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Investigation on insect-specific viruses - West Nile virus interactions: focus on European *Culex pipiens* mosquitoes

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Contents

Declaration of Digital Writing Assistance

Microsoft Word Editor and Grammarly were used for spelling, grammar, and language revision. ChatGPT (OpenAI) was used to support language editing, clarity, and academic phrasing. All suggestions were critically reviewed and edited by the author. These tools were not used as substitutes for the author's original research, analysis, or scholarly judgement, and the author assumes full responsibility for the final content of this dissertation.

Abstract

Arboviruses such as West Nile virus (WNV) and Usutu virus (USUV) represent an increasing public health concern in Europe, where mosquitoes of the *Culex pipiens* complex and *Culex torrentium* serve as principal vectors. Despite their epidemiological relevance, the molecular determinants underlying arbovirus infection and vector competence in *Culex* mosquitoes remain insufficiently understood. In parallel, insect-specific viruses (ISVs), which are restricted to insect hosts, have emerged as important components of the mosquito virome with the potential to modulate arbovirus replication and transmission.

Using a combination of *in vitro* infection assays, small RNA sequencing, virome analysis, and *in vivo* mosquito infection experiments, this study evaluated viral replication kinetics, antiviral RNA interference (RNAi) responses, and potential interference effects between ISVs and medically relevant arboviruses. Two newly established *Cx. pipiens*-derived cell lines (CPE/LULS50 and CPL/LULS56) were characterized using representative viruses from the families *Flaviviridae*, *Togaviridae*, and *Peribunyaviridae*. Both cell lines supported replication of a broad range of arboviruses and ISVs and exhibited robust virus-derived siRNA responses, confirming functional RNAi activity. In contrast to many established mosquito cell lines, no evidence of persistent ISV infections was detected, highlighting their suitability as controlled experimental systems.

Co-infection experiments in cell culture using the insect-specific viruses Eilat virus (EILV) and Niénokoué virus (NIEV) revealed no consistent modulation of arbovirus replication, including WNV and USUV, under both acute and persistent infection conditions. Similarly, *in vivo* experiments demonstrated that prior infection with the insect-specific Agua Salud alphavirus (ASALV) did not significantly alter WNV infection or transmission dynamics in *Cx. pipiens* biotype *pipiens* mosquitoes. Infection studies further indicated that ISV susceptibility is highly dependent on infection route, with efficient infection following intrathoracic injection but limited infection via oral exposure, suggesting midgut-associated barriers.

Collectively, these findings demonstrate that ISV-arbovirus interactions are highly complex and context-dependent, influenced by factors such as virus strain, infection timing, and host background. While ISVs remain promising candidates for innovative vector control strategies, the lack of consistent interference observed in this study highlights the need for comprehensive evaluation across diverse experimental and ecological settings. Furthermore, this work

characterized *Cx. pipiens sensu stricto* cell lines as valuable tools for advancing research on arbovirus-vector interactions in temperate mosquito species.

Kurzdarstellung

Arboviren wie das West-Nil-Virus (WNV) und das Usutu-Virus (USUV) stellen in Europa ein zunehmendes Risiko für die öffentliche Gesundheit dar, wobei Stechmücken des *Culex pipiens*-Komplexes sowie *Culex torrentium* als Hauptvektoren fungieren. Trotz ihrer epidemiologischen Bedeutung sind die molekularen Determinanten der Arbovirusinfektion und der Vektorkompetenz bei *Culex*-Mücken bislang nur unzureichend verstanden. Parallel dazu sind insektenspezifische Viren (ISVs), die ausschließlich in Insekten replizieren, als wichtige Bestandteile des Mückenviroms in den Fokus gerückt, da sie das Potenzial besitzen, die Replikation und Transmission von Arboviren zu modulieren.

