and birds fig. 6. Hereby, the aforementioned dispersal of both viruses from Africa to Europe was most likely achieved by infections of migratory bird species. In fact, WNV and USUV avian host species ranges

are substantially overlapping, including 34 species within 11 orders, 17 of which are to some extent migratory [Nikolay, 2015].

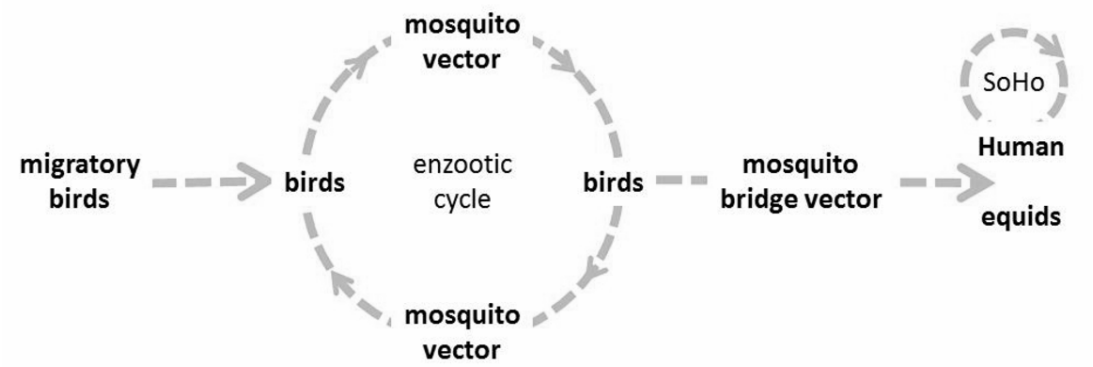


Figure 6: **West Nile virus transmission cycle.** Schematic representation of WNV infections of humans, starting from the introduction with migratory birds. After a local introduction, the virus is maintained in an enzootic cycle between mosquitoes and birds. Spill over events to humans and other animals occur by opportunistically host feeding mosquitoes, or bridge vectors. SoHo: substance of human origin. [ECDC]

For both viruses, the relevant bridge vector mosquitoes are considered to be *Cx. tarsalis* and *Cx. quinquefasciatus* in (Northern) America and *Cx. pipiens* in Europe [Kilpatrick et al., 2006, Colpitts et al., 2012, Reisen et al., 2005, Molaei et al., 2007, Crockett et al., 2012, Zinser et al., 2004, Rizzoli et al., 2015, Fros et al., 2015, Leggewie et al., 2016, Holicki et al., 2020a, Hernández-Triana et al., 2018].

1.3.2 Molecular Characteristics

In general, flaviviruses have a positive sense single-stranded RNA genome in between about 9 kb and 13 kb size [Simmonds et al., 2017b]. The 40 - 60 nm virions are made up by a single capsid protein (C) and are enveloped with two glycoproteins (E and prM or M).

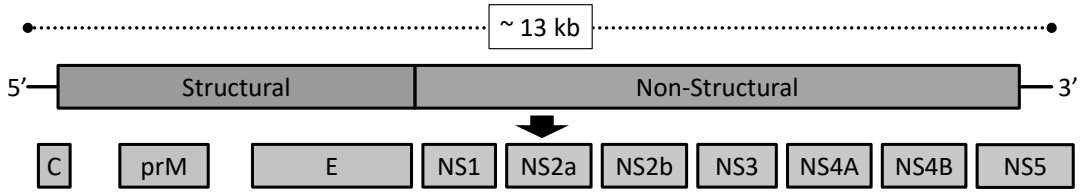


Figure 7: **Flavivirus genome organization.** Schematic representation of the flavivirus genome. It is made up by a positive sense single-stranded RNA, encoding a single ORF. From this a single polyprotein is made, which is cleaved by several viral and cellular proteases into the individual structural and non-structural proteins.

The genomic RNA is 5'-capped and the ORF is flanked by non-coding regions. Trans-

lation occurs cytoplasmic directly into a single, polyadenylated mRNA. It encodes for a single polyprotein which gives rise to the three structural and five non-structural proteins. Replication is facilitated by a serine protease and RNA helicase activity (both NS3), and a RNA-dependent RNA polymerase (RdRp, NS5). They synthesize a genome length negative-strand intermediate as template for genomic RNA production. Virion assembly occurs by budding of intracellular membranes into secretory vesicles.

1.4 Mosquito-Specific Viruses

Likely because of their role as diseases vectors, the metagenomic based expansion of knowledge on the virosphere as a whole, is now not limited to arboviruses alone, but the virome of arthropods in general. And this virome is not only limited to arboviruses, but includes a large variety of mosquito-specific viruses (MSV) [1].

1.4.1 Discovery

Historically, the first glimpse on this matter arose from arbovirus related research. [Stollar and Thomas, 1975] reported that they observed the induction of a cytopathic effect (CPE) when they inoculated cell culture supernatant from an *Ae aegypti* derived cell line onto a *Ae. albopictus* derived one. While it was already known that certain arboviruses can induce CPE on insect cells, outstandingly this effect could not be observed when multiple vertebrate derived cell lines were inoculated. This led the researchers to the conclusion, that the agent they found may be an arbovirus that is adapted to lower culture temperatures of insect cells and therefore is restricted from infecting vertebrate cells, which are cultivated at higher temperatures.

17 years later, the peculiar virus, which was named cell fusing agent virus (CFAV) based on the distinct syncytial phenotype it induces, was further characterized. By nucleotide sequencing, it was recognized that CFAV was indeed related to arboviruses within the *Flaviviridae* [Cammisa-Parks et al., 1992]. But it was also unable to infect vertebrate cell lines at lower culture temperatures. Thus it was supposed that CFAV is a flavivirus that can only infect insects [2].

1.4.2 Prevalence

Even with recent metagenomic data available, it is difficult to ascertain how common infections with MSVs are. Within the arthropods, studies reported that above 80% and

[1] In concurrent scientific publishing on the subject matter, the terms *insect-specific*, *mosquito-specific* or *insect/mosquito associated* are often used interchangeably. Although being epistemological problematic, as it resembles an hardly falsifiable claim, the author will use the term MSV to refer to any virus species in any family that is able to infect certain mosquitoes or their derived cells, but is not known to infect vertebrates or commonly used, derived cell lines.

[2] Today the identification of CFAV is sometimes cited as the "first insect-specific virus" (ISV, ([Öhlund et al., 2019b, Bolling et al., 2015])). Though ISVs were already known at the onset of virology as an academic discipline, namely as a causative agent of certain diseases of silkworms [Glaser, 1918].

below 5% of RNA reads in metagenomic sequencing studies were of viral origins (fig. 8). For studies on mosquitoes in specific, the numbers vary with some reporting as low as 0.01% or up to 37% ([Hameed et al., 2021, He et al., 2021, Pettersson et al., 2019, Öhlund et al., 2019a]). Ultimately, the testing of pools, as performed by the mentioned studies, and reporting ratios of viral reads, give a very limited empiric view to answer the question of MSV prevalence in nature [3].

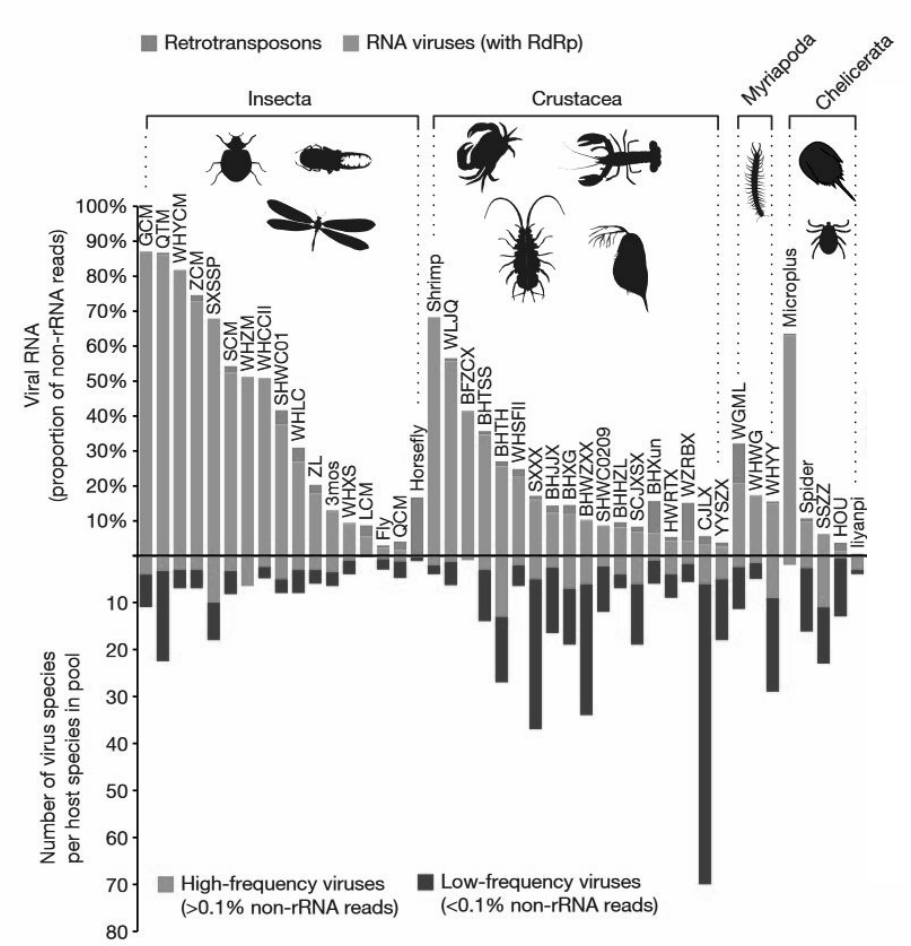


Figure 8: **Frequency and Diversity of viral RNA Transcripts in Invertebrate Transcriptomes.** Plot of non-ribosomal (r)RNA reads that are associated to virus genome (orange), derived from metagenomic sampling pools from different insect, crustacean, myriapods and Chelicerata (top) and normalized number of virus species per pool, subdivided into common (blue) and uncommon (magenta) species. [Shi et al., 2016] [modified]

[3] In a study, performed on over 2000 individually analyzed *Drosophila melanogaster*, virus prevalence varied, depending on the collection side, between >80% and <10%, resulting in a mean of 30% prevalence for the tested virus species, with >6% of individuals carrying more than one virus [Webster et al., 2015]. For comparison, metagenomic pooling of these resulted in between 30% and 5% viral read ratios, putting is close to the ratios reported for mosquito pools.

1.4.3 Identification

In general, RNA virus metagenomics has contributed greatly to our understanding of diversity, evolution and ecology of viruses [Greninger, 2018, Rosario and Breitbart, 2011, Bexfield and Kellam, 2011, Mokili et al., 2012]. However, biological description lacks greatly behind the sheer number of sequence based virus identifications. This development led the ICTV to propose that the recognition of new virus species should be possible even without any biological data accompanying exclusively (meta)genomic reports [Simmonds et al., 2017a]. This however provoked some push back, highlighting the necessity of further characterization in terms of phenotype, biological properties and host associations [Greninger, 2018, Bonning, 2020, Canuti and van der Hoek, 2014].

One way to reliably supplement host species and some phenotypical characteristics, even in the absence of a viable cell culture model and successful virus isolation, can be the use of virus derived small interfering RNAs (see chapter 1.5.3) [Wu et al., 2010]. First, these small RNAs show host-specific characteristics that allow to differentiate whether an identified virus was, at the time of sampling, actively infecting the organism or may just be a contaminant. And second, this data also includes valuable insights into the interactions of the virus with a fundamental part of the hosts immune system.

1.4.4 Characteristics of MSVs

As established above, the term MSV may refer to diverse virus families. Some MSV belong to virus families constituted by known arboviruses like the flaviviruses or alphaviruses (*Togaviridae*) respectively. As such, those MSV share the majority of their molecular characteristics with their related arboviruses. But the identification of other MSVs also led to the establishment of entirely new taxonomic families, like the *Mesoniviridae* and Negeviruses. Examples of MSVs from both categories, as they are relevant to the presented study, are briefly introduced below.

Niénokoué virus Niénokoué virus (NIEV) was isolated from a pool of *Culex* mosquitoes collected in Côte d'Ivoire [Junglen et al., 2017]. In phylogenetic inferences, it clusters with other *Culex* associated classical insect-specific flaviviruses, a paraphyletic taxon to the mosquito-borne flaviviruses, which also encompasses insect-specific flavi-like viruses [Halabi and Mayrose, 2021]. It shows the same genome organization as other flaviviruses (fig. 7).

Eilat virus Eilat virus (EILV) was first isolated in a pool of *Anopheles coustani* collected during an arbovirus survey in the Negev desert, Israel and found to be unable to replicate in vertebrates cells [Samina et al., 1986, Nasar et al., 2012]. Based on sequence similarity, EILV was identified as an alphavirus, closely related to Western Equine Encephalitis virus complex but constituting a distinct phylogenetic branch within the alphaviruses.

Alphaviruses exhibit a positive sense single-stranded RNA genome between 10 and 12 kb length [Chen et al., 2018]. The virions are of 65 - 70 nm size, spherical with a single capsid (C) and enveloped with three glycoproteins (E1, E2 and E3). Their genome is 5'-capped and 3'-polyadenylated. Translation of structural proteins from a single ORF at towards the 3'-end and the non-structural proteins from an ORF beginning at close to the 5'-end. Both are flanked and separated by non-coding regions. Non-structural proteins are directly made from genomic RNA, whereas the structural proteins are made from a subgenomic RNA.

Mesoniviridae The family of *Mesoniviridae* was proposed with the identification of nidovirus (*Nidovirales*) -like viruses found in *Culex* mosquitoes collected in Cote d'Ivoire and Vietnam in 2011 [Zirkel et al., 2011, Nga et al., 2011]. Since then, several more mesoniviruses have been identified, primarily in mosquitoes [Vasilakis et al., 2014, Morais et al., 2022].

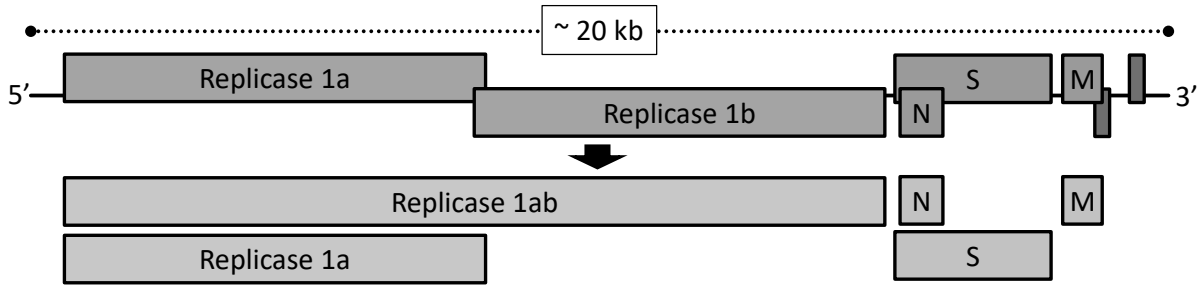


Figure 9: ***Mesoniviridae* genome organization.** Schematic representation of the mesonivirus genome. It is made up by a single strand positive RNA, encoding at least five ORF plus additional small accessory ORFs. From these the replicase polyprotein 1a or by ribosomal frame-shift 1ab, nucleocapsid (N), spike (S) and membrane (M) proteins are produced.

As part of the *Nidovirales*, mesoniviruses share genetic characteristics with *Coronaviridae*, *Arteriviridae* and *Roniviridae* (fig. 9). Virions are about 50 nm, spherical and enveloped with the nucleocapsid (N) and spike glycoprotein (S). Their genome size is about 20 kb positive sense single-stranded RNA with up to seven ORFs, is 5'-capped and 3'-polyadenylated. Translation of the non-structural replication complex occurs from ORF1a and ORF1b by ribosomal frame-shifting. Other ORFs are translated from subgenomic RNAs.

Sandewa- & Nelorpiviruses In 2013 another group of invertebrate infecting viruses was described, this time more related to plant virus taxa of the *Cilevirus*, *Higrevirus* and *Blunervirus* [Vasilakis et al., 2013, Nunes et al., 2017]. The group was named Negeviruses and is subdivided into two polyphyletic groups of Sandewavirus and Nelorpivirus. These

viruses were found in mosquitoes and sand flies (*Phlebotominae*) from the Americas, Europe, Asia and the Pacific.

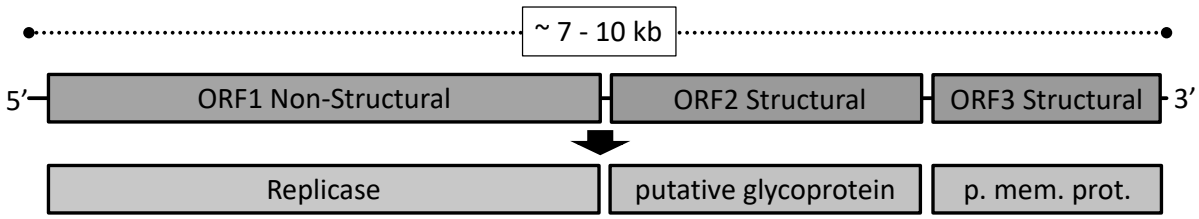


Figure 10: **Negevirus genome organization.** Schematic representation of the negevirus genome. It is made up of a positive sense single-stranded RNA, encoding three ORFs. From these the replicase protein as well as one putative gylco- and one putative membrane-structural protein are produced.

Their virions are of 45 - 55 nm size and hold a 7 - 10 kb sized positive sense single-stranded RNA genomes. It holds 5' and 3' non-coding regions and is 3'polyadenylated. Translation occurs from three ORFs which are separated by short non-coding regions.

1.5 Virus-Host Interactions

Mosquitoes respond with a variety of different mechanisms to infections. These encompass humoral and cellular immune pathways, that have been subject to extensive reviewing [Sim et al., 2014, Blair and Olson, 2014, Tikhe and Dimopoulos, 2021]. Before describing the mechanistics of the pathways relevant to the presented work, a concise depiction on the general outcome of a virus infection on *Culex* spp. mosquito is given here.

Despite reoccurring statements in the scientific literature that "arbovirus establish a persistent infection of mosquitoes" there is -to the authors knowledge- very sparse data to support such a claim, at least in a comprehensive framework that would allow to characterize virus infections on axis like benign/pathogenic or persistent/transient [4].

A somewhat convincing argument for arbovirus persistence in mosquitoes can be made, yet not from experimental *in vivo* infections under controlled conditions, but from a more ecological approach by sampling mosquito populations outside the transmission season. Virus positive mosquitoes during that time have been reported for WNV in *Cx. pipiens*, JEV in *Cx. tritaeniorhynchus* and *Cx. pipiens*, Saint Louis encephalitis virus (SLEV, *Flaviviridae*) in *Cx. pipiens* and *Cx. tarsalis*, Western quine encephalomyelitis virus (*Togaviridae*) in *Cx. tarsalis*, and Sindbis virus (SINV *Togaviridae*) in *Cx. pipiens*, indicating the establishment of a persistent infection of the host, probably as a result

[4]The field of evolutionary infection ecology has tried to establish certain general principles on the matter of *resistance* or *tolerance* phenotypes towards infections [Simms and Triplett, 1994, Råberg et al., 2007]. Both strategies use specific organismic systems to achieve certain evolutionary trade-offs between the infectious agent and its host. Despite a comment conception of an evolutionary "arms race" in host-parasite relationships, a stable equilibrium of tolerance is more likely to occur on evolutionary time scales [Råberg et al., 2009, Carval and Ferriere, 2010, Ayres and Schneider, 2012].

of pathogen tolerance [Rudolf et al., 2021, Nasci et al., 2001, Hayashi and Al, 1975, Lee, 1971, Bailey et al., 1978, Reeves et al., 1958, Blackmore and Winn, 1956, Bergman et al., 2020]. One experimental study with *Cx. tarsalis* was able to show that infectious SINV was still present close to the end of the animal's life span [Theilmann et al., 1984]. Likewise, published data on persistent MSV infections of mosquitoes is currently limited to a single report of a Culex flavivirus (CxFV, *Flaviviridae*) positive laboratory colony of *Cx. pipiens* with continuous infection of its progeny [Bolling et al., 2011].

Regarding *in vitro* models, extensive evidence for the ability of a variety of arboviruses and MSVs alike to establish a persistent infection in aedine cells has been published [Burivong et al., 2004, Kanthong et al., 2010, Scallan and Elliott, 1992, Newton et al., 1981, Elliott and Wilkie, 1986, Fujita et al., 2018, de Oliveira et al., 2021, Weger-Lucarelli et al., 2018, Peleg, 1969, Karpf et al., 1997, Franzke et al., 2018]. For Culex derived cells, the line of evidence is comparably smaller. It has been shown that two commonly used cell lines, CT derived from *Cx. tarsalis* and HSU derived from *Cx. quinquefasciatus* harbor different, and in the case of CT multiple, MSVs [Göertz et al., 2019, Weger-Lucarelli et al., 2018, Rückert et al., 2019]. It was also shown that SLEV can establish a persistent infection in CT cells, and WNV in HSU cells [Randolph and Hardy, 1988, Rückert et al., 2019]. These findings implicate that in general, at least on a cellular level, virus infections of mosquitoes can be tolerated by the host.

Although there may not be conclusive evidence whether MSV infections of mosquitoes are, in general, more transient or persistent in nature, the line of evidence described above makes the assumption of MSVs persistence at least plausible.

As a simplistic framework for the presented study, the author will therefore refer to *acute* infections as the phase in proximity to inoculation and *persistent* infections for phases in which a sustained viral infection can be observed bona fide (thus, a *transient* infection would be characterized as the failure to establish a persistent infection, whether due to the cell/organism succumbing to the infection or by clearance of the infectious agent by the host).

1.5.1 Virus Infection Modulating Pathways

As mentioned earlier, multiple mechanisms that interfere with viral infections in *Culicidae* have been reported, primarily based on evidence from *Aedes* spp. Studies of the immune response upon viral infection of Culex mosquitoes mainly investigated changes at the transcript level. In *Cx. pipiens* infections with WNV, the involvement of Jak-STAT, TOLL, IMD signal transduction pathways, ubiquitination, apoptosis and RNA interference (RNAi) pathways are indicative [Zink et al., 2015, Núñez et al., 2020]. Also, in WNV infections, *Cx. quinquefasciatus* and HSU cells show involvement of the Jak-STAT and RNAi pathways, as well as the involvement of the NF-$_K$B homolog Rel2, the pro-

tein kinase Akt, and the proposed cytokine Vago [Paradkar et al., 2012, Paradkar et al., 2014, Ahlers et al., 2019]. The RNAi pathways have been extensively described for aedine mosquitoes and characterized as the major antiviral immune response [Blair, 2022, Wei et al., 2020, Sanchez-Vargas et al., 2004]. For Culex mosquitoes, the general presence and activity of the RNAi pathway is indicative, but experimental insights are sparse [Walsh et al., 2022, Rückert et al., 2019, Göertz et al., 2019, Altinli et al., 2023].

1.5.2 Mosquito RNAi

As a post-transcriptional repression pathway, RNAi is conserved throughout the eukaryotes and was initially described as being triggered by the presence of double stranded (ds)RNA [Hannon, 2002]. These dsRNA molecules are not only produced by viruses with a dsRNA genome, but also by both positive and negative sense single stranded RNA viruses as well as DNA viruses [Chen and Hur, 2022]. Moreover it was discovered that the RNAi system is also involved in processing endogenous sources of RNAs [Murchison and Hannon, 2004, Parhad and Theurkauf, 2019]. Based on the distinct size and properties of the so called small (s)RNAs that are produced from the RNA substrate, the RNAi pathway is subdivided into three branches: the micro (mi), short-interfering (si) and Piwi-interacting (pi)RNA pathways. All three parts of the RNAi system are well described in a variety of animal plant organisms but hereinafter the focus will be on what is already known about the involved mechanisms from Culex mosquitoes.

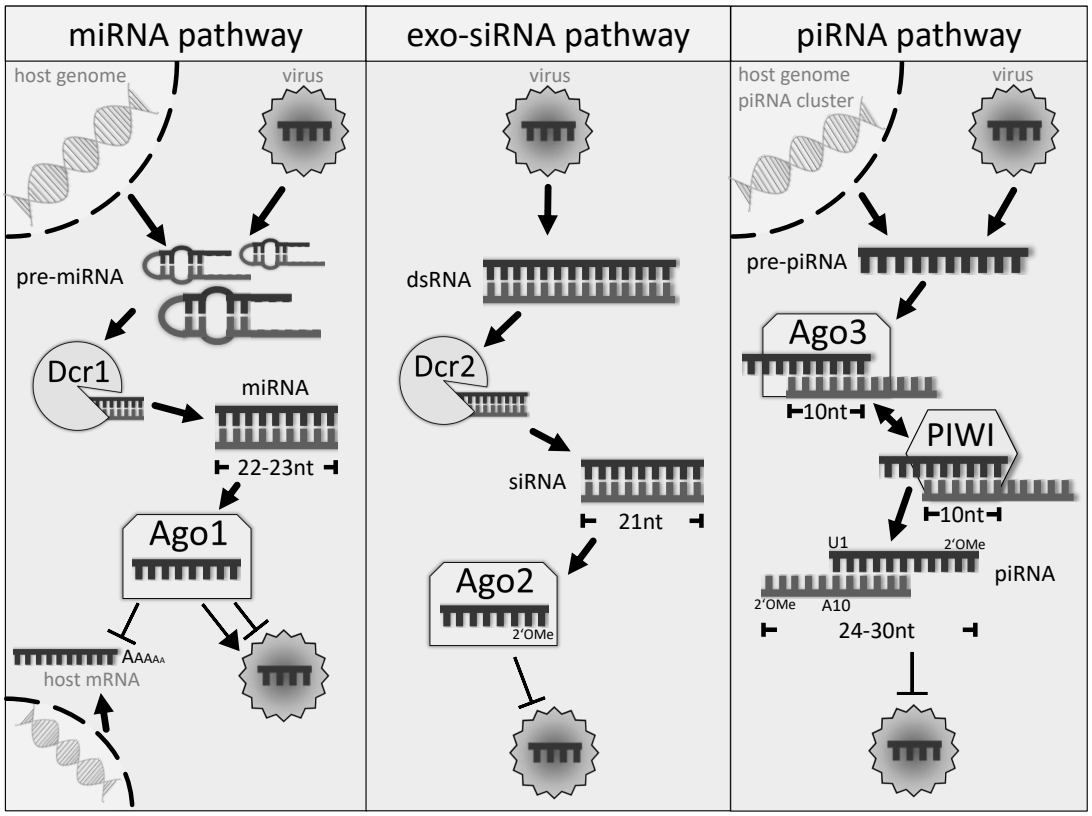


Figure 11: **Mosquito RNAi pathways in viral infections.** Schematic representation of RNA interference pathways. The miRNA pathway (left) is triggered by the occurrence of dsRNA molecules (pre-miRNA) of host or viral origin. Dcr1 converts the pre-miRNA into 22 - 23 nt long duplexes. The miRNA becomes bound by Ago1, which is able to bind complementary target RNA. The exogenous-siRNA pathways (middle) is triggered by the presence of dsRNA molecules, which are cleaved by Dcr2 into 21 nt long siRNA duplexes. A single strand of the duplex is incorporated by Ago2, leading to the degradation of complementary viral RNA. The piRNA pathway (right) is triggered by single stranded pre-piRNA molecules. These can be bound by Ago3 or other PIWI proteins in an amplification cycle between the two parts. As a result, the produced 24 - 30 nt long piRNAs may show either a U1 and/or A10 bias, as well as an 10 nt overlap pattern.

miRNA The effector molecules of the miRNA pathway are approximately 22 - 23 nt long and canonically produced from cellular miRNA genes [Monsanto-Hearne and Johnson, 2020]. The single stranded primary (pri-)miRNA is processed by Drosha and Pasha already in the nucleus. This precursor (pre-)miRNAs are transported into the cytoplasm and further processed by the RNase Dicer-1 (Dcr1) into a duplex. The miRNA becomes bound to the protein Argonaut-1 (Ago1) and this complex binds parts of mRNA targets. As a result these are either degraded, their translation repressed, or they even become stabilized [Huntzinger and Izaurralde, 2011, Vasudevan, 2012]. These gene expression mediating processes primarily play a role in the development of multiple insect organ systems, but have also been shown to be involved in the context of virus infections [Lucas et al., 2015, Asgari, 2013, Hussain et al., 2013, Monsanto-Hearne and Johnson, 2018].

In aedine mosquitoes, multiple miRNAs that influence infections with WNV and DENV, have been identified [Slonchak et al., 2014, Hussain and Asgari, 2014, Avila-Bonilla et al., 2017, Yan et al., 2014, Zhou et al., 2014]. Also, viruses, like WNV, produce miRNAs that target host transcripts [Hussain et al., 2012, Aguado and tenOever, 2018].

siRNA The exogenous (exo)-siRNA pathway is triggered by the presence of exogenous dsRNA molecules. These structures are recognized by Dicer-2, which cleaves them into, in the case of mosquitoes, 21 nt long duplexes. Again, one of the strands is degraded while the other is bound by Argonaut-2 (Ago2). The siRNAs loaded onto Ago2 also become 3' methylated (2'OMe). The production of virus derived (v)siRNA is observed upon infection for a multitude of viruses, in both *Aedes* and *Culex* cell culture and organismic systems. In both CT and HSU cells, the production of vsiRNAs early in infection with WNV have been reported [Rückert et al., 2019]. Furthermore the presence of vsiRNA in a persistent infection with WNV in HSU, as well as Phasi Charoen-like phasivirus (PCLV, *Bunyavirales*), Calbertado virus (CLBOV, *Flaviviridae*), Flock House virus (FHV, *Nodaviridae*), Culex narnavirus 1 (CxNV, *Narnaviridae*), all considered to be MSVs, of CT cells was shown [Rückert et al., 2019, Göertz et al., 2019]. For WNV the production of vsiRNAs has been shown in both *Cx. pipiens* and *Cx. quinquefasciatus* [Fros et al., 2015, Rückert et al., 2019].

piRNA Unlike the other two pathways, the piRNA pathway seems not to be induced by dsRNA structures. Canonically the piRNA converse resistance against transposons [Brennecke et al., 2007, Duc et al., 2019, Thomson and Lin, 2009, Saito and Siomi, 2010]. Hence the name of the proteins associated with the pathway, from P-element Induced WImpy testis (PIWI), with P-elements being the transposable elements in the *D. melanogaster* genome [Lin and Spradling, 1997]. Contrary to other eukaryotes, where the piRNA pathway is primarily active in germline cells, mosquitoes show activity of the pathway also in somatic tissues and in response to virus infections [Varjak et al., 2018]. The effector molecules of the piRNA pathway have a broad size range from between 24 - 30 nt. But piRNAs may share four further general characteristics, if they are produced from the so called 'ping-pong' amplification cycle. The piRNA molecules that are produced by this cycle may exhibit a nucleotide bias for an uridine at the first position (U1) on one strand and a 10 nt overlap pattern to a complementary strand, resulting in a bias for adenosine at the 10th position (A10) and are 3'terminal methylated (2'OMe). As the piRNA precursor is a single strand, virus derived (v)piRNAs originate, with exceptions, from the virus genome strand and, in contrast to vsiRNAs, are produced from more distinct genome loci. Production and possible amplification of piRNA production is Dcr-independent but involves Argonaut-3 and PIWI proteins.

The same studies that reported vsiRNA production described above also looked for the presence of piRNAs. For both CT and HSU cells production of vpiRNA-like molecules

have been described for infection with WNV, as well as PCLV and Merida virus (MERDV, *Rhabdoviridae*) [Rückert et al., 2019, Göertz et al., 2019]. Though the characteristics of the induced vpiRNA-like molecules appear to differ between WNV and PCLV/MERDV, with the latter exhibiting signs of 'ping-pong' amplification. Interestingly there were no sRNAs that resemble vpiRNAs detected from the other co-infecting MSVs (CLBOV, FHV, CxNV), from which vsiRNA production was observed, and WNV derived piRNA production also was not consistent in all studies. Yet, in WNV infected *Cx. pipiens* production of piRNA sized sRNAs was shown, but without signs of ping-pong dependent amplification, while in *Cx. quinquefasciatus* no such presence was indicated [Fros et al., 2015, Rückert et al., 2019].

1.6 Virus-Virus Interactions

From an anthropocentric point of view, the most relevant implication for research on MSVs is the question of how they interact with arbovirus infection and transmission, given the reasonable assumption that a MSV infection is already acquired and maintained in a mosquito by the time of arbovirus infection. Virus co-infections are a phenomenon commonly observed throughout all domains of life [DaPalma et al., 2010]. And in general, these interactions, either as simultaneous or sequential co-infections, can be described as either competitive, cooperative or benign coexistence. Regarding the diverse genetic backgrounds of hosts, arboviruses and MSV, it should not invoke surprise in finding all these kinds of outcomes reflected in the Culex-Flavivirus-MSV systems.

In *Culex tritaeniorhynchus* derived CTR cells that were inoculated with CxFV and passaged multiple times before challenge showed reduced titers of JEV at four days post infection, corresponding to a decline in JEV positive cells. At the same time, DENV infection only showed a decreased growth of titers with no decline of DENV infected cells [Kuwata et al., 2015]. Another study involved a colony of *Cx. pipiens* with continuous CxFV infections by vertical transmission, showed a slightly delayed dissemination rate in the early phase of WNV infection [Bolling et al., 2012]. *Culex annulirostris* that were intrathoracally injected with PCV and received an oral WNV infection a week later, showed reduced infection and transmission rates, but no reduction in WNV titers [Hall-Mendelin et al., 2016]. In a study with CxFV and WNV, effects in *Cx. quinquefasciatus* one strain showed no alteration of WNV infection, replication, dissemination or transmission. In contrast, in another strain, obtained from a different geographic location, WNV transmission rates were increased in acute co-infections but not sequential infections [Kent et al., 2010]. The same study that reported a negative effect of Nhumirim virus on WNV in simultaneous infections of *Cx. quinquefasciatus* but no such effect was observable in *Cx. pipiens* [Goenaga et al., 2015]. There are also two reports on an ecological association of CxFV and WNV in *Culex* spp. mosquitoes, with one reporting an increased likelihood of CxFV infections in WNV infected pools and the other finding no such correlation

[Newman et al., 2011, Crockett et al., 2012]. And a considerable portion of experimental data suggesting negative interference of MSVs with Flaviviruses were obtained from infections of C6/36 cells [Goenaga et al., 2020, Goenaga et al., 2015, Hobson-Peters et al., 2013, Kenney et al., 2014, Bolling et al., 2012] [5].

For both Negeviruses and Mesoniviruses, not much is known about their interactions with arboviruses. For two Negeviruses, Negev virus and Piura virus, a reduction of replication for a variety of alphaviruses during parallel infections in *Aedes albopictus* derived C7/10 cells have been reported [Patterson et al., 2021]. For the Mesonivirus Yichang virus (YicV), a reduction of DENV and ZIKV titers in parallel and sequential (12 hpi) infections in C6/36 has been reported [Ye et al., 2020]. DENV titers were also reduced in parallel YicV infections of Aag2 cells, but ZIKV titers were not significantly affected. For both set-ups, no effect in the titers of JEV could be detected. In the same study, *Ae. albopictus* that received a 1:1 YicV-DENV containing blood-meal showed reduced DENV genome copies in their heads as well as reduced infection and transmission rates at 7 and 14 dpi.

1.7 Agenda

From the atrocity of the Atlantic slave trade, responsible for the emergence of YFV and DENV in the Americas, to today's neglect of tropical diseases to the ongoing obstruction of ecosystems on a global scale and the impending drastic change of the planetary climate system, the emergence of zoonotic diseases, like arboviruses, are and will, as accompanying factor of the economically motivated exploitation of humans and the planet remain a burden for most societies [Friedman, 2021, Weaver and Reisen, 2010, Liang et al., 2015, Robert et al., 2020, Franklinos et al., 2019, Jamrozik and Selgelid, 2016, Wilder-Smith et al., 2017, Aguirre and Tabor, 2008, Jones et al., 2008]. Studying arboviruses and MSVs will be helpful in understanding the dynamics of transmission cycles [Vasilakis and Tesh, 2015]. Additionally, technological applications of MSVs for reducing arbovirus disease burden by altering vector competence of mosquitoes, or as novel vaccine platforms have been discussed [Bolling et al., 2015, Öhlund et al., 2019b, Nouri et al., 2018, Patterson et al., 2020, Hobson-Peters et al., 2019].

As outlined above, knowledge of the general biology of MSVs, their interactions with both the host immune system and arboviruses, particularly if they belong to non-arbovirus families, lags behind their mere identification. In order to advance the understanding of MSVs, as well as to give a comprehensive example of approaching related research questions from identification and characterization towards the establishment of experimental

[5] Though results from these experiments appear to be less relevant compared to the aforementioned, as C6/36 are derived from *Aedes* spp. mosquitoes and, even more concerning, show a radically different genetic host background as they exhibit a truncation of Dcr2, resulting in a siRNA deficient phenotype [Brackney et al., 2010, Morazzani et al., 2012].

systems as a basis for possible applications, the presented work included the following objectives:

- Analysis of a viral specimen from field samples and reconstruction of genomes and phylogenetic relationships.
- Characterization of the MSV interactions with the mosquito RNAi system during the early and late phases of infection.
- Establishment of both *in vitro* and *in vivo* models to study the interactions of MSVs and arboviruses.
- Experimental evaluation on the effects of MSV co-infections in both the acute and persistent phase on arbovirus infections and the hosts RNAi system.

2 Materials & Methods

2.1 Materials

Table 2: **Chemicals, Kits, Consumables, Devices, Antibodies, Viruses, Mosquitoes, Cell lines, Bacteria & Plasmids**

Chemical	Manufacturer / Supplier
2x MEM	Gibco / Thermo Fisher Scientific Inc., Waltham, MA, USA
2x L-15	Gibco / Thermo Fisher Scientific Inc., Waltham, MA, USA
3M sodium acetate	Invitrogen / Thermo Fisher Scientific Inc., Waltham, MA, USA
Agarose	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ampicillin	Gibco / Thermo Fisher Scientific Inc., Waltham, MA, USA
Borate buffer	Thermo Fisher Scientific Inc., Waltham, MA, USA
Chloroform 99 %	Sigma-Aldrich / Merck KGaA, Darmstadt, Germany
Crystal violet	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich / Merck KGaA, Darmstadt, Germany
DNA gel loading dye	Thermo Fisher Scientific Inc., Waltham, MA, USA
dNTP Set	Thermo Fisher Scientific Inc., Waltham, MA, USA
DPBS	PAN-Biotech GmbH, Aidenbach, Germany
Dulbecco's Modified Eagle Medium (DMEM)	PAN-Biotech GmbH, Aidenbach, Germany
Ethanol 99 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ethidiumbromide	AppliChem GmbH, Darmstadt, Germany
Fetal calf serum (FCS)	Biochrom AG, Berlin, Germany
Formaldehyde	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
GeneRuler 1 kb Ladder	Thermo Fisher Scientific Inc., Waltham, MA, USA
GeneRuler 100 bp Ladder	Thermo Fisher Scientific Inc., Waltham, MA, USA
Glycerol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Glycogen	Thermo Fisher Scientific Inc., Waltham, MA, USA
Isopropanol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
KCl	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
KH2 PO4	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Leibovitz's L-15 medium	Gibco / Thermo Fisher Scientific Inc., Waltham, MA, USA
Na2 HPO4	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
NaCl	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Oligo(dT)18 primer	Thermo Fisher Scientific Inc., Waltham, MA, USA
Penicillin/Streptomycin	Gibco / Thermo Fisher Scientific Inc., Waltham, MA, USA
Phosphate Buffered Saline (PBS)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Random Hexamer Oligonucleotides	Thermo Fisher Scientific Inc., Waltham, MA, USA
RNAse inhibitor	Promega Corp., Fitchburg, WI, USA
Sodium acetate	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodium periodate	Sigma-Aldrich / Merck KGaA, Darmstadt, Germany
Tragacanth	Sigma-Aldrich / Merck KGaA, Darmstadt, Germany
TRIS	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Triton X 100	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
TRIzol Reagent	Invitrogen / Thermo Fisher Scientific Inc., Waltham, MA, USA
Trypsin / EDTA	PAN-Biotech GmbH, Aidenbach, Germany
Tryptose phosphate broth	Gibco / Thermo Fisher Scientific Inc., Waltham, MA, USA
LB medium	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
LB agar	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
DharmaFECT2	Horizon Discovery Ltd., Cambridge, UK
Opti-MEM	Thermo Fisher Scientific Inc., Waltham, MA, USA
Kit	
GoTaq DNA polymerase	Promega Corp., Fitchburg, WI, USA
M-MLV reverse transcriptase	Promega Corp., Fitchburg, WI, USA
Monarch RNA Cleanup Kit 50 µg	NEB, Ipswich, MA, USA
NucleoSpin Gel and PCR Clean-up	Macherey-Nagel, Düren, Germany
QuantiTect SYBR Green PCR kit	Qiagen, Hilden, Germany

QuantiTect Probe PCR Master Mix	Qiagen, Hilden, Germany
NucleoSpin Plasmid EasyPure	Macherey-Nagel, Düren, Germany
Consumable	
25 ml pipetting reservoir	Argos Technologies / Cole-Parmer GmbH, Wertheim, Germany
96 well microplate, PS, F-bottom	Greiner Bio-One GmbH, Frickenhausen, Germany
Adhesive PCR seal	Roche, Basel, Switzerland
Biosphere Filter Tips 10 µL - 1000 µL	Sarstedt AG & Co. KG, Nümbrecht, Germany
Cell culture flask T25, T75	Greiner Bio-One GmbH, Frickenhausen, Germany
Cell scraper 25 cm	Sarstedt AG & Co. KG, Nümbrecht, Germany
Conical Centrifugation tube 15 mL, 25 mL	Sarstedt AG & Co. KG, Nümbrecht, Germany
Cryo Tube vials	Thermo Fisher Scientific Inc., Waltham, MA, USA
Disposal bags	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Eppendorf tubes	Thermo Fisher Scientific Inc., Waltham, MA, USA
LightCycler 480 Multiwell plate 96, white	Roche, Basel, Switzerland
Multiply-µStrip Pro 8-strip PCR tubes	Sarstedt AG & Co. KG, Nümbrecht, Germany
Parafilm	Bemis, Oshkosh, NE, USA
Serological pipets 5, 10, 25 ml	Corning Inc., Glendale, AZ, USA
Syringe (1, 2, 10 ml)	B. Braun Melsungen AG, Melsungen, Germany
TC plate 6, 12, 24 wells	Sarstedt AG & Co. KG, Nümbrecht, Germany
Device	
Agarose Casting stand MultiCast	PEQLAB / VWR, Darmstadt, Germany
Agarose gel chamber	PEQLAB / VWR, Darmstadt, Germany
Centrifuge 5415D	Eppendorf, Hamburg, Germany
Centrifuge ct15 himac	VWR, Darmstadt, Germany
Centrifuge Labofuge 400 R	Heraeus Holding GmbH, Hanau, Germany
Centrifuge Mini Star	VWR, Darmstadt, Germany
Centrifuge Pico 17	Thermo Fisher Scientific Inc., Waltham, MA, USA
Erlenmeyer flask 1000 mL	VWR, Darmstadt, Germany
Evos FL Fluroescenc Microcsope	Thermo Fisher Scientific Inc., Waltham, MA, USA
FlexCycler	Analytik Jena AG, Jena, Germany
Imaging System ChemiDoc Touch	Bio-Rad Laboratories GmbH, München, Germany
Incubator Function line	Heraeus Holding GmbH, Hanau, Germany
Incubator Heraeus 6000	Heraeus Holding GmbH, Hanau, Germany
Incubator innova co 170	Thermo Fisher Scientific Inc., Waltham, MA, USA
Metal Block Thermostat MBT 250	ETG, Ilmenau, Germany
Microscope AE2000	Motic, Wetzlar, Germany
Mini see-saw rocker SSM4	Stuart / Cole-Parmer, UK
Mr. Frosty Cryo Freezing Container	Thermo Fisher Scientific Inc., Waltham, MA, USA
Multipette 5 µL - 50 µl / 50 µL - 300 µl	PZ HTL S.A., Poland
NanoDrop 1000 Spectrophotometer	peqLab Biotechnologie GmbH, Erlangen, Germany
Neubauer counting-chamber	P. Marienfeld GmbH & Co. KG, Lauda Königshofen, Germany
Pipetus	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany
PowerPac 300	Bio-Rad Laboratories GmbH, München, Germany
Research Plus Pipettes 10 µL, 100 µL, 1000 µL	Eppendorf AG, Hamburg, Germany
Scale Scout Pro	OHAUS Europe GmbH, Switzerland
Thermomixer comfort	Eppendorf, Hamburg, Germany
Vortex Genie 1 & 2	Scientific Industries, Bohemia, USA
Watherbath WNB45	Memmert, Schwabach, Germany
Antibodies	**Reference**
3G1.1 dsRNA Hybridoma	[O'Brien et al., 2015]
AlexaFluor 488 goat anti-mouse IgG	Invitrogen / Thermo Fisher Scientific Inc., Waltham, MA, USA
Virus	**Source, strain, Accession**
WNV lineage 1	[Rossini et al., 2011], TOS-09 Ferrara, HM641225.1
WNV lineage 2	[Ziegler et al., 2019], Germany 2018, MH924836.1
USUV	[Cadar et al., 2017], BNI-491, KY113092.1
NIEV	[Junglen et al., 2017], B51/CI/2004, NC 024299.2
EILV	[Nasar et al., 2012], EO329, JX678730.1
Mosquito	**Reference**
Culex quinquefasciatus	Malaysia, Bayer AG
Culex pipiens molestus	Mol S, [Leggewie et al., 2016]

Cell lines / Bacteria	Species, Reference
CT	*Culex tarsalis*, [Chao and Ball, 1976]
HSU	*Culex quinquefasciatus*, [Hsu et al., 1970]
C6/36	*Aedes albopictus*, [Igarashi, 1978]
Aag2-AF5	*Aedes aegypti*, [Varjak et al., 2017]
Aag2-AF319	*Aedes aegypti*, [Varjak et al., 2017]
Aag2-AF525	*Aedes aegypti*, [Scherer et al., 2021]
Vero ATCC CCL-81	*Chlorocebus aethiops*, [Y, 1963]
Escherichia coli XL-10 Gold	Tetr Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F'proAB lacIq ZΔM15 Tn10 (Tetr) Amy Camr], Stratagene, La Jolla CA, USA
Escherichia coli DH5α	F- Φ80lacZΔ M15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ-, Thermo Fisher Scientific Inc., Waltham, MA, USA
Plasmids	**Type, Reference**
pPUb-myc-Ago2	*Aedes aegypti* Ago2, [Varjak et al., 2020]
pPUb-myc-Dcr2	*Aedes aegypti* Dcr2, [Varjak et al., 2017]
pPUb-myc-eGFP	eGFP, [Varjak et al., 2017]
pPUb-myc-Dcr2 D1198A, E1548A, D1444A, E1548A (mtR3)	*Aedes aegypti* Dcr2*RNase IIIA/B, [Gestuveo et al., 2022]
pPUb-myc-Dcr2 P1464L	*Aedes aegypti* Dcr2*RNase IIIB, [Gestuveo et al., 2022]
pPUb-myc-Dcr2 K39N	*Aedes aegypti* Dcr2*helicase motif I, [Gestuveo et al., 2022]
pPUb-myc-Dcr2 G488R	*Aedes aegypti* Dcr2*helicase motif VI, [Gestuveo et al., 2022]
pPUb-myc-Dcr2 Y232G	*Aedes aegypti* Dcr2*helicase motif IV, [Gestuveo et al., 2022]

Table 3: **Stock Solutions**

Buffer	Amount	Reagent
TAE 10X	242 g	TRIS
	57.1 mL	5.7 % acetic acid
	100 mL	EDTA (pH 8.0) / 0.5 M stock solution
	ad 1000 mL	water
PBS 10X	8 g	NaCl
	0.2 g	KCl
	1.44 g	Na2 HPO4
	0.24 g	KH2 PO4
	ad 1000 mL	water
PBST	3 mL	Triton X 100
	60 mL	PBS 10X
	ad 600 mL	water
Blocking solution	1 mL	FCS
	9 mL	PBST
Crystal violet 10X	10 g	Crystal violet
	50 mL	Formaldehyde (37 %)
	100 mL	Methanol
	ad 340 mL	water

2.2 Bioinformatics

2.2.1 small RNA sequence analysis

To analyze sRNA sequencing data a workflow within the Galaxy RNA framework was constructed (available at: `https://rna.usegalaxy.eu/u/jonny/w/srna-v4`, fig. 12).

The fastq formatted data obtained from BGI were already quality checked and trimmed. The reads were mapped on the respective reference genomes using BWA [Li and Durbin, 2010] fig. 12. Uniquely mapped reads were sorted in three separated libraries using samtools [Danecek et al., 2021], RSeQC [Wang et al., 2012] deeptools [Ramírez et al., 2016] and bedtools [Quinlan and Hall, 2010]; Library I containing all 18 - 31 nt long reads were counted to create the read size distribution plots. Library II, containing only 21 nt reads and Library III containing 26 - 30 nt reads were used to create bed graphs of read genome coverage distributions. Additionally Library III reads were used to create a nucleotide versus cycle plot to search for nucleotide position biases and to compute sequence overlaps [Antoniewski, 2014].

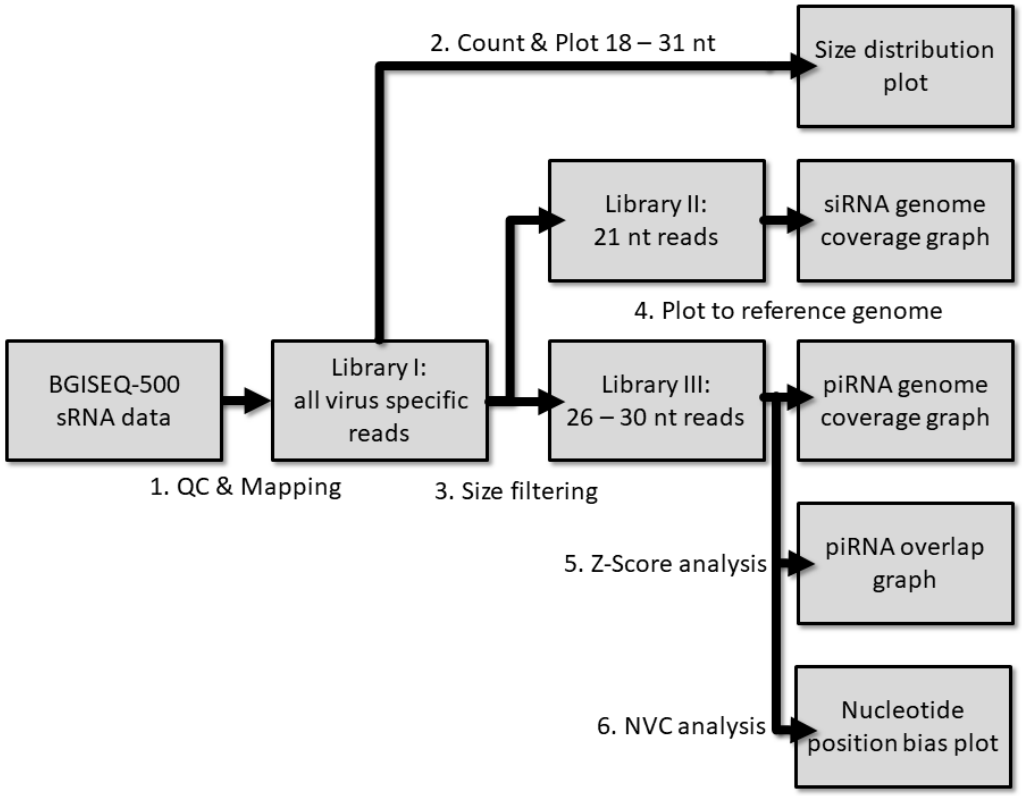


Figure 12: **Schematic flow chart of sRNA analysis workflow.** Size-fractionated RNA reads were (1.) filtered by phred-scores, mapped against the reference genome and again filtered by mapping quality. (2.) All sRNA reads contained in Library I, in the range of 18 nt to 31 nt, were counted and plotted. (3.) All reads in library I. were split into two libraries by size discrimination into only 21 nt reads (Library II) and 26 nt to 30 nt reads (Library III). (4.) Reads of Library II and Library III were plotted against the reference genome individually. (5.) Library 3 reads were used to create overlap graphs and (6.) nucleotide versus cycle plots.

2.2.2 Virus discovery

Paired-end reads were obtained in fastq format and adapter trimmed using trim galore [Krueger, 2021] fig. 13. The primary viral sequence read library was created by mapping the obtained data against the C6/36 reference genome (GCA 001876365.2 [Miller et al., 2018]) using BWA-MEM2 ([Li, 2013]) and discarding all mapped reads. The remaining reads were used as input for trinity ([Grabherr et al., 2011]) using standard settings to obtain potential viral genome sequences. All sequences with sizes greater than 2 kb were identified using BLAST. Highest ranking blast hit species were used as reference to map the reads of the primary library, creating a secondary library for each identified virus species by retaining reads that mapped to the respective viral genome and discarding reads that mapped to other hits. These secondary libraries were subsequently used with the genome-guided model of trinity to obtain the final virus genome sequences.

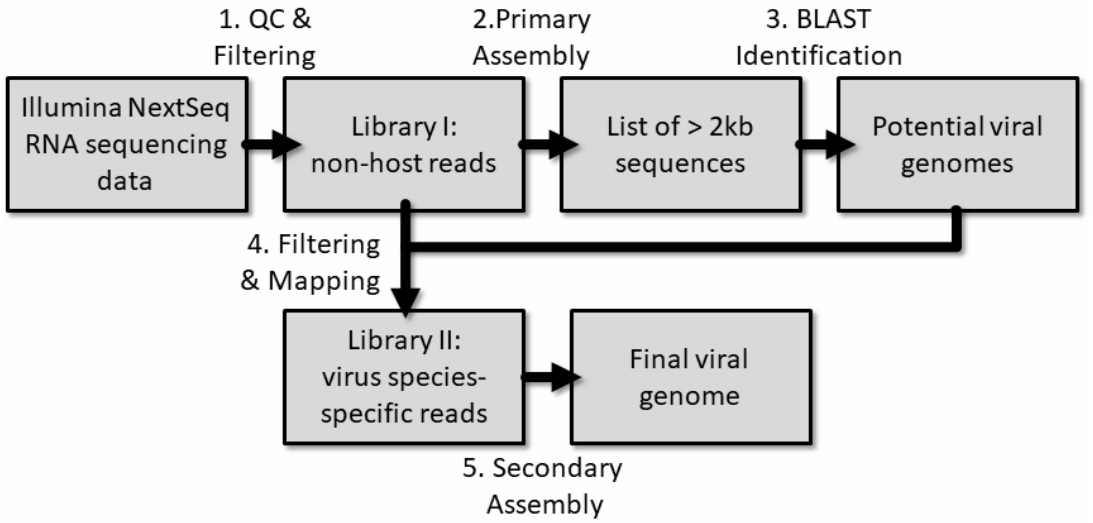


Figure 13: **Schematic flow chart of virus discovery work flow.** Total RNA reads were (1.) quality filtered and mapped against the host cell transcriptome. Mapped reads were removed to create Library I. (2.) de-novo transcript assembly was performed. (3.) Obtained transcript of more than 2 kb size were annotated. (4.) Annotated transcripts were used to filter Library I for identified potential viruses and remaining reads were mapped against the identified transcripts to create Library II (5.) Reads in this library were used for a second iteration of transcript assembly, to create the final viral genome.

2.2.3 Molecular Phylogeny

For virus genome sequences, open reading frames (ORFs) were identified and translated into amino acid sequences. Conserved protein domains were identified and concatenated. Alignment and photogenic inference was performed using MEGA X.

For the NegeV, methyltransferase, helicase and RNA-dependent RNA-polymerase (RdRp) domains were used. LG substitution model with gamma distribution and invariant sites was chosen.

For MesoV, $3CL_{PRO}$, RdRp and helicase were used. LG+F substitution model with gamma distribution was chosen.

Both Inferences were based on 1000 replicates.

2.3 Mosquito

2.3.1 Breeding

Culex quinquefasciatus (Malaysia, Bayer AG) and *Culex pipiens molestus* (Mol S, [Leggewie et al., 2016]) were held in an insectarium at the BNITM at 28°C and 75% humidity with an 12h diurnal light cycle. Adults mosquitoes were supplied with 8% fructose solution on cotton pads *ad libitum*. To induce egg production, adults mosquitoes were offered an artificial blood meal (equal parts of human erythrocyte concentrate and FCS with 0.5% fructose) also on cotton pads. For experiments, adult mosquitoes were aspired from rear-

ing cages and anesthetized with CO_2 to be transferred to vessels in groups of 10 to 40 individuals.

2.3.2 Infection

In general, mosquitoes were starved for one day prior to infection. After infection, mosquitoes were provided with fructose feeding solution on cotton pads *ad libitum*. Incubation was performed in climate chambers with either 27°C +/-5°C (over 24h), 70% RH when mosquitoes were infected with $1 \cdot 10^7$ TCID50/mL WNV, or 27°C, 70% RH in any other infection assay.

Per Os For viral exposure of mosquitoes by the oral route, cotton sticks were soaked in a the respective feeding containing solution $1 \cdot 10^7$ TCID50/mL of the respective virus and inserted into the vessel. After one to two hours at room temperature, individuals were anesthetized with CO_2 and inspected. Visually engorged mosquitoes were kept, non-fed ones were sacrificed.

Per Intrathoracic Injection For direct inoculation of mosquitoes, virus stock solution was diluted to target titers in PBS. Under concurrent CO_2 anesthetization mosquitoes were injected 60 nL solution containing 70 TCID50/mL of the respective virus into the dorsolateral region of the thorax. Injections were performed using the Drummond Nanoject II (Drummond Scientific Company, USA) with self crafted glass capillaries.

2.3.3 Homogenate Preparation

To obtain samples from whole mosquitoes, they were anesthetized with CO_2, transferred to individual vessels and frozen at -80°C at least over night. Afterwards homogenization of mosquitoes was performed in 500 µL PBS per mosquito, using an electric pestle.

2.4 Cell

2.4.1 Culture

Mosquito Cells *Culex tarsalis* derived CT, *Culex quinquefasciatus* derived HSU, *Aedes albopictus* derived C6/36 and *Aedes aegypti* derived AF5 cells as well as their derived Dcr2 knock-out line AF319 [Varjak et al., 2017] and Ago2 knock-out line AF525 [Scherer et al., 2021] were cultivated at 28°C in Leibovitz's L-15 Medium supplemented with 10% fetal calf serum (FCS), 1 % penicillin & streptomycin (P/S) and 10% tryptose phosphate broth.

Mammalian Cells African green monkey (*Chlorocebus aethiops*) kidney epithelia derived cells Vero were cultivated at 37°C in an 5% CO_2 atmosphere in Dulbecco's modified Eagle Medium (DMEM) supplemented with 5% FCS and 1% P/S.

2.5 Virus

2.5.1 Propagation

WNV lineage 1 [Rossini et al., 2011], lineage 2 [Ziegler et al., 2019] and USUV [Cadar et al., 2017] were grown on Vero cells. Sample 8345 [Boerstler, 2016], NIEV strain B51/CI/2004 [Junglen et al., 2017] and EILV [Nasar et al., 2012] were grown on C6/36. To a monolayer of cells in a T25 flask with 5 mL culture medium 5 µL virus solution was added and incubated until a pronounced CPE was observable. Afterwards the medium was centrifuged for 5 min at 1000 x g. The supernatant was stored at -80°C. Titers estimation by TCID50 was performed using the same cell types.

2.5.2 Plaque Isolation

For plaque isolation, $2 \cdot 10^5$ C6/36 in 1 mL medium per well were seeded onto a 12 well plate. The media was removed and the cells were inoculated with 200 µL of a serial dilution of virus stock in L15. After 1 h incubation at respective culture conditions, the cells were covered with 2 mL 4% tragacanth 1:1 4% media (2x L-15 with 4% FCS) solution. After onset of CPE, single plaques were picked and transferred to $4 \cdot 10^4$ C6/36 in 200 µL medium on a 12 well plate.

2.5.3 TCID50

For TCID50 $4 \cdot 10^4$ of either Vero or C6/36 cells in 180 µL of L15 or DMEM culture medium respectively per well were seeded onto a 96 well plate. The next day the cells were inoculated with 20 µL virus stock solution of a serial dilution in L15 or DMEM respectively. After the appropriate incubation period with regular culture conditions, the cells were fixated with 8% formaldehyde for 30 - 60 min. After incubation the plates were washed with tap water. Then the cells were stained with 100 µL crystal violet. The CPE status of the dilutions steps was evaluated and the TCID50 computed according to the Spearman-Kaerber algorithm [Hierholzer and Killington, 1996].

2.6 Molecular

2.6.1 End-Point PCR

End-point PCR was performed for amplification of specific target sequences. For a single reaction the reagents and volumes as specified in table 4 were mixed with the primer pairs listed in table 10. Subsequent amplification cycling was performed using the FlexCyler (analytikjena) thermocycler with the parameters as specified in table 5.

Table 4: **End-point PCR Master Mix.**

Reagent	**Volume (µL)**
Template DNA	2
Forward primer (10 pmol)	2.5
Reverse primer (10 pmol)	2.5
dNTPs (10mM)	1
GoTaq polymerase	0.25
GoTaq 5x buffer	10
ddH_2O	ad 50

Table 5: **GoTaq PCR Cycling Parameters.**

Temperature (°C)	**Time (sec.)**	**Repeats**
95	120	
95	30	35
T_M-2	30	
72	3 per 100 nt	
72	420	

2.6.2 Real-Time PCR

For quantification of RNA levels Quantitect SYBR Green PCR Kit (Qiagen) and Quantitect Probe PCR Kit (Qiagen) were used. The reagents used for a single reaction are listed in table 6 and table 7, and were mixed with primer pairs and probes listed in table 10. Sample amplification and detection was performed with a LightCycler 480 (Roche) using the cycling protocol specified in table 8 for SYBR Green and in table 9 for probe based detection systems.

Table 6: **SYBR Real-Time PCR Mastermix.**

Reagent	**Volume (µL)**
2x QuantiTect SYBR Green PCR Master Mix	5
Primer Mix 0.3 µM fw & rv	0.6
ddH_2O	3,4
cDNA	1

Table 7: **Probe Real-Time PCR Mastermix.**

Reagent	**Volume (µL)**
2x QuantiTect Probe PCR Master Mix	5
Primer Mix 0.4 µM fw & rv	0.8
ddH_2O	2.9
cDNA	1

Table 8: **SYBR Green Real-Time PCR Cycling Parameters**

Name	**Temperature (°C)**	**Time (Sec.)**	**Analysis Mode**	**Repeats**
Inition	95	900		
Cycling	94	15	Quantification	45
	60	30		
	72	30		
Melting Curve	95	5	Melting curve	
	50	15		
	95	1		
End	40	∞		

Table 9: **Probe Real-Time PCR Cycling Parameters**

Name	**Temperature (°C)**	**Time (Sec.)**	**Analysis Mode**	**Repeats**
Inition	95	900		
Cycling	94	15	Quantification	45
	60	60		
End	40	∞		

2.6.3 RNA Isolation

Isolation of total RNA from cells was performed using Trizol or from mosquito homogenates and cell culture supernatants using Trizol L/S Reagent. 1 mL Trizol was added to cells. To 0.6 mL Trizol L/S, 0.2 mL homogenate was added. After 5 min incubation at room temperature, 200 µL chloroform were added and mixed in thoroughly. Afterwards the sample was centrifuged for 15 min at 10500 rpm at 4°C. The aqueous phase was transferred to a new tube and 500 µL isopropanol was added and incubated for 10 min at room-temperature. For samples prepared for sRNA sequencing, isopropanol precipitation was performed with glycogen (10 mg/ml) added. The solution was centrifuged

Table 10: **Oligonucleotide Primers and Probes used for PCR reactions.**

Name		**Sequence** (5' - 3')
DeziV	fw	GGTTCTGCTGCCACGTATTT
	rv	ATTTTTCGAACCTCGGTGTG
DaesV	fw	GCATTGTCCCGAAGTTTTGT
	rv	GTCGTGCTGGATCTCTGTCA
MERDV	fw	AACACCTAGCCATTGCCCTC
	rv	ACAGGGGCACATAGAACAGC
EILV	fw	TCCAGTGACAGACAATCCGC
	rv	GAAGGACGAGCAGACTGTGA
YicV	fw	ATTGCCTCCACCAAGAGAGC
	rv	ATGGCGTCTAGAGTCTCGGT
Cx-Dcr2	fw	GTAGGCTGTTGACTTGTGCG
	rv	ACCGAGGAACGAAGTCCTCT
Cx-Ago1	fw	CACCTTCCCCCAAGAAACCTT
	rv	ATTGTTTGCCTCGCATGTCC
Cx-Ago2	fw	CAGATGGACAAGGTTGGGGT
	rv	CAGTTTGGAGCCAAAGACCAC
Cx-Ago3	fw	CTTGGCACGATCACCCAGT
	rv	CGGAATCTCGTATTCGGAGCA
Cx-Piwi2	fw	CACAAGTCCGGGCGTGAA
	rv	GGGGGAAGATTCTGGTTACGG
Cx-Vago	fw	CCCTGGAGTCGGCGAAACTGC
	rv	CACGAGCACGGAACCGCAAGT
Aae-S7	fw	CCAGGCTATCCTGGAGTTG
	rv	GACGTGCTTGCCGGAGAAC
Aae-Ago2	fw	GGCTGCTCACCCAATGTATCAAGA
	rv	AACCGTTCGTTTTGGCGTTGAT
Aae-Dcr2	fw	CGGGCAAACCCTGTTACATC
	rv	TGTTGGATCCTGCGCAAAC
NIEV	fw	CATGTGGAGTGGGCGGAATA
	rv	TGGGCCAGCTCTAACAGGAA
	pr	Cy5-CCAACCAGTGTTCTTTCCTAGCGATTTCTTC-BHQ2
USUV	fw	CTGAGAAGGGAGGAAAAG
	rv	GCCACAATGAGTGTTATG
	pr	FAM-CGTCCGGCAACTCTTCAAGG-BHQ1
WNV	fw	AGTAGTTCGCCTGTGTGAGC
	rv	GCCCTCCTGGTTTCTTAGA
	pr	FAM-AATCCTCACAAACACTACTAAGTTTGTCA-BHQ1
Cx-GAPDH	fw	TCAAGCAGAAGGTCAAGGAAG
	rv	GTTGTCGTACCAGGAGATGAG
	pr	ROX-TCGACTACACCGAGGAGGAGGTCGTCTCCA-BHQ1

for 10 min at 10500 at 4°C and the supernatant was discarded. 500 µL 70% ethanol was added and centrifuged for 5 min at 8500 at 4°C. Afterwards the ethanol was discarded and the procedure was repeated once. The sample was dried, resolved in H_2O and stored at -20°C.

2.6.4 Gel Electrophoresis

Analysis of DNA fragments was performed with 1% agarose in 1x TAE supplemented with ethidium bromide. Samples were mixed with loading dye. Gels were analyzed using the GelDoc Imaging System (BioRad).

2.6.5 Nucleotide Quantification

The concentration and quality of DNA/RNA was measured and calculated by photospectrometry using 'NanoDrop' (Thermo).

2.6.6 cDNA Synthesis

Synthesis of cDNA from total RNA was performed using M-MLV reverse transcriptase (Promega) according to the manufacturers specifications. In short, 1 µg of RNA sample was mixed with 1 µL random hexamer solution and water to 15 µL. The solution was incubate at 70°C for 5 min and subsequently cooled to 4°C for 1 min and transferred on ice. To the solution 5 µL 5x M-MLV buffer, 1.25 µL 10 mM dNTP mixture, 1 µL RNase inhibitor, 1 µL M-MLV and 1.75 µL water was added. The solution was incubated for 1 h at 37°C and subsequently heat inactivated for 10 min at 70°C.

2.6.7 Beta-Elimination

Methylation status of small RNAs was determined using beta-elimination assay as previously described (Scherer et al. 2021). In short, isolated total RNA samples were equally divided into two portions of 10 µL and 5 µl of 20x borate buffer (50 mM, pH 8.5), 100 µl water and 12.5 µl of sodium periodate (200 mM) for the verum or water for mock treatment were added. After 15 min, 10 µl of glycerol was added and incubated for a further 15 min at room temperature. Afterwards, Ethanol precipitation was performed with 1µL glycogen (10 mg/mL) as carrier. Subsequently, precipitated, dried pellets were resuspended in borate buffer (55 mM, pH 9.5) followed by 90 min incubation at 45° C. RNA was purified with Monarch RNA Cleanup kit (50 µg) (New England Biolabs, Inc., Ipswich, MA, USA) according to the manufacturer's protocol.

2.6.8 Microbiological Transformation and Plasmid Preparation

Transformation of chemo-competent *E. coli* XL-10 gold or DH5α was performed with 50 µL bacterial solution with plasmid solution. The solution was incubated for 30 minutes in

ice and afterwards heat shocked at 42°C for 30 seconds. Samples were cooled for two min on ice and afterwards mixed with 500 µL LB medium. After at least one hour incubation at 37°C with 500 rpm on a rotational shaker, bacteria were plated on selective LB-Agar plates and incubated over night at 37°C.

Amplification of plasmids was performed by picking a single bacterial colony and transferring it to 1.8 mL LB medium with the respective antibiotic. The suspension was incubated over night at 37°C with 250 rpm. Plasmid DNA was extracted with the NucleoSpin Plasmid EasyPure Kit (Machery-Nagel, Düren, Germany) according to the manufacturers protocol.

2.7 Setup of In Vitro Experiments

2.7.1 In Vitro Infections

For both single and co-infections of cell cultures, $2{\cdot}10^5$ cells were seeded in 1 mL L-15 medium into a single well of a 24-well plate. One day after seeding, the medium was removed and replaced with 200 µL infectious medium at the indicated MOI (in case of sample 8345 the titer of the complete sample, not individual virus species was used). After one hour of incubation, the infectious medium was removed, the culture washed with 500 µL medium and further incubated with 1 mL complete medium.

2.7.2 Plasmid Transfection

For transfection of plasmids $2 \cdot 10^5$ cells were seeded onto 24-well plates. One day later a solution containing 500 ng plasmid, 1 µL Dharmafect2 in 100 µL OptiMEM was prepared by vigorous mixing. After 30 minutes, the solution was carefully applied to an individual well.

3 Results

3.1 Discovery and Characterization of MSV from Field Caught Mosquitoes

In a preceding study, [Boerstler, 2016] screened mosquitoes caught from the field in Germany for various viruses. In a pool of three *Coquilletidia richiardii*, collected in 2014, at Kühkopf, Germany, the presence of a mesonivirus was indicated by a pan-*Mesoniviridae* PCR. The sample was used to inoculate C6/36 and after observation of CPE, passaged once more on C6/36. This specimen is referred to as sample 8345.

3.1.1 Next-Generation Sequencing based Discovery of Three Virus Species

Next-generation sequencing (NGS) generated data of the obtained sample was processed to allow for an unbiased discovery of RNA virus genomes. The sample 8345 was processed by the BNITM NGS core facility for RNA sequencing with the Illumina NextSeq system using Mag-MAX Viral RNA Isolation Kit. After random RT-PCR amplification of the RNA, the extracted viral DNA and RNA were subjected to library preparation using a QIAseq FX DNA Library Kit and sequenced using (2×150 bp paired-end) MiSeq Reagent Kits v3. After adapter trimming, the obtained paired-end read data set is composed of mostly 149 nt long reads with an average Phred score of 34. The data set was then mapped against the C6/36 reference transcriptome (GCF_001876365.2). The unmapped reads were retained and further processed by trinity to yield potential viral genomes. This approach suggested the presence of three viruses, two members of the Negevirus (NegV) taxon and one member of the Mesonivirus (MesoV) family. To improve the quality of the genomic information, the initial library was additionally filtered against a reference library of either MesoV or NegV genomes respectively. The remaining reads were aligned to the reference genomes identified in the first step to improve the genome assembly using trinitys genome guided mode. This iterative approach yielded the final three viral genomes in the sample that were used in further analysis.

The first of the two NegV found was identified as Dezidougou virus (DeziV), which is part of the Sandewavirus group, with a global nucleotide sequence identity of 83.1% to the closest isolate (JQ675604.1). The second NegV was in the Nelorpivirus group and identified as Daeseongdong virus (DaesV). It shared 90.8% of global nucleotide identity with its closest known relative (KU095841.1). The concatenated amino acid sequences of the conserved domains of the helicase and RdRp were used to infer their phylogenetic relation within the NegV taxon. Both DeziV and DaesV clustered closely together with their respective species relatives and were therefore considered to be of the same species and can be referred to as DeziV and DaesV strain 8345 respectively (fig. 14 B).

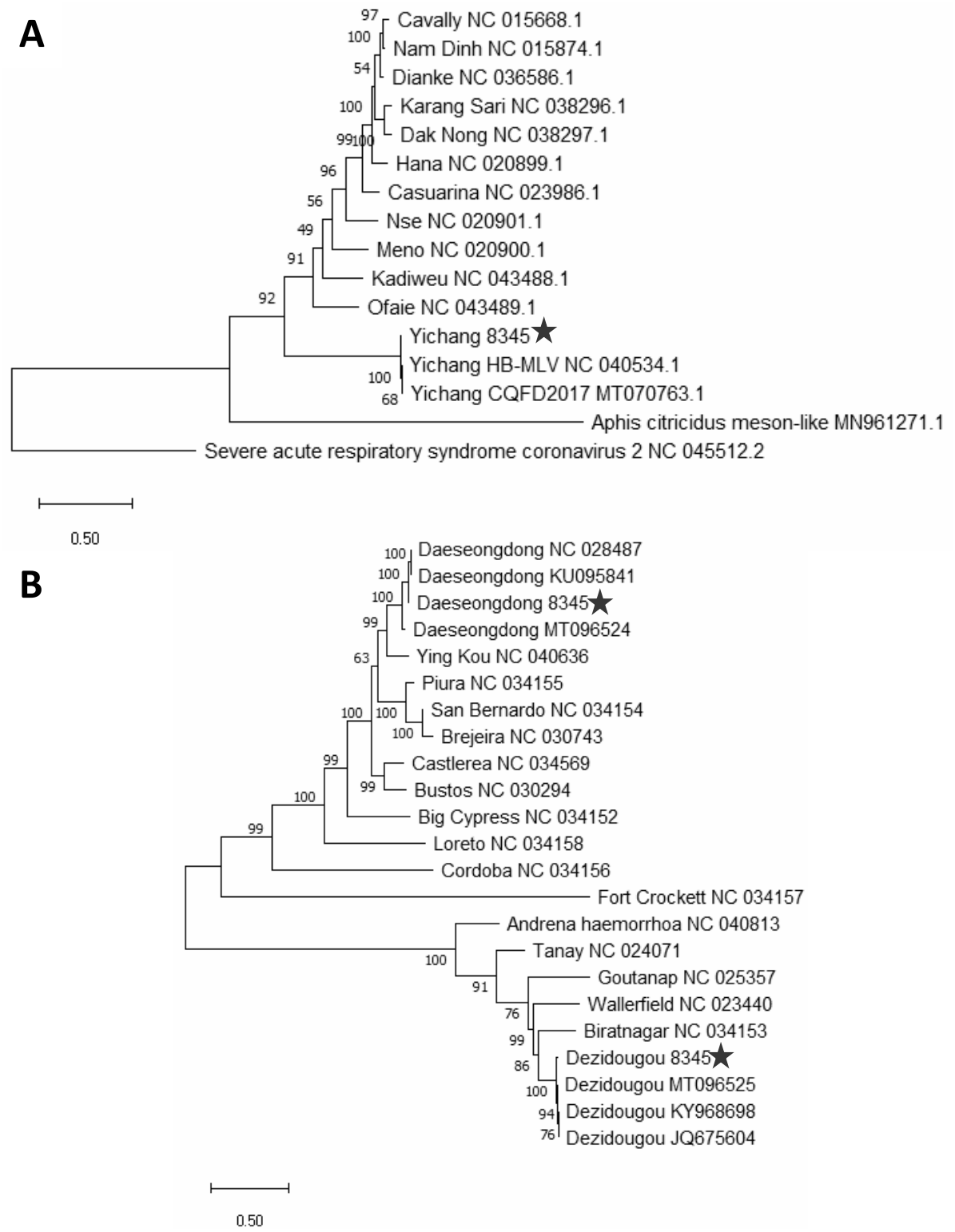


Figure 14: **Maximum likelihood molecular phylogeny of MesoV and NegV.** (A) The phylogenetic inference of MesoV was based on a concatenated amino acid alignment of the conserved regions of the 3CLpro, RdRp and helicase domains. (B) The phylogenetic inference of NegV was based on a concatenated amino acid alignment of the conserved regions of the methyltransferase, helicase and RdRp domains. The virus species identified in this study are marked with a red star. The bootstrap values represent 1000 replicates.

The identified MesoV shared a 96.5% global nucleotide identity with Yichang virus (YicV, MT070763.1). Phylogenetic interference was performed based on the concatenated amino acid sequences of the conserved parts of the $3CL_{PRO}$ and RdRp. A Paired Evolutionary Distance (PED) analysis performed on the same data set resulted in a value of .014 (to NC 040534.1), well under the proposed cut-off value for species demarcation within the *Mesoniviridae* [Cowley and Walker, 2007, Vasilakis et al., 2014]. Consequently, the identified virus was considered to be of the same species and can be referred to as YicV strain 8345 (fig. 14 A). All three identified viruses are the first records of their occurrence in central Europe.

3.1.2 Establishment of DeziV, DaesV and YicV Persistently Infected Cell Lines

As previously shown ([Agboli, 2021], Agboli & Schulze et al., *in prep.*), DeziV, DaesV and YicV can infect both CT and HSU cells. In order to study the effect of both acute and persistent co-infections on arboviruses, the capability of these viruses to establish a persistent infection was investigated.

To this end *Cx. tarsalis* derived CT cells as well as *Cx. quinquefasciatus* derived HSU cells were inoculated with YicV/DaesV/DeziV and passages multiple times (CT 3 passages, HSU 11 passages). At these time points, total RNA was extracted, reverse transcripted and analyzed by end point PCR using DeziV, DaesV and YicV specific primers. Both DeziV and DaesV showed a strong signal in samples from both the CT and HSU cells (fig. 15 left and middle). YicV only produced a faint signal in the sample from the HSU cells, while no amplification occurred in the CT samples. This indicated that both DeziV and DaesV can readily induce a persistent infection in these cell types. At the same time, YicV seems incapable to establish persistence in CT cells but may be able to replicate at a low level in HSU cells.

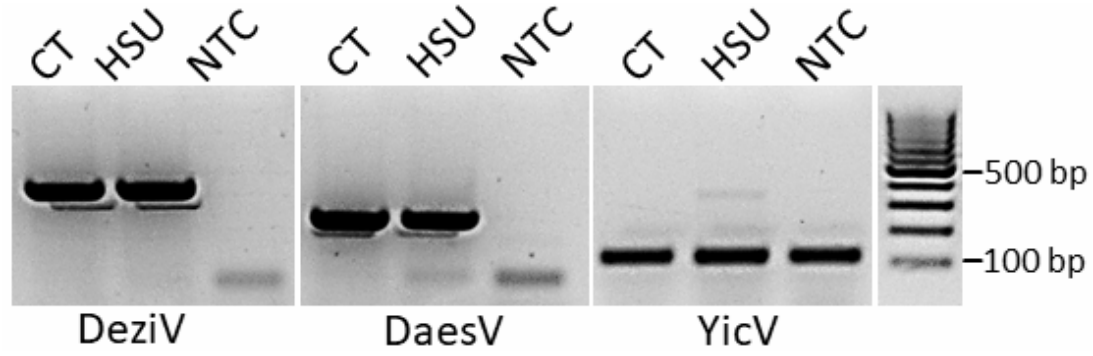


Figure 15: **PCR amplification of DeziV, DaesV and YicV.** CT and HSU cells were inoculated with YicV/DaesV/DeziV MOI=10 and passaged three or eleven times, respectively. Total RNA was extracted, reverse-transcribed and used as template for end-point PCR with specific primer pairs for DeziV, DaesV and YicV.

In order to further characterize the three virus species individually, as well as make them accessible for co-infection experiments, a plaque isolation on C6/36 cells was attempted. The viruses did not induce sharply differentiable plaques. Therefore, it was attempted to pick plaques stochastically at 24 hpi. The samples were subcultured, and after on-set of CPE, total RNA was extracted and analyzed by end point PCR targeting DaesV and DeziV. During multiple iterations of the procedure, it was not possible to obtain samples that were only positive for one of the viruses. Therefore further experiments had to be conducted using the original mixed DeziV, DaesV and YicV sample.

3.1.3 Interactions of DeziV, DaesV and YicV with the Mosquito siRNA System

In a first order to characterize the interactions of Yicv/DeziV/DaesV with the mosquito immune system, namely the siRNA pathway, the replication of YicV in *Ae. aegypti* Aag2 derived AF5 cells and two knock-out cells lines, the Dcr2 deficient AF319 and Ago2 deficient AF525 were performed[6]. The experiments and data collection was kindly performed by C. Scherer.

Respective cells were inoculated with MOI = 0.1 YicV/DeziV/DaesV, and total RNA was isolated 48 hpi. Viral RNA levels were estimated by real-time PCR and normalized to ribosomal S7. In both AF525 and AF319, the levels of YicV vRNA are strongly and significantly reduced compared to AF5, with -6log_2 and -7log_2 reduction, respectively (fig. 16, A).

To further investigate this rather unintuitive result, the expression levels of Dcr2 and Ago2 in infected AF5 cells were analyzed. AF5 cells were inoculated with MOI = 1 YicV/DeziV/DaesV, and total RNA was isolated 48 hpi. Levels of Dcr2 and Ago2 transcripts were estimated by real-time PCR and normalized to ribosomal S7. Compared to expression levels in mock infected AF5, levels of both Dcr2 and Ago2 transcripts were significantly elevated in YicV/DeziV/DaesV infected cells (fig. 16, B).

[6] As the presence of DeziV and DaesV were not known by the time, no quantification of these were performed.

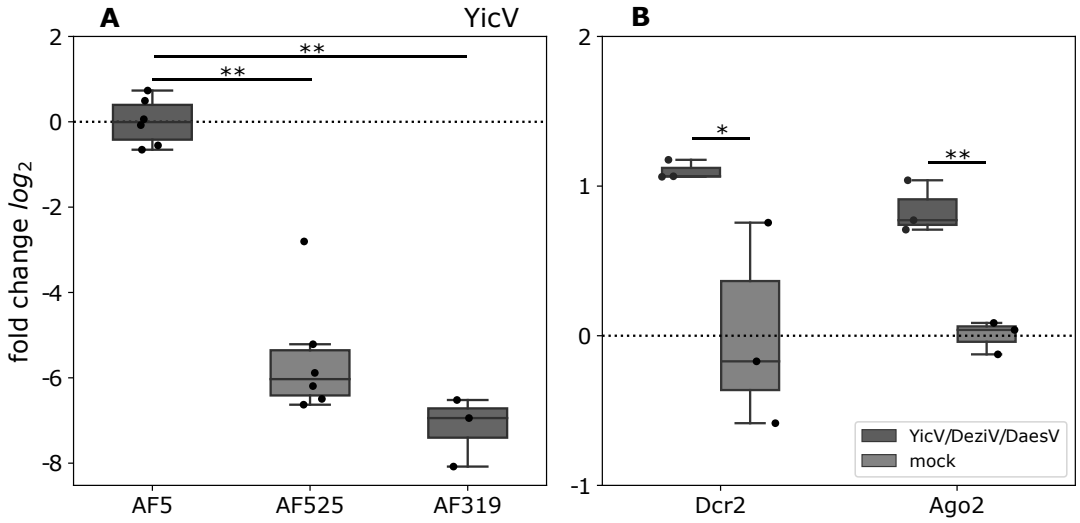


Figure 16: **YicV and Dcr2 & Ago2 levels in *Aedes aegypti* derived cells.** Shown are box plots with individual observations (black circles) of fold change values normalized to ribosomal S7. (A) AF5, AF319 and AF525 cells were inoculated with YicV/DeziV/DaesV MOI = 0.1. At 48 hpi total RNA was isolated and levels of YicV and ribosomal S7 were estimated by real-time PCR. YicV fold change values are expressed relative to AF5. AF5 and AF525 experiments were repeated five, AF319 three times with technical triplicates. (B) AF5 cells were inoculated with YicV/DeziV/DaesV MOI = 1. At 48 hpi total RNA was isolated and levels of Dcr2, Ago2 and ribosomal S7 were estimated by real-time PCR. Dcr2 and Ago2 fold change values are expressed relative to the mock infected controls. Results of three experimental repetitions are shown. Statistical analysis was performed by students t-test for unpaired, two-sided data. Single asterisk (*) indicate p-values ≤ 0.05, double (**) $p \leq 0.01$. Experiments and data collection was performed by C. Scherer.

To solidify the observations in *Ae. aegypti* derived Dcr2 and Ago2 knock-out cell lines, rescue experiments were performed. Therefore AF319 and AF525 cells were transfected with either pPUb-myc-Dcr2 (expressing *Ae. aegypti* Dcr2), pPUb-myc-Ago2 (expressing *Ae. aegypti* Ago2) or pPUb-myc-eGFP as control. Additionally, recently described Dcr2-mutant constructs (**??**) were transfected into AF319 to be able to infer the role of the functional domains involved in the observed reduction of YicV in the absence of Dcr2. The experiments and data collection involving Dcr2 were performed by S. Röder under supervision of the author.

AF319 or AF525 cells were transfected with the respective construct and inoculated with MOI = 1 YicV/DeziV/DaesV the following day. At 48 hpi total RNA was isolated, and real-time PCR performed to estimate YicV levels relative to ribosomal S7. Results were further normalized to pPUb-myc-eGFP transfected cells. For both the Dcr2 in AF319 and Ago2 in AF525 rescues, the levels of YicV were elevated in comparison to control GFP transfected control cells (fig. 17, A & B). The Dcr2 construct holding the RNaseIIIA/B (mtR3, P1464L) and helicase motif I and VI (K39N, G488R) mutations showed a significant reduction of YicV levels compared to wild type Dcr2, while the

helicase motif IV mutant (Y232G) did not show a significant difference (fig. 17, A).

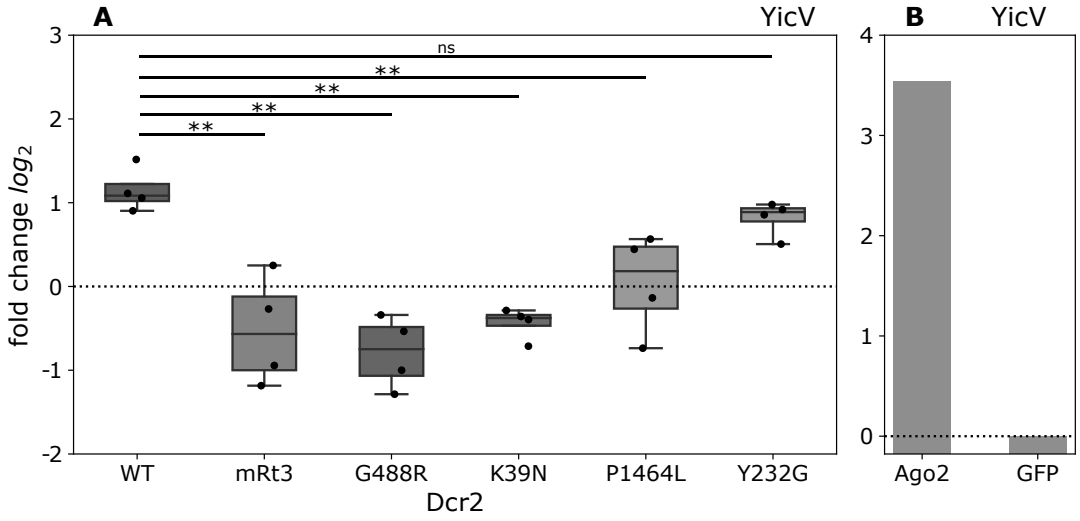


Figure 17: **Effect of Dcr2 wt, Dcr2 mutants and Ago2 complementation in *Aedes aegypti* derived knock-out cell lines on YicV levels.** (A) Box plots with individual observations (black circles) of fold change values of YicV normalized to ribosomal S7 and relative to levels in GFP transfected controls (dashed line). AF319 cells were transfected with the indicated Dcr2 construct and inoculated with YicV/DeziV/DaesV MOI = 1 the next day. At 48 hpi total RNA was extracted and levels of YicV and ribosomal S7 were estimated by real-time PCR. Results of four experimental repetitions are shown. (B) Bar plot of fold change value of YicV normalized to ribosomal S7 and relative to levels in GFP transfected controls. AF525 cells were transfected with pPUb-myc-Ago2 and inoculated with YicV/DeziV/DaesV the next day. At 48 hpi total RNA was extracted and levels of YicV and ribosomal S7 were estimated by real-time PCR. The results of a single experiment is shown. Statistical analysis was performed by students t-test for unpaired, two-sided data. Single asterisk (*) indicate p-values ≤ 0.05, double (**) p ≤ 0.01, ns p ≥ 0.05. Experiments and data collection under (A) was performed by S. Röder under supervision of the author.

3.1.4 small RNA profiles from YicV, DaesV & DeziV infected cells

To further investigate the interactions of YicV, DaesV and DeziV with the mosquito RNAi system, small RNA (sRNA) profiles from different cell lines of multiple mosquito species were compared. Data sets were mapped to the above described virus genomes. This allows inferring a few general characteristics: the origin of vsiRNA and vpiRNA-like molecules, the proportion of vsiRNA and vpiRNA-like reads, as well as specific characteristics of a possible ping-pong cycle dependent amplification of vpiRNAs which can be detected by looking for nucleotide frequency and read overlaps.

Samples created during a previous study ([Agboli, 2021]) were re-analyzed to compare the characteristics of the RNAi response to the three viruses between CT and *Aedes aegypti* derived Aag2 cells. The samples had been inoculated with YicV/DaesV/DeziV, and total RNA had been isolated at 24 hpi.

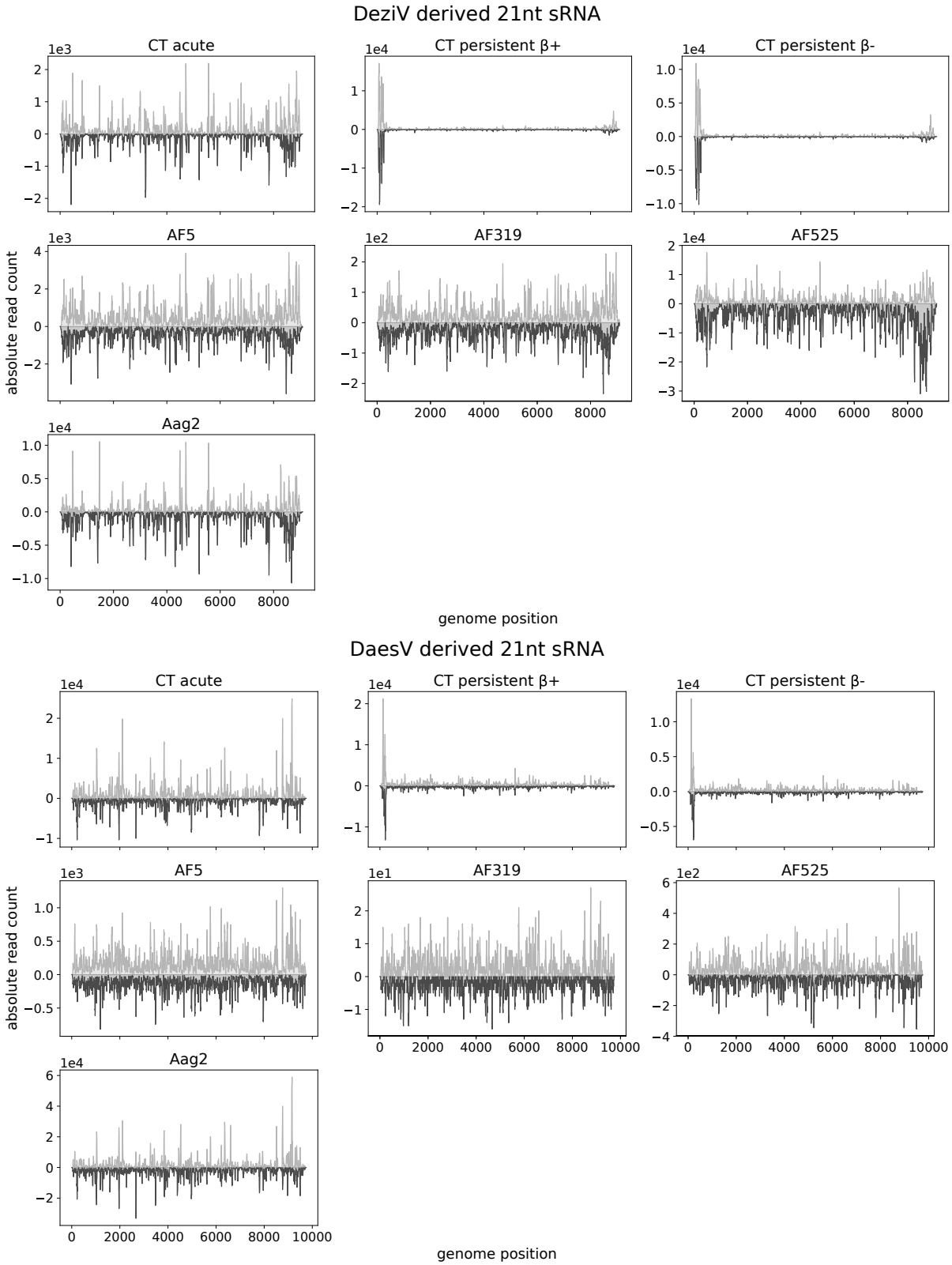


Figure 18: **Analysis of DeziV and DaesV derived 21nt sRNAs from various mosquito cell lines.** Indicated cells lines were inoculated with YicV/DaesV/DeziV MOI=10 and total cellular RNA samples were collected and transferred for sRNA sequencing. Shown are the distributions of 21nt sRNAs along the DeziV (top) and DaesV (bottom) genome. CT acute, AF5, AF319, AF525 and Aag2 Samples were collected at two days post infection, CT persistent were passaged three times and subjected to either an active beta-elimination or mock eliminated. Green, positive values indicate genomic, purple, negative values antigenomic origin. Data for CT acute and Aag2 represent mean values from two sequencing runs, the rest from a single run.

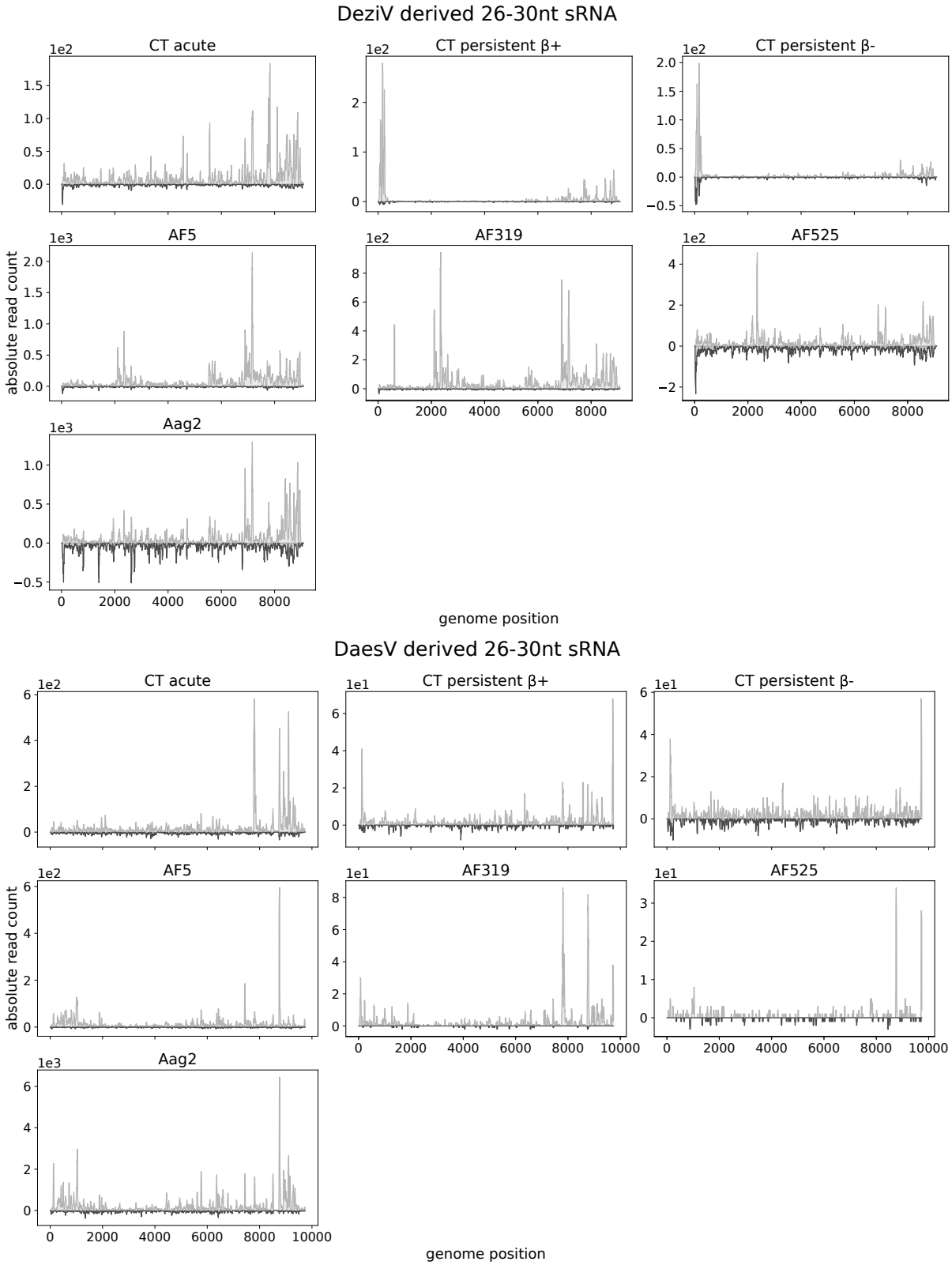


Figure 19: **Analysis of DeziV and DaesV derived 26-30nt sRNAs from various mosquito cell lines.** Indicated cells lines were inoculated with YicV/DaesV/DeziV MOI=10 and total cellular RNA samples were collected and transferred for sRNA sequencing. Shown are the distributions of 26-30nt sRNAs along the DeziV (top) and DaesV (bottom) genome. CT acute, AF5, AF319, AF525 and Aag2 Samples were collected at two days post infection, CT persistent were passaged three times and subjected to either an active beta-elimination or mock eliminated. Green, positive values indicate genomic, purple, negative values antigenomic origin. Data for CT acute and Aag2 represent mean values from two sequencing runs, the rest from a single run.

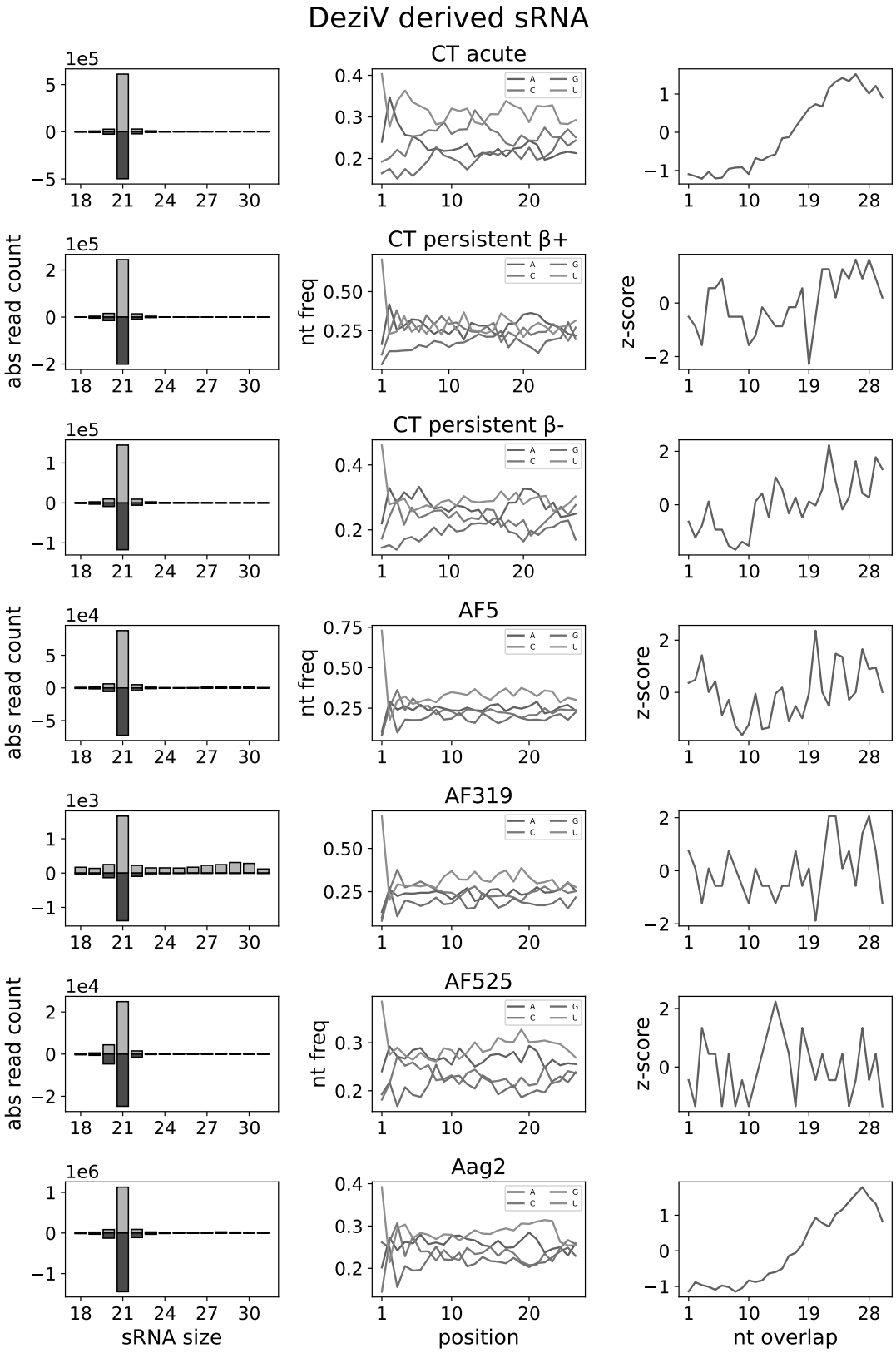


Figure 20: **Analysis of DeziV derived sRNAs from various mosquito cell lines.** Indicated cells lines (top left) were inoculated with YicV/DaesV/DeziV MOI=10 and total cellular RNA samples were collected and transferred for sRNA sequencing. Shown are the amount of reads of specified size (left), the nucleotide frequency per position of 26-30nt sRNAs (middle) and the z-score of overlap probability per size of overlap (right) of DeziV derived sRNA reads. CT acute, AF5, AF319, AF525 and Aag2 Samples were collected at two days post infection, CT persistent were passaged three times and subjected to either an active beta-elimination or mock eliminated. Green, positive values indicate genomic, purple, negative values antigenomic origin. Data for CT acute and Aag2 represent mean values from two samples, the rest from a single sample.

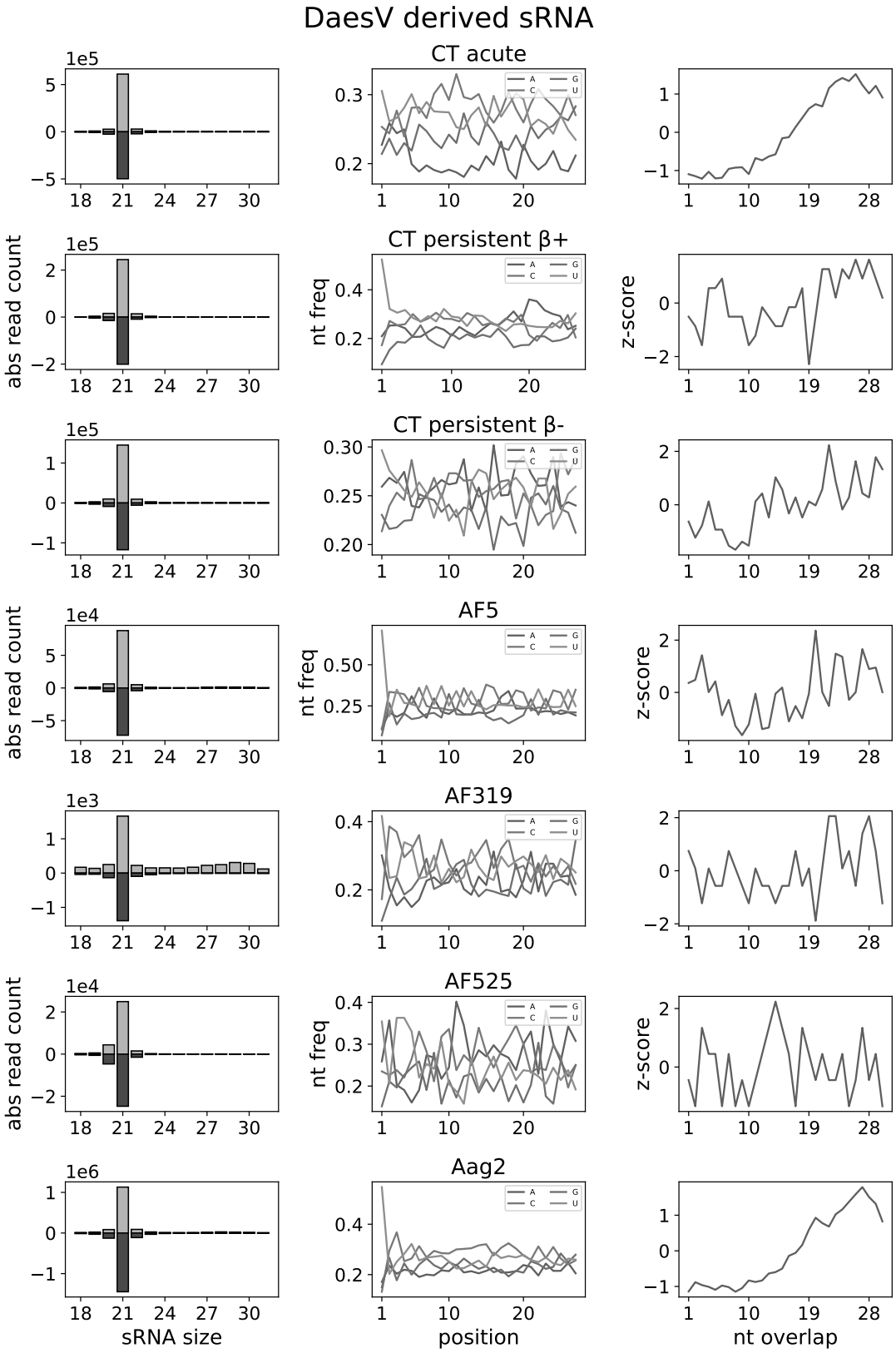


Figure 21: **Analysis DaesV derived sRNAs from various mosquito cell lines.** Indicated cells lines (top left) were inoculated with YicV/DaesV/DeziV MOI=10 and total cellular RNA samples were collected and transferred for sRNA sequencing. Shown are the amount of reads of specified size (left), the nucleotide frequency per position of 26-30nt sRNAs (middle) and the z-score of overlap probability per size of overlap (right) of DaesV derived sRNA reads. CT acute, AF5, AF319, AF525 and Aag2 Samples were collected at two days post infection, CT persistent were passaged three times and subjected to either an active beta-elimination or mock eliminated. Green, positive values indicate genomic, purple, negative values antigenomic origin. Data for CT acute and Aag2 represent mean values from two samples, the rest from a single sample.

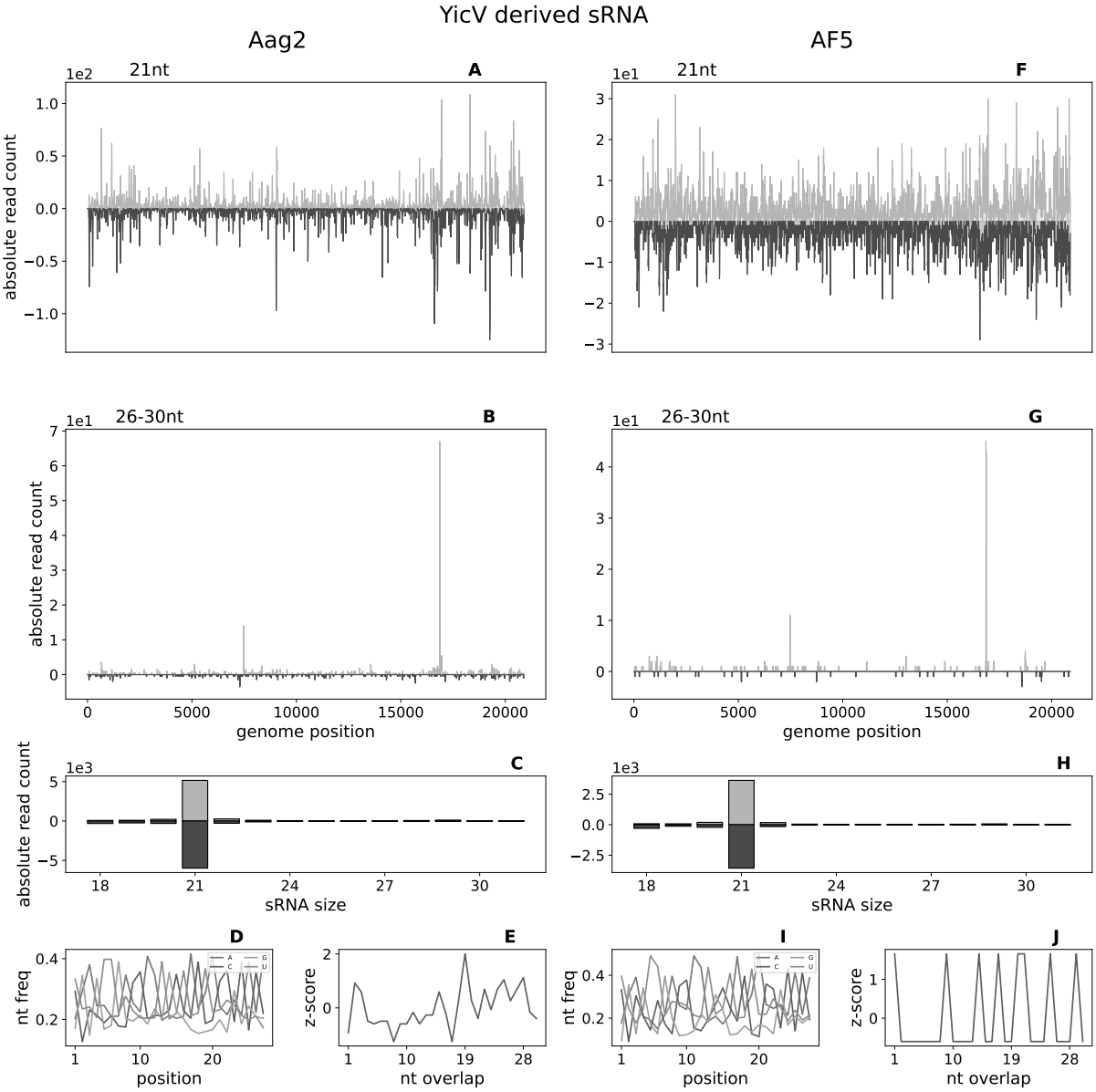


Figure 22: **Analysis YicV derived sRNAs from *Aedes aegypti* derived cell lines.** Aag2 (left) or AF5 (right) cells were inoculated with YicV/DaesV/DeziV MOI=10 and total cellular RNA samples were collected and transferred for sRNA sequencing. Shown are the distributions of 21nt sRNAs (A, F), 26-30nt sRNAs (B, G), amount of reads of specified size (C, H), the nucleotide frequency per position of 26-30nt sRNAs (D, I) and the z-score of overlap probability per size of overlap (E, J) of YicV derived sRNA reads. Samples were collected at two days post infection. Green, positive values indicate genomic, purple, negative values antigenomic origin. Aag2 represent mean values from two samples runs, AF5 from a single sample.

In both the Aag2 and CT cells, sRNAs derived from DaesV and DeziV could be detected, while YicV-specific reads were only present in the Aag2, not the CT sample table 11. In both Aag2 and CT cells, DaesV specific reads were the most abundant, with 12.1-12.2% and 4.5-6.1% respectively. The ratio of DeziV derived reads was 3.6-3.7% in Aag2 and 0.6-0.9% in CT cells. YicV reads were 0.05% in Aag2.

All three viruses induced the production of 21 nt long siRNAs as the majority of sRNA reads between 71.5%-81% in Aag2 and 81.2-87.3% in CT. These did not exhibit a pronounced bias towards a specific region of the genome, in both Aag2 and CT cells (figs. 20, 21 and 22). In Aag2 cells, but not CT cells, a pronounced bias of antigenomic origin for DeziV derived siRNAs was observed (table 11). No such strong bias was present in Aag2 and CT cells for YicV and DaesV siRNAs. Both DaesV and DeziV additionally induced the production of piRNA sized (26 - 30 nt) sRNAs in Aag2 and CT cells, while YicV only produced neglectable amounts of such piRNA-like sRNAs (figs. 20, 21 and 22). Proportions of piRNA sized sRNAs were considerably smaller than siRNA sized. Again DeziV derived piRNA sized sRNAs were more abundant with 0.4-0.5% in Aag2 and 0.04% in CT. DaesV piRNA like reads amounted to 0.2% in Aag2 and 0.02% in CT cells. The YicV derives piRNA sized sRNAs in Aag2 only made up 0.002% of all reads. For DaesV in Aag2 and CT as well as DeziV in CT, these piRNA-like sRNAs originated mostly from the genomic strand. At the same time, in Aag2 the DeziV derived piRNA-like sRNAs also originated to a considerable amount from the anti-genome. In all samples, the piRNA-like sRNAs originated markedly from the 3' third of the genome. In both cell lines, DeziV derived piRNA-like sRNAs exhibited a slight U1, but no A10 bias (fig. 20). This bias was also strongly present in DaesV derived piRNA-like sRNAs, but only in Aag2 and not in CT cells. None of the samples piRNA-like sRNAs exhibit a 10 nt overlap signature (fig. 21).

Next, sRNA samples from the CT cells with persistent DaesV and DeziV infections described above were taken to allow a comparison between the involvement of the RNAi system in acute and persistent infections. Total RNA was isolated as described. Additionally, the samples were divided into a beta-elimination and a control, mock treated group. The beta-elimination reaction on sRNAs allows for the detection of 3' methylation in RNA sequencing by excluding non-3'-methylated sRNAs from beeing sequenced. In animals, this modification can be found in both siRNA and piRNAs that were loaded onto an Ago protein [Katharina Elmer, 2014]. Beta-elimination treatment enriches such sRNAs in RNA sequencing libraries by preventing non-methylated sRNAs from being sequenced.

In the control group, patterns of both DeziV and DaesV derived sRNAs were remarkably altered in the persistent infected CT cells compared to acute infections (figs. 18 and 19). Production of siRNAs was almost exclusively restricted to a single region close to the 5' end, more pronounced in DeziV than for DaesV. Likewise piRNA-like sRNAs showed a similar distribution pattern for DeziV derived reads. The total amount of DaesV

derived piRNA-like reads was reduced by an order of magnitude. The remaining reads showed two distinct hot spots at the 5' and 3' end. The DeziV derived piRNA-sized sRNAs showed a more pronounced U1 bias, compared to the acute infection, but also did not exhibit an overlap pattern (fig. 20).

The beta-elimination treatment did not change the sRNA patterns of DeziV derived sRNAs. For DaesV, only a more pronounced U1 bias of piRNA-sized reads was detected.

In order to elucidate the role of the two major effector proteins of the siRNA pathway, Dcr2 and Ago2, knock-out cell lines were used. These cells derive from a sub-clone of Aag2, AF5, from which the Dcr2-KO cell line AF319 and the Ago2-KO cell line AF525 were created [Varjak et al., 2017, Scherer et al., 2021]. Samples, kindly provided by C. Scherer, were drawn from AF5, AF319 and AF525 at 2 dpi, inoculated with DeziV/DaesV/YicV MOI = 10 and processed as described above.

In AF5 cells DeziV specific reads were the most abundant of all three viruses, with 0.02% of all reads, followed by DaesV with 0.007%, while YicV only made up 0.0003% of all reads (table 11). For all three viruses, the vsiRNA fraction made up the major proportion of reads, ranging from 76% for DeziV to 81% for YicV and DaesV (figs. 20, 21 and 22). The distribution of these vsiRNAs was in a usual pattern of cold and hot spots from about similar amounts from virus genome and anti-genome along the whole genome length (figs. 18, 18 and 22). The vpiRNA-like fraction made up from 4% of reads for YicV to 8% and 10% for DeziV and DaesV respectively. For all three viruses, these were heavily biased towards genomic origin, and with few hot spots (figs. 18, 19 and 22). Both DeziV and DaesV derived piRNAs showed a distinct U1 bias which was absent in YicV, most likely to the low overall abundance of YicV specific piRNAs (figs. 20, 21 and 22). None of the viruses exhibited an A10 or 10 nt overlap pattern.

In the Dcr2 knock-out cell line AF319, amounts of DeziV and DaesV specific reads were reduced to 0.0024% and 0.0002%, respectively while YicV reads could not be detected (table 11). Interestingly vsiRNAs were still the majority of reads, with 43% for DeziV and 50% of DaesV specific reads (figs. 20 and 21). The proportion of vpiRNA-like sRNAs did increase, though to 30% and 21%. Both vsiRNA and vpiRNA-like sRNAs from DeziV and DaesV were similarly distributed to the genomes as in AF5 cells (figs. 18 and 19). While the U1 bias for DeziV retained as in AF5, it was much less distinct for DaesV in AF319 (figs. 20 and 21).

In the Ago2 knock-out cell line AF525 YicV derived sRNAs were also absent. The proportion of DeziV specific reads was remarkably increased to around 0.1%, while DaesV reads were with 0.003% lower than in the parental AF5 cell line (table 11). The fraction of vsiRNAs for DeziV and DaesV was similar to that in AF5 with 78% and 77%. The vpiRNA-like sRNA fraction was remarkably reduced to 0.5% for DeziV and only 0.006% for DaesV.

Table 11: **General analysis of sRNA sequencing reads obtained of YicV/DaesV/DeziV MOI=10 inoculated Aag2, AF5, AF319, AF525 and CT cells.** Shown are the total reads in the of the individual samples, the total virus specific reads and the ratio of virus specific reads to total reads. Further, the total and relative amounts as well as the ratio of genomic to antigenomic reads of both 21 nt and 26 - 30 nt reads are shown. Finally the ratio of 21 nt to 26 - 30 nt sRNAs is displayed.

cell/condition	clean reads	virus	total reads	% reads	% 21 nt all	21 nt (+)/(-)	% 26-30 nt all	26-30 nt (+)/(-)	piRNA:siRNA
Aag2 I	27381110	DeziV	1010705	3,691	71,48	0,569	0,214	1,279	0,081
		DaesV	3347954	12,227	77,22	0,767	0,507	4,000	0,054
		YicV	14779	0,054	80,99	0,880	0,002	2,368	0,043
Aag2 II	27179670	DeziV	966738	3,557	73,07	0,593	0,16	1,509	0,061
		DaesV	3281237	12,072	78,25	0,796	0,409	4,963	0,043
		YicV	12859	0,047	80,26	0,841	0,001	1,825	0,034
AF5	29129112	DeziV	589298	0,020	76,22	1,192	6,47	8,298	0,085
		DaesV	197478	0,007	81,16	1,214	3,49	9,863	0,043
		YicV	8914	0,000	80,64	1,021	2,48	3,885	0,031
AF319	26670720	DeziV	64462	0,002	43,36	0,930	30,06	14,640	0,69
		DaesV	6075	0,000	50,24	1,196	21	32,750	0,418
		YicV	nd						
AF525	25985764	DeziV	3225968	0,124	78,21	0,334	0,453	1,204	0,006
		DaesV	64601	0,002	76,9	1,007	0,006	3,953	0,007
		YicV	nd						
CT acute I	24906587	DeziV	231465	0,929	82,98	1,193	0,020	7,233	0,026
		DaesV	1526190	6,128	87,32	1,250	0,044	3,701	0,008
		YicV	nd						
CT acute II	22576201	DeziV	132209	0,586	81,22	1,046	0,021	7,188	0,044
		DaesV	1021041	4,523	86,67	1,195	0,045	3,688	0,012
		YicV	nd						
CT persistent mock eliminated	25501394	DeziV	202915	0,796	82,59	1,189	0,007	2,918	0,011
		DaesV	312077	1,224	83,98	1,236	0,005	2,420	0,005
		YicV	nd						
CT persistent betaeliminated	26665422	DeziV	301687	1,131	85,59	1,173	0,008	15,104	0,008
		DaesV	517535	1,941	86,013	1,218	0,004	3,190	0,002
		YicV	nd						

3.2 *In vitro* Analysis of MSV - Arbovirus Interactions

As described above, it was not possible to isolate either of the three identified viruses. Therefore the study had to be continued with different, isolated MSV species. Two viruses were chosen, Niénokoué virus (NIEV) and Eilat virus (EILV). As both viruses belong to different families, NIEV to the flaviviruses and EILV to the alphaviruses, it would allow a direct comparison of the interactions of MSV that are either genetically more closely related to or more divergent from flavi-arboviruses.

3.2.1 Infectability of *Culex* spp. Derived Cell Culture Model Systems

For neither WNV and USUV nor NIEV and EILV, much is known about their infectivity of *Culex* spp. derived cells. In order to identify viable systems to study virus-virus interactions, first individual continuous growth kinetics of WNV and USUV, as well as NIEV and EILV were performed. This was done using the *Cx. tarsalis* derived CT and the *Cx. quinquefasciatus* derived HSU cell lines.

CT cells were inoculated with WNV1, WNV2 and USUV MOI=10, HSU cells were inoculated with WNV1 MOI=10. Cell culture supernatants were collected at 0, 1, 2 and

3 dpi and virus concentrations determined by TCID50 titration on Vero cells. In CT cells, WNV1 and USUV titers were steadily increasing over the three days time period, WNV1 reaching about $1 \cdot 10^7$ TCID50/mL and USUV $1 \cdot 10^8$ TCID50/mL (fig. 23 A). WNV2 already reached its peak titer at 2 dpi with $5 \cdot 10^9$ TCID50/mL declining towards $1.6 \cdot 10^9$ TCID50/mL 3 dpi. In HSU cells, WNV1 titers slightly declined from 0 to 1 dpi and remained stable at around $1 \cdot 10^3$ TCID50/mL up to 3 dpi. As HSU cells appear to be either non-infected or showing a delayed infection kinetic, they were not used for further experiments.

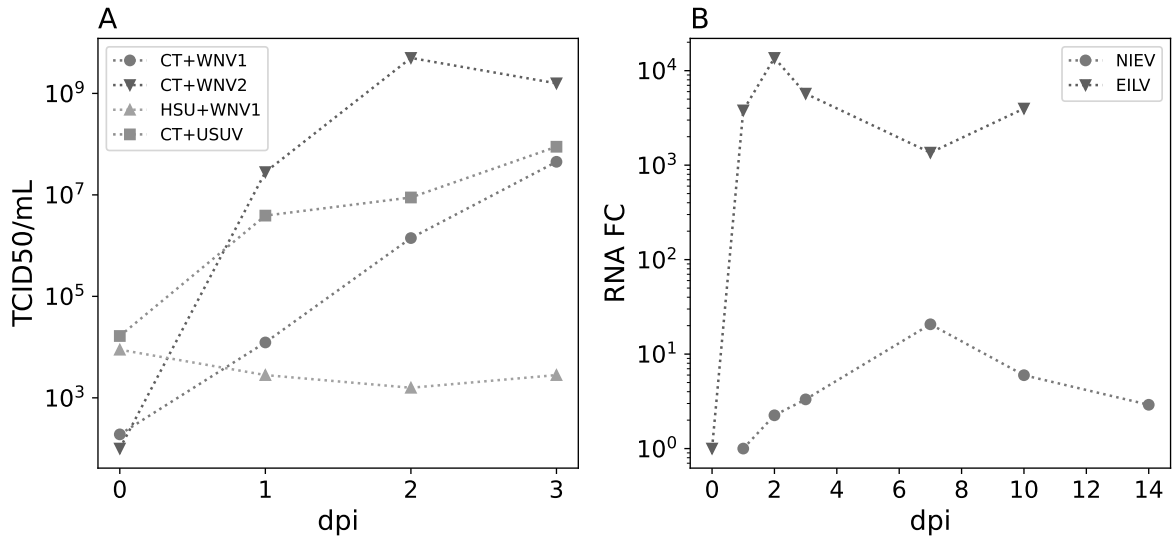


Figure 23: **Growth kinetics of single infections of WNV1, WNV2, USUV, NIEV and EILV in *Culex* derived cells.** (A) HSU cells were infected with WNV1, CT cells with either WNV1, WNV2 or USUV, all MOI=10. Cell culture supernatants were collected at the indicated time points, and their titers were determined as TCID50/mL. (B) CT cells were infected with either NEIV MOI=0.1 or EILV MOI=0.1, and total cellular RNA was extracted at the indicated time points. Viral replication was estimated using real-time PCR with virus- and GAPDH specific primers, expressed in fold change values relative to the initial Ct. Results of a single experiment are shown. TCID50s were performed in technical quadruplicates, real-time PCR in triplicates.

In order to investigate whether both NIEV and EILV are able to induce a persistent infection, sampling was performed for a longer period. For both NIEV and EILV, CT cells were inoculated with MOI=0.1. Total RNA was isolated 1, 2, 3, 7. 10 and 14 dpi from NIEV and 0, 1, 2, 3, 7 and 10 dpi from EILV inoculated cells. (fig. 23 B & C). Viral RNA levels were determined by real-time PCR relative to GAPDH. Throughout the sampled time frame, CT cells remained positive for both viruses.

The results indicate that CT cells are favorable over HSU cells regarding their ability to allow for quick growth of WNV and USUV and can tolerate both NIEV and EILV infections without signs of clearance.

3.2.2 Co-infections Assays in CT Cells

To be able to compare the effects of both an acute, and parallel infection as well as an established, persistent infection on arbovirus replication, a NIEV and an EILV persistent infected CT cell lines were established.

CT cells were initially infected with a MOI=1 with either NIEV (CT-NI) or EILV (CT-EI). Both cultures were passages ten times. At this point, total RNA was extracted, reverse transcribed and amplification of NIEV, EILV and GAPDH by end-point PCR was performed. Both CT-NI and CT-EI cells showed a distinct signal of NIEV and EILV amplification, respectively, indicating the successful establishment of a persistent infection. (fig. 24).

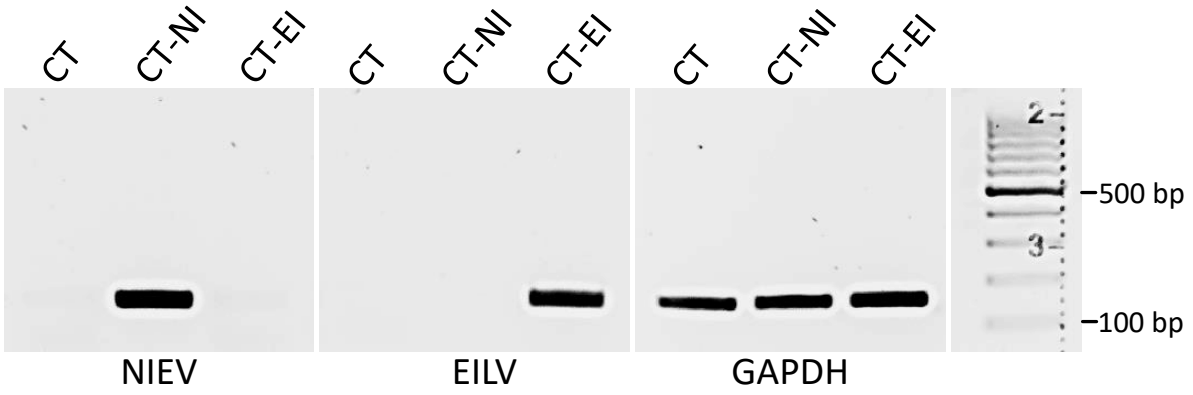


Figure 24: **Establishment of NIEV and EILV persistent infections in CT cells.** CT cells were initially infected with an MOI=1 with either NIEV (CT-NI) or EILV (CT-EI) and passaged ten times. To assess whether both viruses established a persistent infection, total cellular RNA was extracted, reverse-transcribed and analyzed by end-point PCR with NEIV, EILV and GAPDH specific primers.

In order to elucidate the interactions of NIEV and EILV in both acute and persistent co-infections, these newly established persistently infected cell lines were used along with naive CT cells for co-infection assays with WNV2. Non-infected CT cells were inoculated with either MOI=0.1 WNV2 alone, or both WNV2 and NIEV or EILV, also with an MOI=0.1. CT-NI and CT-EI were likewise inoculated with WNV2 MOI=0.1. At the indicated time point, cell culture supernatant was collected, and WNV titers were estimated via TCID50 on Vero cells.

For NIEV co-infections, a significant reduction in WNV2 titers between the single WNV infection and persistent co-infection was detected at 1 & 2 dpi (fig. 25 A). This trend of decreased WNV replication in persistently infected cells was also observable at 3 & 4 dpi, although not enough samples were collected to infer statistical testing. WNV titers in acute co-infected cells were similar to single infected cells.

In EILV co-infected cells, a trend for decreased WNV titers was observed for acute but not persistent co-infections at 1 & 2 dpi, again not statistically significant due to a too small sample size (fig. 25 B). A reduction of WNV titers for both acute and persistent EILV co-infection was also detected at 3 dpi, while at 4 dpi WNV titers in acute co-infected cells appeared to be higher than in single or persistent infected cells.

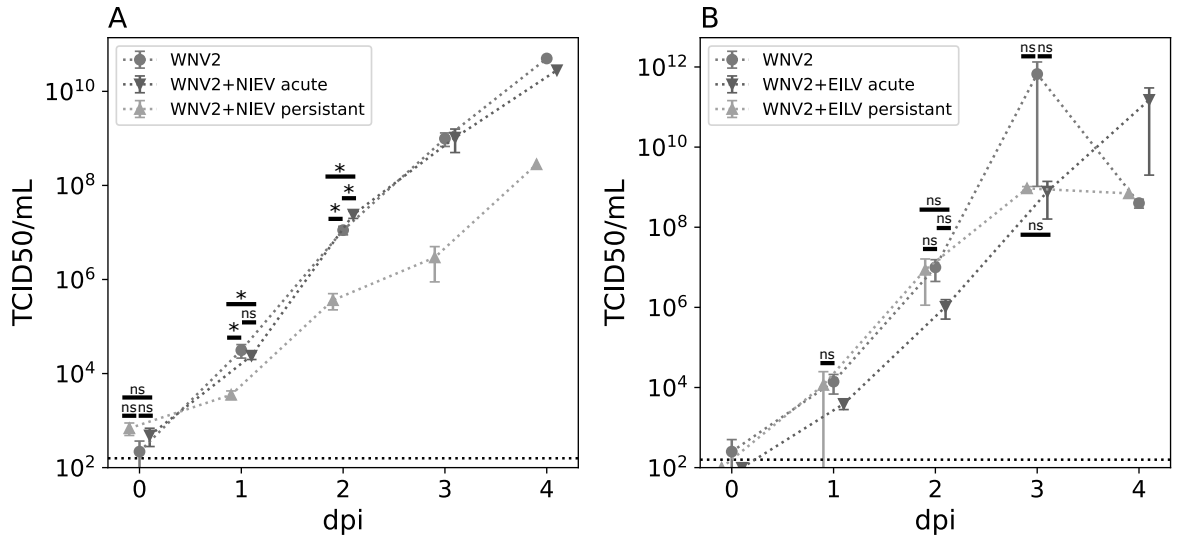


Figure 25: **Growth kinetics of co-infections of WNV2 and NIEV or EILV in acute or persistent infection of CT cells.** Infections with WNV2 with a MOI=0.1 were performed either in parallel infections in naive CT cells or with pre-established NIEV (A) or EILV (B) infections. Cell culture supernatants were collected at the indicated time points, and WNV2 titers determined as TCID50/mL. Data represents one to three repetitions of experiments with technical triplicates. Time points with three samples were used for statistical testing using students t-test for unpaired, two-sided data. Asterisks indicate p-values ≤ 0.05, ns ≥ 0.05, and pairings with no indicators were not tested due to $N \leq 2$. Individual number of experimental repeats (n), see table 12.

Similar experiments were performed to test the influence of NIEV on an USUV infection. In order to test whether the initial amount of infectious virus particles has an influence on resulting USUV titers, experiments with either a low (MOI=0.1) and high (MOI=10) or both USUV and NIEV were performed.

First, naive CT cells were infected in parallel with either USUV MOI=0.1 or 10 and NIEV MOI=0.1 or 10. Cell culture supernatants were collected and USUV titers were estimated by TCID50 on Vero cells.

In the low USUV MOI group, both low and high NIEV MOI appeared to result in lower USUV titers at 1, 2 & 3 dpi (fig. 26 A). When inoculated with a higher MOI, a slight reduction of USUV replication could be observed only for the high MOI NIEV group at 1 & 2 dpi, while the lower MOI NIEV resulted in higher USUV titers at 2 & 3 dpi.

Furthermore, naive and NIEV persistent CT cells were infected with USUV MOI=5

and cell culture supernatants were collected daily up to 5 dpi and titrated (fig. 26 B). USUV titers were already reduced in the co-infected samples at 0 & 1 dpi, but rose to equal levels at 2 dpi and were elevated at 3 and 5 dpi.

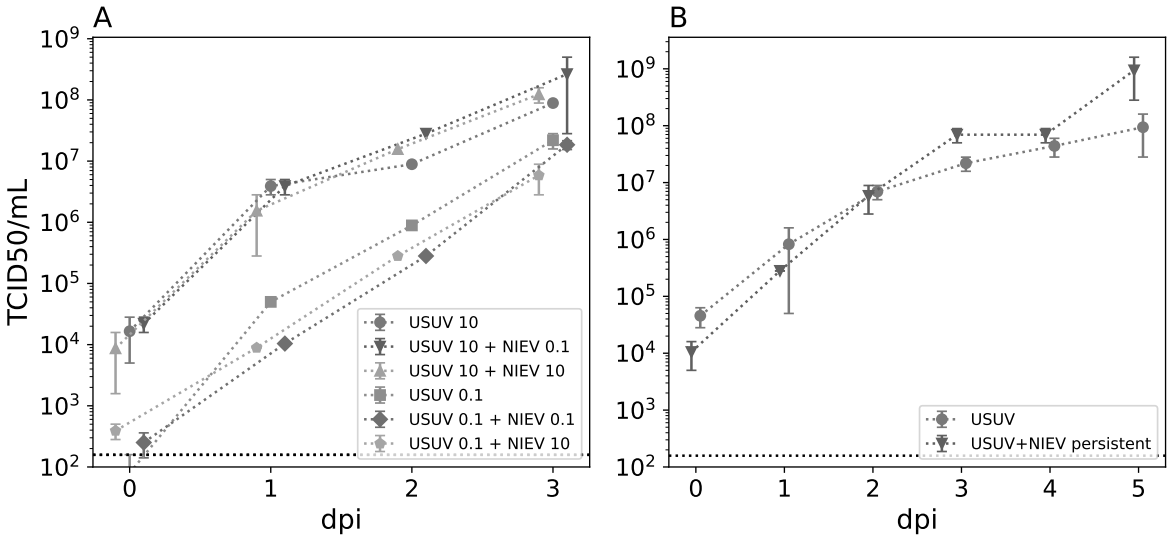


Figure 26: **Growth kinetics of co-infections of USUV and NIEV in acute or persistent infection of CT cells.** CT cells were infected with USUV with MOI=0.1 or 10 and NIEV MOI=0.1 or 10 in parallel (A). NIEV persistent CT and CT cells were infected with USUV MOI=5 (B). Cell culture supernatants were collected at the indicated time points, and USUV titers determined as TCID50/mL. Data represents one to two experimental repetitions of technical triplicates. Individual number of experimental repeats (n), see table 12.

3.2.3 Involvement of RNAi Gene Transcript Levels in WNV-EILV Co-Infections

Numerous studies have investigated changes in the transcriptome upon arbovirus infections [Etebari et al., 2017, Bonizzoni et al., 2012, Shrinet et al., 2017, Colpitts et al., 2011, Li et al., 2021, Li et al., 2020a, Henderson et al., 2022, Girard et al., 2010, Núñez et al., 2020]. Though these were mostly performed with aedine mosquitoes and single arbovirus infection. By utilizing the recent identification of RNAi genes in *Cx. quinquefasciatus* [Altinli et al., 2023, Walsh et al., 2022], real-time PCRs targeting Ago1, Ago2, Ago3 and Dcr2 were performed to test if they can be used on *Cx. tarsalis*. RNA was extracted from cell samples created during the EILV/WNV co-infection experiment described above. Results of quantitative PCR for Dcr2, Ago2, Ago1 and Ago3 for 0 & 2 dpi, as fold change values normalized to GAPDH are presented (fig. 27).

No samples showed a considerable change in transcript levels (FC $\geq$ 2 or $\leq$ 0.5) compared to mock infected samples. Though all virus inoculated cells showed an overall reduction of transcript levels at 1 hpi. For Dcr2, Ago2 and Ago3, this trend persisted at 48 hpi while Ago1 fold change values scattered around 1.

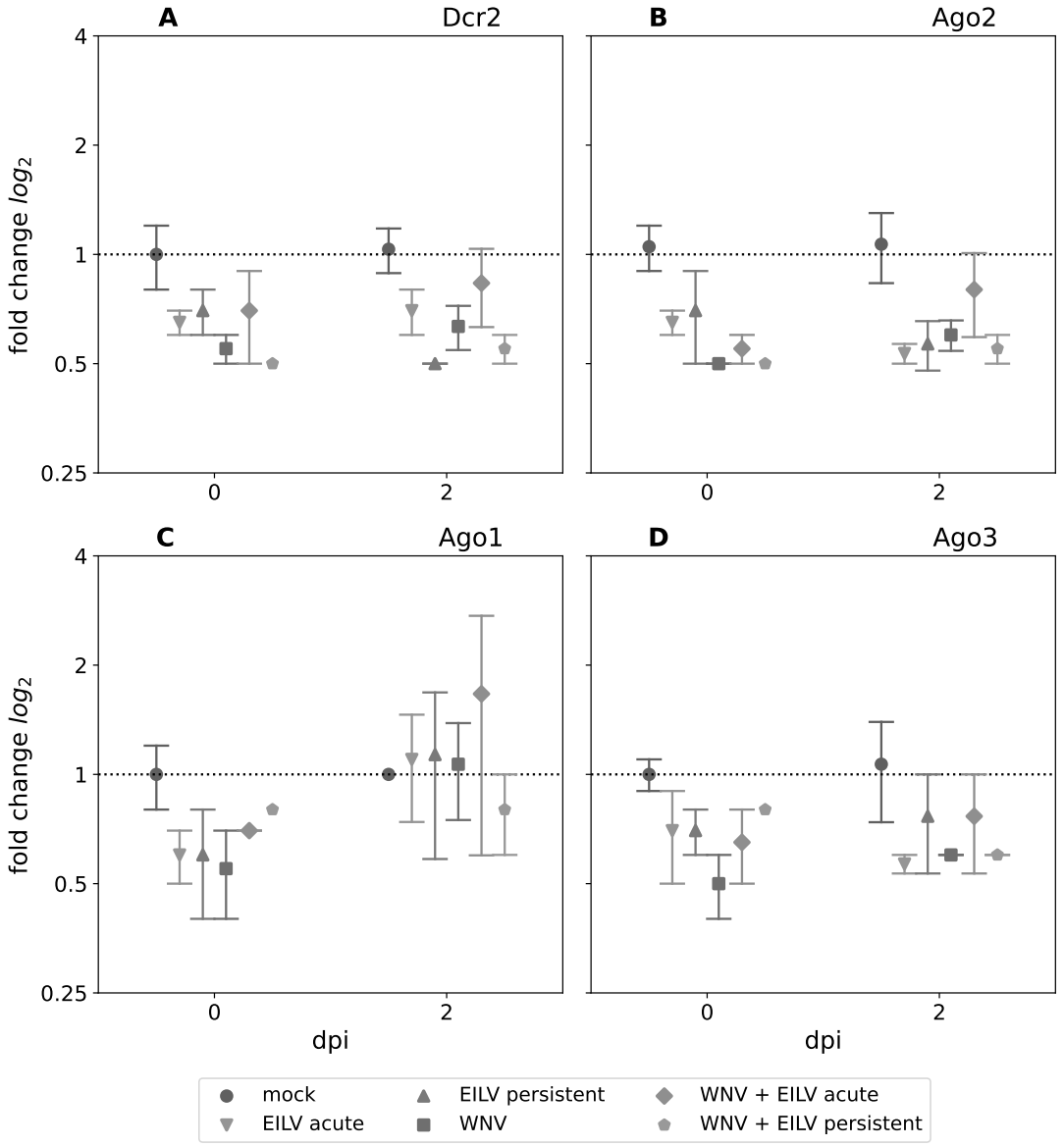


Figure 27: **Real-time PCR analysis of RNAi gene expression in CT cells co-infected with WNV2 and EILV.** Real-time PCRs on transcripts of homologs of the RNAi effector proteins Dcr2, Ago2, Ago1 & Ago3 of *Cx. tarsalis* and GAPDH were performed on samples from the WNV/EILV co-infection experiments. At the indicated time points, total RNA was extracted and levels Dcr2 (A), Ago2 (B), Ago1 (C) and Ago3 (D) were estimated and fold change values were computed after normalization to GAPDH. Results of one to three independent experimental repetitions with technical triplicates are shown. Individual number of experimental repeats (n), see table 12.

3.3 *In Vivo* Analysis of Laboratory Reared *Culex* spp. and MSV - Arbovirus Interactions

3.3.1 Infectability of Arboviruses

In addition to the experiments in the *Cx. tarsalis* derived cell line, *in vivo* experiments were performed using laboratory reared colonies of *Cx. pipiens molestus* and *Cx. quinque-*

fasciatus. First, the infectability for WNV1, WNV2 and USUV was evaluated. Female *Cx. pipiens molestus* were offered a blood meal containing $1 \cdot 10^7$ TCID50/mL either WNV1 or WNV2. Blood engorged individuals were selected and sampled 7 and 14 days post exposure. During this period, the mosquitoes were held at 27°C +/-5°C over 24h with 70% RH. The infection status was evaluated by real-time PCR of the homogenated mosquito body. No WNV1 was detectable at either 7 or 14 dpi, while WNV2 could be detected in 14% (1/7) 7 dpi and 9% (1/11) 14 dpi (fig. 28 A, B). Female *Cx. quinquefasciatus* were offered blood meals either containing USUV or WNV2 and also subjected to real-time PCR testing of homogenated mosquito bodies at 7, 14 and for USUV also 21 dpi. These were held under identical conditions to *Cx. pipiens molestus*. No USUV could be detected at any of the indicated time points (fig. 28 C). WNV2 was detected in 45% (9/20) at 7 dpi and 56% (10/18) at 14 dpi (fig. 28 D).

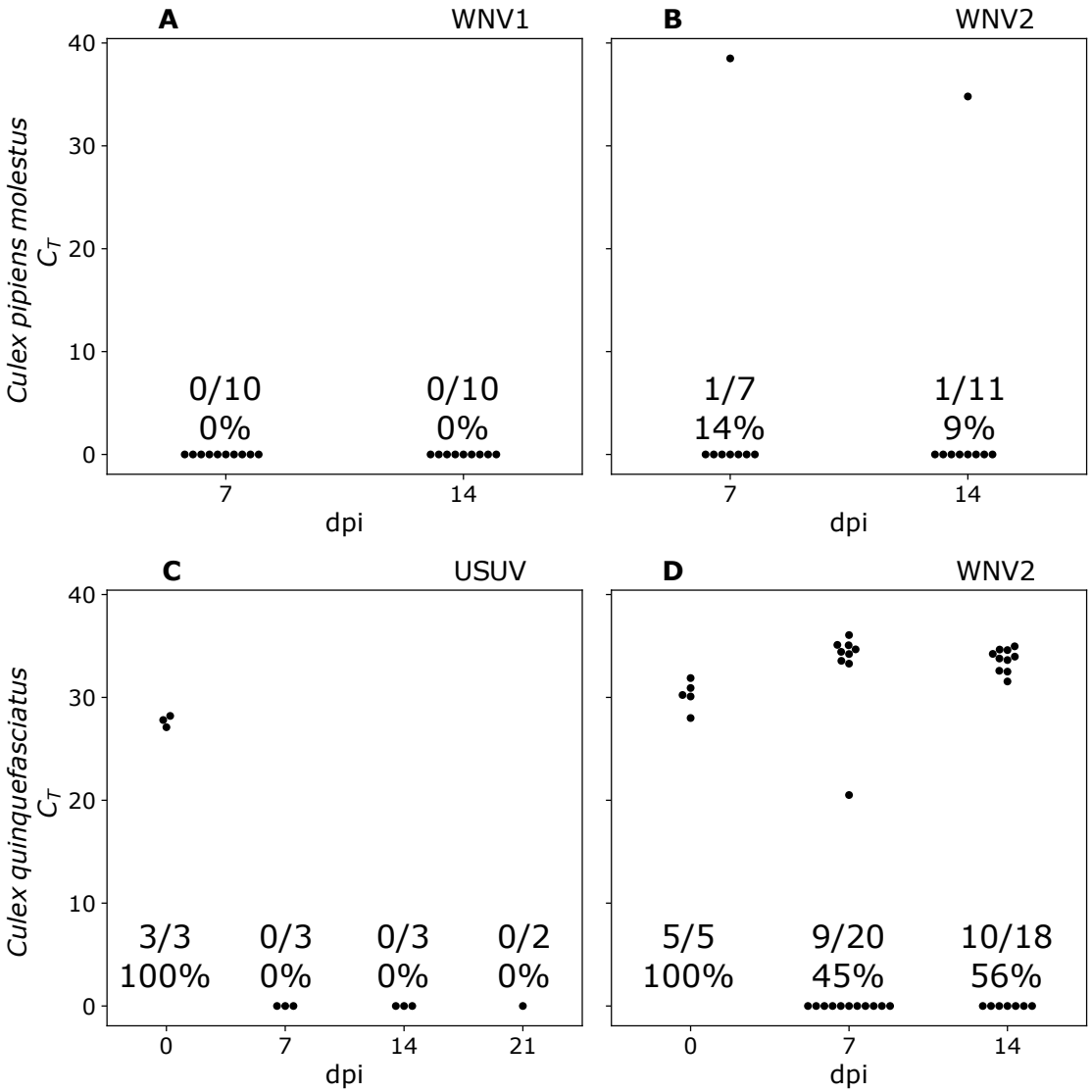


Figure 28: **Infection rates of *Cx. pipiens molestus* and *Cx. quinquefasciatus* after a blood meal with WNV1, WNV2 or USUV.** One to four days old female *Cx. pipiens molestus* (A, B) or *Cx. quinquefasciatus* (C, D) were starved for one day and afterward offered an artificial blood meal containing $1 \cdot 10^7$ TCID50/mL of either WNV1 (A), WNV2 (B, D) or USUV (C). At the indicated time points, individual mosquitoes were sacrificed and total RNA was extracted. Infections status was determined by virus-specific real-time PCR.

3.3.2 Infectability of MSVs

To check whether the effects of NIEV and EILV co-infections on WNV could also be observed *in vivo*, first, a single infection with these viruses had to be established. Previous work (ML, personal communication) had shown that NIEV is incapable of infecting *Cx. quinquefasciatus per os*. Therefore adult female mosquitoes were intrathoracally injected with 70 TCID50/mL of NIEV. Mosquitoes were held at 27°C, 70% RH and were sacrificed at 3, 7, 10 and 14 dpi when the presence of NIEV RNA was measured by real-time PCR.

At 3 dpi, half of the mosquitoes were NIEV positive (10/20), by 7 dpi the infection rate dropped to 20% (6/20) and further decreased at 10 and 14 dpi to 6% (1/15) and 5% (1/18), respectively (fig. 29 C). This steady decline in infection rates rendered *Cx. quinquefasciatus* as an unsuitable model to study co-infections.

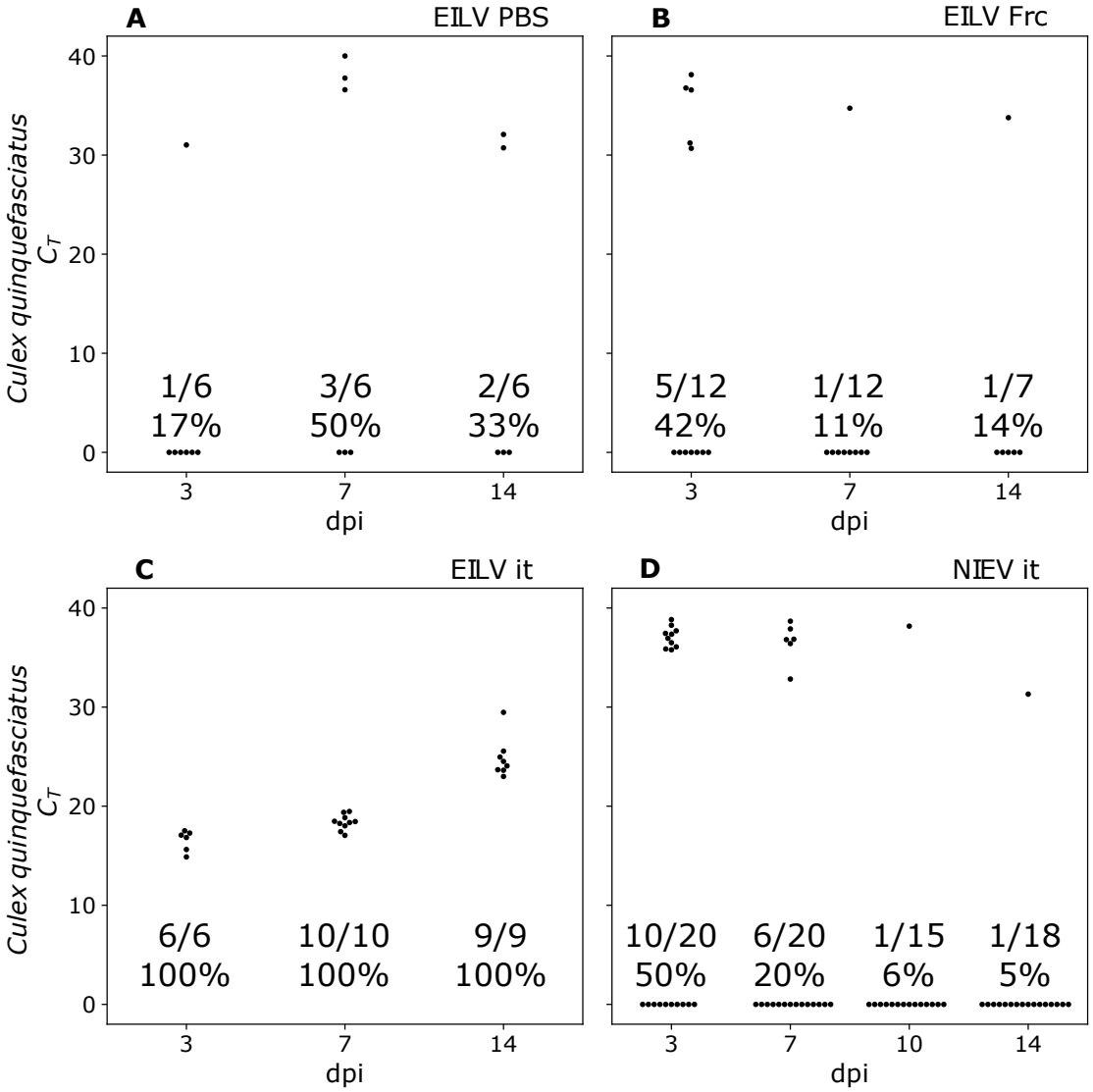


Figure 29: **Infection rates of *Cx. quinquefasciatus* with EILV or NIEV.** One to four days old female *Cx. quinquefasciatus* were starved for one day and afterwards were either offered PBS (A) or Fructose solution (B) containing $1 \cdot 10^7$ TCID50/mL EILV or were intrathoracally injected with 70 TCID50/mL EILV (C) or NIEV (D). At the indicated time points, individual mosquitoes were sacrificed, and total RNA was extracted. Infections status was determined by virus specific real-time PCR.

In the case of EILV, the literature suggests that *Cx. quinquefasciatus* can not be infected orally but by injection [Nasar et al., 2014]. To check whether this also proves to be correct for the *Cx. quinquefasciatus* colony used, 1-4 days old adult female *Cx. quinquefasciatus* were either offered a PBS or Fructose solution (FRC) containing $1 \cdot$

10^7 TCID50/mL EILV *ad libitum* for three days or were injected with 70 TCID50/mL EILV intrathoracally. Mosquitoes were held under the same conditions as in the NIEV infections. Individual mosquitoes were sacrificed 3, 7 & 14 dpi, homogenized and their EILV infection status was determined by PCR from total RNA extract. It showed that at all three time points, all injected mosquitoes were EILV positive (fig. 29 D). In contrast, the infection rates in mosquitoes which took up EILV over the oral route were lower at all time points (fig. 29 A, B). At 3 dpi 17% of the PBS and 42% of the FRC group were EILV positive. 7 dpi 50% (PBS) and 11% (FRC), and 14 dpi 33% and 14% were EILV positive, respectively.

3.3.3 WNV - EILV Co-Infection

In order to transfer the *in vitro* co-infection experiments to an *in vivo* model, adult female *Cx. quinquefasciatus* were first injected with either 70 TCID50/mL EILV or PBS or left untreated (mock) and two days later offered a blood meal containing $1 \cdot 10^7$ TCID50/mL WNV2, as described above for the single infections, and again held at 27°C +/-5°C over 24h with 70% RH. At 0, 7, 14 & and 21 dpi, whole mosquitoes were sacrificed and homogenized. No virus could be detected at 7 dpi fig. 30. At 14 dpi, only 1/9 mosquitoes of the EILV group was positive, while PBS and mock treated were completely negative. Also, at 21 dpi, no WNV positive mosquito from the PBS group was detected and only 1/5 from the EILV and 1/8 mock groups.

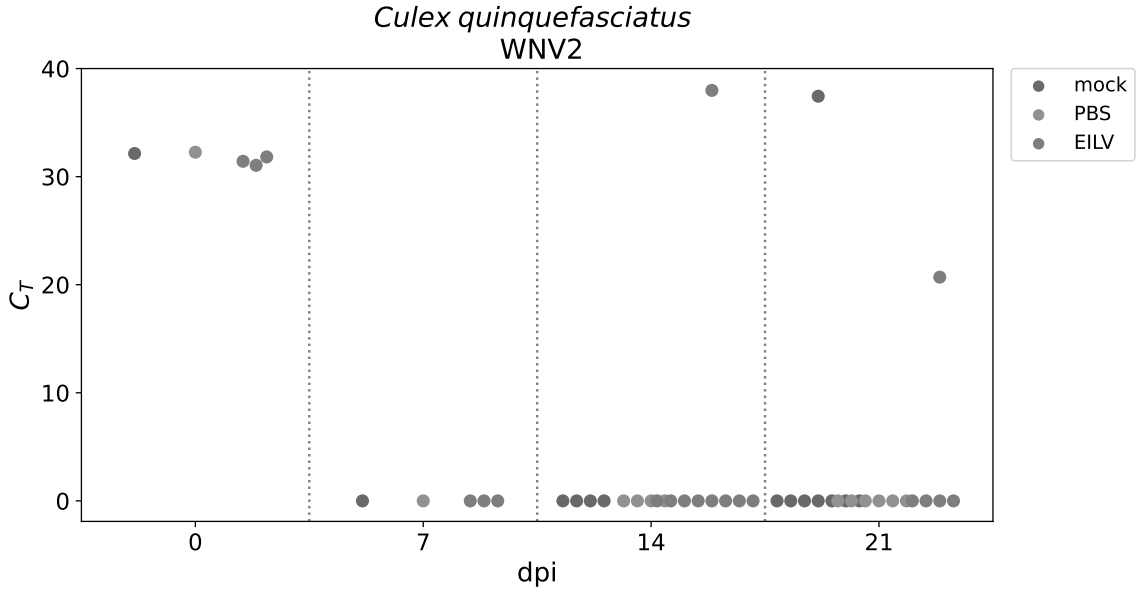


Figure 30: **WNV infection rates of *Cx. quinquefasciatus*.** One to four days old female *Cx. quinquefasciatus* were intrathoracally injected with 70 TCID50/mL EILV and starved for one day and afterward offered an artificial blood meal containing $1 \cdot 10^7$ TCID50/mL of WNV2 one week after the initial treatment. At the indicated time points, individual mosquitoes were sacrificed and total RNA was extracted. Infections status was determined by real-time PCR for WNV and GAPDH as positive control.

4 Discussion

Both mesoniviruses and negeviruses are recently established groups of RNA viruses. Mesonivirus species have been, by now, identified mostly in mosquito species collected on multiple continents. Yet they do not show signs of species segregation based on geographic distances, nor species specific host patterns [Morais et al., 2022]. Both points appear to be reflected in the identification of YicV described in this study. The two other known YicV species isolates originated from *Culex* spp. mosquitoes, collected in China, whereas the YicV strain identified in the presented study came from mosquitoes of the genus *Coquillettidia* from Germany [Wang et al., 2017]. Whether this distribution of closely related mesonivirus species in distant geographic areas is an artifact of human activity by accidental translocation, as it has been suggested for Nam Dinh virus, remains speculative [Vasilakis et al., 2014]. More sampling, also in other insect species, as well as re-analysis of historic samples for the presence of mesoniviruses will be necessary. It can facilitate our understanding of the distribution and evolution of these viruses by reducing the current bias in the genomic data sets towards (arbovirus vector) mosquitoes host from only the last 15 years.

Most of these points also apply to negeviruses. Dezidougou virus samples have been obtained from *Ae. aegypti*, *Ae. africanus*, *Cx. pipiens*, *Cx. quinquefasciatus* and *Ochlerotatus communis* collected in Africa, Europe and South America [Vasilakis et al., 2013, Gil et al., 2022, Truong Nguyen et al., 2022, da Silva Ferreira et al., 2020, Shrivastava et al., 2017]. Daeseongdong virus has been by now only identified from *Cx. pipiens* collected in East Asia and Europe [Hang et al., 2016, Gil et al., 2022]. Given geographic distribution and host range this wide, it is likely that both mesonivirus and negevirus infections of mosquitoes are a commonly occurring incident. And as for both virus families experimental data on possible adverse interference with arboviruses exists, it is indicative to investigate how the presence of these viruses in mosquito populations influence arbovirus transmission cycles [Patterson et al., 2021, Ye et al., 2020].

The data presented here is the first characterization of the sRNA response to negeviruses and mesoniviruses in mosquito cells. Comparable studies have been performed with the mesoniviruses Aphis citridus mesnoi-like virus (AcMSV) detected in an Aphid and three negeviruses, Nelorpivirus dungfly 1 (NVD1) and Sandewavirus dungfly 1 (SVD1) identified in a dungfly and Bactrocera dorsalis negev-like virus (BDNV) from Fruit Flies [Chang et al., 2020, Lu et al., 2020, Zhang et al., 2020]. AcMSV , NVD1, SVD1 and BDNV all appear to be targeted by the siRNA pathway in a similar fashion to YicV, DeziV and DaesV respectively in these insects. Strikingly NVD1 and SVD1 infected the same animal at the same time point. Another study also found members of the Nelopri- and Sandewavirus groups, also including DaesV and DeziV, as well as a mesonivirus, on

baited FTA cards [Birnberg et al., 2020]. Since the YicV, DeziV and DaesV variants described here were also found in a single pool of just three individual mosquitoes, it appears to be suggestive to ask whether these findings are merely coincidences based on a possible high abundance of these viruses, or a closer relationship between them may exist.

This study is also the first characterization of the interactions of MSVs and the RNAi system in an experimentally induced persistent infection. Göertz et al. reported the sRNA response of CT and Aag2 cells towards Calbertado virus (CALBOV), Flock House virus (FHV), Culex narna-like virus 1 (CxNV1), Phasi-Charoen-like virus (PCLV) and Aedes densovirus (AeDNV), respectively [Göertz et al., 2019]. These viruses all maintain a persistent infection in these cell lines. Interestingly the authors only reported piRNA production for the negative RNA genome PCLV but not for any of the positive RNA genome viruses, whereas DeziV and DaesV readily induce the production of piRNA-like sRNAs. In the case of the dsRNA genome Culex Y virus (CYV) production of piRNA sized sRNAs in Aag2 was reported [Franzke et al., 2018]. A possible explanation may be exemplified by the absence of YicV derived sRNAs in acute infected CT (as well as rather low read counts in Aag2) while still detectable by PCR, hinting at a sensitivity issue of sRNA sequencing techniques, especially in cells that are infected with multiple viruses and for regularly low abundant piRNAs. However, it was possible to confirm that the piRNA:siRNA ratio is also higher for both DeziV and DaesV in Aag2 compared to CT cells, like the findings reported for PCLV and Rift Valley Fever virus [Dietrich et al., 2017]. The strong over-representation of siRNAs from the near 5' region in persistent infections with DeziV and DaesV begs the question of whether targeting only this region is sufficient enough to control the infection, or if the accessibility of the virus genomes alters in the course of an infection. Available sRNA profiles of persisting virus infections show that CYV, FHV, CxNV1, PCLV and AeDNV do not show such characteristics, while the flaviviruses CALBOV and CFAV also exhibit a 3'UTR bias. These observations may have intersting implications on the mechanistics of persistance phenotypes of different virus families.

The Dcr2 and Ago2 knock-out cells AF319 and AF525 are valuable resources, used to study the interactions of viruses with the mosquito RNAi system [Varjak et al., 2017, Altinli et al., 2022, Parry et al., 2019, Scherer et al., 2021]. Two studies using AF319 described a complete loss of siRNA production upon infection with Semliki Forest virus (SFV) and Aedes albopictus densovirus in conjunction with an increased production of vpiRNAs [Varjak et al., 2017, Parry et al., 2019]. In a study on Agua Salud alphavirus (ASALV) vsiRNAs were still produced in decreased numbers but originated mostly from the 3' end of the virus genome [Altinli et al., 2022]. Numbers of vpiRNAs were also elevated, and with an U1 bias and 10 nt overlap patterns. For both DeziV and DaesV, a slight reduction of vsiRNA, which remained to be the dominant sRNA species, and

an increase in piRNA-sized RNAs occurred. Interestingly, the overall read count for both viruses decreased remarkably. In contrast, most studies report an increase in virus replication in AF319 cells. Based on the presented work, the probable cause for this rather unintuitive observation remains speculative. Since negeviruses have the capability to replicate to rather high titers, in conjunction with the observed loss of YicV replication may implicate two scenarios. Either because the samples were taken at 2 dpi and by this time most cells have succumbed to the exaggerated infection due to the loss of Dcr2. But as the overall sRNA read counts in the AF5 and AF319 samples were roughly the same it may be more likely that in the case of a co-infection with two highly replicating virus species in immune deficient cells leads to an over-usage of cellular resources causing a reduced replication for all co-infecting virus species. Ultimately, having isolates of YicV, DeziV and DaesV to perform infection experiments individually will be necessary to explain the observations made.

Changes in the sRNA profiles in the Ago2 deficient AF525 cell line have also been described for experimental infections with SFV and ASALV as well as on the persisting MSVs PCLV and CFAV [Scherer, 2021, Scherer et al., 2021, Altinli et al., 2022]. For these, an overall increase in the production of sRNA, mostly vsiRNAs, was observed. Interestingly, here this increase was only observed for DeziV while DaesV derived reads were reduced, and YicV also being undetectable. This again hints towards the ability of negeviruses, or at least DeziV, to out-compete other viruses in cells with an impaired siRNA response. Though, as the initial ratios of YicV/DeziV/DaesV in the inoculum are unknown, it is not possible to ascertain whether this effect is due to a greater portion of DeziV in the sample or to intrinsic properties of this virus.

Besides of the sRNA data, YicV levels in AF319 and AF525 were also significantly reduced compared to AF5, and it was possible to elevate YicV levels again upon Dcr2 and Ago2 rescue, respectively. This generally suggests some sort of involvement of the host immune system in the virus-virus interactions. This was also reflected in the observation of increased expression of Dcr2 and Ago2 in AF5 cells upon YicV/DeziV/DaesV infection. An induction of especially these central anti-viral immune genes has been reported in several insect species infected with a variety of viruses [Lozano et al., 2012, Li et al., 2020b, Niu et al., 2016, Garbutt and Reynolds, 2012, Wang et al., 2016, Fan et al., 2022, Liu et al., 2013, Yang et al., 2020, Torri et al., 2020]. Though the data hints towards a highly dynamic system of gene regulation in regards to dose and timing of infection. And some effects on these genes also appear to be merely stress induced by experimental animal handling, while other effects are only observed on protein but not transcript levels. But due to it being a highly conserved pathway, similar effects have also been reported in fungi and plants [Chiba and Suzuki, 2015, Ando et al., 2021]. Only a few studies have reported data on levels of RNAi gene expression in the context of virus infections. No change in Dcr1, Dcr2 or Argonaut genes have been observed during ZIKV infections of

A. aegypti [Etebari et al., 2017]. In contrast, a correlation between Dcr2 levels and viral loads has been reported in DENV infections of *A. albopictus* [Wu et al., 2022]. The same study also showed an influence of temperature or seasonality on Dcr2 expression. In a similar manner, infections of *A. aegypti* with CHIKV regulation of Dcr2 also appeared to be temperature dependent [Wimalasiri-Yapa et al., 2021]. These rather heterogeneous findings are also reflected in the data presented in this study; induction of the Dcr2 and Ago2 occurred upon multiple MSV infections of aedine derived cells (fig. 16), but infection of Culex derived cells with EILV and/or WNV resulted even in a slight reduction of Dcr2, Ago2 and Ago3 mRNA levels (fig. 27). In conjunction, these findings indicate that immune gene regulation may be an important but under-observed variable in mosquito virus infection studies and should be considered in future studies to support our understanding of the complex host-virus-environment relationships and how they shape processes like vector competence.

In order to gain a more mechanistic understanding of the regulation of viral infection by the RNAi system, a study recently undertook the effort to perform analysis of mutants of Dcr2 [Gestuveo et al., 2022]. These mutations targeted two functional domains of the protein, the RNase and helicase. They identified sites on both domains that are crucial for silencing in RNAi reporter assays, as well as infections with luciferase reporter expressing SFV. In the context of the reduced YicV levels under Dcr2 knock-out conditions, S. Röder observed in her work similar effects whereas Dcr2 mutants which showed no silencing activity also did not elevated YicV levels. Interestingly though, the RNase and two of the helicase mutants showed even a further reduction of YicV levels compared to the unmitigated Dcr2 knock-out phenotype. To reiterate, the observed effects on YicV under Dcr2 knock-out may be explained by extensive replication of the negeviruses.Something that would have to be investigated in further studies, it remains entirely unclear why a loss of function of single Dcr2 domains has a more detrimental outcome on YicV replication compared to the complete knock-out.

For EILV, a negative effect on viral titers of alpha-arboviruses (Estern-, Western- & Venezuelan-Equine Encephalitis virus, Chikungunya virus and Sindbis virus) in acute co-infections in aedine models have been reported [Nasar et al., 2015]. In contrast, the data presented in this study suggests that neither acute nor persistent co-infections with EILV have a significant detrimental effect on WNV2 in a culine cell line, suggesting that the observed interference effects may be limited to either alphaviruses or aedine mosquitoes. Further research on this matter is therefore needed to allow a proper assessment of the potential capabilities of using MSV, such as EILV, as agents to reduce arbovirus transmission.

On the other hand, a persistent co-infection of NIEV had a significant effect on WNV titers, while acute co-infections had no effect. Interestingly acute, but probably not per-

sistent NIEV infection showed a trend in reducing USUV titers. No experimental data on its interactions with other viruses is available. Studies on other mosquito-specific flaviviruses have relied on cells that neither reflects the host pattern of the co-infecting arbovirus, are notably immune deficient or only investigated the effect of acute phase co-infections [Baidaliuk et al., 2019, Goenaga et al., 2015, Romo et al., 2018a, Kenney et al., 2014, Hobson-Peters et al., 2013, Bolling et al., 2012, Kent et al., 2010]. Notably, also enhancing effects of CxFV, a mosquito-specific flavivirus for which a negative interference with arbovirus infections has repeatedly been reported in C6/36, on DENV replication and titers has been shown in a *Ae. aegypti* derived Aa20 cell line [Zhang et al., 2017]. Therefore, the establishment of cell culture systems that can reflect both the relevant host species and can be used to study the complex virus-virus-host interactions are of great benefit. These can also be an advantage to the more technical challenging use of laboratory reared colonies or field caught mosquitoes for such experiments with human pathogens like WNV. These *in vitro* models also give a more stable and controllable experimental basis than mosquitoes, in which virus infections can have drastically different out-comes with only slightly different rearing conditions, environmental factors, genetic backgrounds and microbiome/virome [Tabachnick, 2013, King, 2020, Carvajal-Lago et al., 2021, Jupatanakul et al., 2014]. These factors may also be the main confounding factor that could explain the differing results of the WNV2 infections experiments in *Cx. quinquefasciatus* in this study. As both experiments were performed at different time points, mundane factors in animal handling and nutrition, population densities during rearing as well as seasonal changes in the populations biology, which even occurs under controlled physical rearing conditions could fundamentally change the animal's phenotype to an arbovirus infection and can hardly be accounted for at all times in experimental research or would be exaggeratedly intricate to control for.

The vector competence of mosquitoes for WNV has been exhaustively studied (see two recent reviews on the matter [Vogels et al., 2017, Rochlin et al., 2019]). Three studies on the vector competence of *Cx. pipiens molestus* for WNV have been performed. Using WNV2 (strain Greece 2010), infection rates at 14 dpi were at 24% after incubation at 18°C and 23°C, but only 14% at 28°C [Vogels et al., 2016]. Similarly, using field caught *Cx. pipiens molestus* and also WNV2 (strain Germany 2018, same as used in the presented study) showed that infection rates were higher after 18°C for 14/15 dpi and 20/21 dpi with 83% compared to 65%/67% 14/15 dpi and 77%/67% 20/21 dpi for 25°C or 28°C respectively [Holicki et al., 2020b]. In the same studies, two laboratory colonies were compared, resulting in infection rates of 100% and 7% for one, and 65% and 15% at days 14/15 and 20/21 doe at 25°C incubation respectively. Finally, using WNV1 (strain TOS-09, same as used here) and the same *Cx. pipiens molestus* colony used in the presented study found infection rates to be 0% 14 dpi at 18°C, 24°C and 27°C and 3% for 18°C and 24°C, and 6% for 28°C 21 dpi [Jansen et al., 2019].

Though direct comparison between *in vivo* studies is not only difficult due to the reason described above, they also can vary along methodological lines regarding the source and composition of the blood meal, feeding methods, the cell type used for virus propagation (mosquito or mammal) and virus titers. Given these caveats, the low infection rates observed in the presented study may be explained by both the relatively high incubation temperature and mosquito colony. As it would still be valuable to have vector competence data that directly compares both WNV lineages, not only different mosquito populations, to guide the understanding of their respective characteristics (also in regard to MSV co-infections), future studies should be performed using generally lower incubation temperatures. The trend toward higher infection rates at lower temperatures found in these studies is also interesting in regard of the influence to the mosquito RNAi system discussed above.

Studies on *Cx. quinquefasciatus* and WNV are numerous and suggest, in general, that WNV infection can be detected continuously and in relative high rates under a variety of conditions [Alto et al., 2014, McGregor et al., 2021, Eastwood et al., 2011, Micieli et al., 2013, Anderson et al., 2010, Richards et al., 2010, Richards et al., 2014, Jansen et al., 2008, Romo et al., 2018b, Richards et al., 2012, Leggewie et al., 2016]. In that regard *Cx. quinquefasciatus* appears to be a rather suitable model to study experimental MSV co-infections with WNV compared to *Cx. pipiens* if the focus of the investigation is aimed at the virus-virus-host interactions specifically. Though they are not present in Europe yet, *Cx. quinquefasciatus* still remains one of the major culprits of arbovirus transmission, especially in the global south. Furthermore, given that the annual average global temperature will likely exceed the 1.5°C of the Paris Agreements, and will be hard to limit to below 2°C, models of future habitats under a warming climate well include parts of Europe [IPCC, 2023, Samy et al., 2016]. Which may lead to parts of Eurpe becoming a suitable habitat for *Cx. quinquefasciatus*. Therefore it remains to be an important target organism for identifying MSVs that are capable of reducing arbovirus disease burden.

As for WNV, data on the infectability of Culex mosquitoes for USUV are varying. Though a general vector competence for *Cx. pipiens* s.l. can be assumed [Abbo et al., 2020, Hernández-Triana et al., 2018, Fros et al., 2015, Cook et al., 2018, Holicki et al., 2020a, Abbo et al., 2021, Bates et al., 2021]. For *Cx. quinquefasciatus*, only limited data from two studies is available. Oral infections of *Cx. quinquefasciatus* from a laboratory colony, originated from North America with USUV (SAAr1776), resulting in 93% and 70% infections rates at 7 and 14 dpi, respectively [Cook et al., 2018]. Using two more recent USUV isolates, strains Netherlands 2016 and Uganda 2012, again with *Cx. quinquefasciatus* lab colony originated from North America, resulting in 50% infection rates at 14 dpi for USUV Netherlands 2016 and 19 % for USUV Uganda 2012 [Kuchinsky et al., 2022]. Incubation conditions were similar to those in the presented study. The disparities in the published data and the apparent refractory or low infection rate phenotype observed here

may therefore be best explained by genetic differences of the USUV strains used. With its continuous circulation in Africa and Europe and the potential of dispersal towards the Americas and Asia, and therefore into unadapted host populations, USUV is a looming threat to human and animal health that should receive surveillance on research attention accordingly.

For most MSVs, modes of transmission and host ranges are unknown. EILV has previously been shown to infect *Ae. aegypti*, *Ae. albopictus*, *An. gambie* and *Cx. quinquefasciatus* with 100% infection rates at 7 dpi. Though these rates considerably dropped when the virus was transmitted by a blood meal, with now *Ae. aegypti* 87%, *Ae. albopictus* 15%, *An. gambie* 43% and *Cx. quinquefasciatus* with 30% infection rates, indicating a possible strong restriction of infection by the mid gut barrier. Yet the reasoning for feeding MSVs in a blood based medium, a practice that is somewhat frequent in published studies, remains enigmatic to the author. Here it was attempted to infect *Cx. quinquefasciatus* with mediums that resemble a possible environmental source of infectious virus by co-feeding on shared water or nectary mediums. Though fructose containing media resulted in lower rates compared to a solely water based medium indicates that infection by co-feeding on flowers may be a less likable transmission route for EILV. Interestingly, as infection rates in the water-based medium were similar to those of the blood-based medium, as negative influence of blood on oral infection also seems to be unlikely. But the presence of EILV in larvae indicates that infection may be acquired transovarial or occur at an early life stage and be maintained by transstadial transmission [Bennouna et al., 2019].

For NIEV, it was currently only known to be able to infect Culex mosquitoes, as it was isolated from a pool of *Cx. spp.*[Junglen et al., 2017]. The data presented here shows that *Cx. quinquefasciatus* are not a likely host, as infection rates drop rather quickly after direct injection. But as it was shown that NIEV can replicate in *Cx. tarsalis* derived cells, it may be more promising to try to establish infection using these mosquitoes, as *Cx. tarsalis* are not endemic to NIEVs region of origin this might ultimately fail too. As NIEV persistent infections showed some promising results regarding the ability to infer with an WNV infection *in vitro*, it would be worthwhile to further investigate its host range.

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Acknowledgments

First I have to thank Prof. Dr. Esther Schnettler for taking me back in her lab to do my PhD study and providing me with lots of academic freedom during this time. Also thanks to the Bundesministeriums für Ernährung und Landwirtschaft for providing the funding for my studies and all the collebarations partners of the Culifo2 project. A Special thanks to Ute Ziegler, Giada Rossini, Sandra Junglen and Scott C. Weaver for providing their virus isolates.

Thanks to the whole lab group, Mine, Mayke, Marlis, András and Bernhard for all the little things that made this experience the way it was. Also thanks to other colleagues of the institute Anna, Steph and all the other people who keep our mosquito colonies alive, Katharina Höhn for her work on the TEM as well as Daniel Cadar and his team for the services of the NGS facility.

Big shout out to the Ivory Tower crew for beeing helpfull allies during all of this. First of all, Christina, thank you for talking me into this in the first place and keeping up with me throughout this adventure, you are awesome. Not to forget all the other great crew members, Alex, Lennart, Nadine, Caro, Rashwita, Magda, Steph, Clarissa. You all played your part in keeping me sane and creating a safe haven, thanks a ton. And a special shout out to Konsti for your emotional support. Also thank you Florian for the talks and input during these years.

"Nicht im Sinn des bloß betrachtenden Verstands, der die Dinge nimmt, wie sie gerade sind und stehen, sondern des beteiligten, der sie nimmt, wie sie gehen, also auch besser gehen können."

Das Prinzip Hoffnung
Ernst Bloch / 1959

5 Appendix

5.1 Affidavit

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Ich versichere, dass dieses gebundene Exemplar der Dissertation und das in elektronischer Form eingereichte Dissertationsexemplar und das bei der Fakultät zur Archivierung eingereichte gedruckte gebundene Exemplar der Dissertationsschrift identisch sind.

Hamburg, der 20.05.2023

Jonny Schulze

5.2 Supplements

Table 12: Number of experimental repeats.

dpi	WNV2	WNV2/NIEV acute	WNV2/NIEV persistent	WNV2	WNV2/EILV acute	WNV2/EILV persistent
0	3	3	3	2	2	2
1	3	3	3	3	2	3
2	3	3	3	3	3	3
3	3	2	2	3	3	3
4	1	1	1	2	2	2
	USUV 0.1	USUV 10	USUV 0.1/NIEV 0.1	USUV 0.1/NIEV 10	USUV 10/ NIEV 0.1	USUV 10/NIEV 10
0	2	2	2	2	2	2
1	2	2	2	2	2	2
2	1	1	1	1	1	1
3	2	2	2	2	2	2
	mock-dcr2	EILV acute-dcr2	EILV persistent-dcr2	WNV-dcr2	WNV + EILV acute-dcr2	WNV + EILV persistent-dcr2
0	2	2	2	2	2	1
2	3	3	3	3	3	2
	mock-ago2	EILV acute-ago2	EILV persistent-ago2	WNV-ago2	WNV + EILV acute-ago2	WNV + EILV persistent-ago2
0	2	2	2	2	2	1
2	3	3	3	3	3	2
	mock-ago1	EILV acute-ago1	EILV persistent-ago1	WNV-ago1	WNV + EILV acute-ago1	WNV + EILV persistent-ago1
0	2	2	2	2	2	1
2	1	3	3	3	3	2
	mock-ago3	EILV acute-ago3	EILV persistent-ago3	WNV-ago3	WNV + EILV acute-ago3	WNV + EILV persistent-ago3
0	2	2	2	2	2	1
2	3	3	3	3	3	2

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