Mithilfe einer Kombination aus in vitro-Infektionsassays, Small-RNA-Sequenzierung, Viromanalyse sowie in vivo-Infektionsexperimenten wurde in dieser Arbeit die virale Replikationskinetik, antivirale RNA-Interferenz-(RNAi)-Antworten sowie mögliche Interferenzeffekte zwischen ISVs und medizinisch relevanten Arboviren untersucht. Zwei neu etablierte, aus *Cx. pipiens* abgeleitete Zelllinien (CPE/LULS50 und CPL/LULS56) wurden unter Verwendung repräsentativer Viren aus den Familien *Flaviviridae*, *Togaviridae* und *Peribunyaviridae* charakterisiert. Beide Zelllinien unterstützten die Replikation eines breiten Spektrums von Arboviren und ISVs und zeigten ausgeprägte virusabgeleitete siRNA-Antworten, was auf eine funktionelle RNAi-Aktivität hinweist. Im Gegensatz zu vielen etablierten Mückenzelllinien konnten keine persistierenden ISV-Infektionen nachgewiesen werden, was ihre Eignung als kontrollierte experimentelle Systeme unterstreicht.

Koinfektionsexperimente in Zellkultur mit den insektenspezifischen Viren Eilat-Virus (EILV) und Niénokoué-Virus (NIEV) zeigten keine konsistente Modulation der Replikation von Arboviren, einschließlich WNV und USUV, weder unter akuten noch unter persistenten Infektionsbedingungen. Ebenso zeigten in vivo-Experimente, dass eine vorherige Infektion mit dem insektenspezifischen Agua-Salud-Alphavirus (ASALV) die Infektion und Transmission von WNV in *Cx. pipiens* biotyp *pipiens*-Mücken nicht signifikant beeinflusste. Weitere Untersuchungen deuteten darauf hin, dass die Suszeptibilität gegenüber ISVs stark vom Infektionsweg abhängt, wobei nach intrathorakaler Injektion effiziente Infektionen beobachtet wurden, während eine orale Aufnahme nur zu einer eingeschränkten Infektion führte, was auf das Vorhandensein von Barrieren im Mitteldarm hinweist.

Zusammenfassend zeigen diese Ergebnisse, dass Interaktionen zwischen ISVs und Arboviren hochkomplex und kontextabhängig sind und durch Faktoren wie Virusstamm,

Infektionszeitpunkt und Wirtskontext beeinflusst werden. Obwohl ISVs vielversprechende Kandidaten für innovative Strategien zur Vektorkontrolle darstellen, verdeutlicht das Ausbleiben konsistenter Interferenzeffekte in dieser Arbeit die Notwendigkeit umfassender Untersuchungen unter unterschiedlichen experimentellen und ökologischen Bedingungen. Darüber hinaus wurden in dieser Arbeit *Cx. pipiens sensu stricto*-Zelllinien als wertvolle Werkzeuge zur Erforschung von Arbovirus-Vektor-Interaktionen in temperaten Mückenarten etabliert.

List of abbreviations

%	- percent
(RT-)qPCR	- (reverse transcription) quantitative PCR
Ae.	- *Aedes*
approx.	- approximately
arbovirus	- arthropod-borne virus
as	- antisense
BNITM	- Bernhard Nocht Institute for Tropical Medicine
bp	- basepair
BSL	- biosafety level
C	- capsid protein
CPE	- cytopathic effect
CT	- *Culex tarsalis* cell line
Cx.	- *Culex*
D.	- *Drosophila*
dd	- double-distilled
DMEM	- Dulbecco's Modified Eagle Medium
DNA	- deoxyribonucleic acid
dNTP	- deoxynucleotide triphosphate
dpi	- days post infection
dpt	- days post transfection
dsRNA	- double-stranded ribonucleic acid
eGFP	- enhanced green fluorescent protein
FBS/FCS	- fetal bovine serum / fetal calf serum
GMEM	- Glasgow Minimum Essential Medium
hpi	- hours post infection
hpt	- hours post transfection
kb	- kilobase
L-15	- Leibovitz L-15 medium
miRNA	- microRNA
MOI	- multiplicity of infection
mRNA	- messenger RNA
n	- number
ns	- non-significant
nsP	- non-structural protein
nt	- nucleotide
p	- p-value
PBS	- Phosphate-buffered saline
PCR	- polymerase chain reaction
PFU	- plaque forming units
piRNA	- PIWI-interacting RNA
PIWI	- P-element induced Wimpy testis
pmol	- picomol
RNA	- ribonucleic acid
RT	- room temperature
s	- sense
siRNA	- small interfering RNA
ssRNA	- single-stranded ribonucleic acid
TCID50	- 50 % tissue culture infective dose

Tm	- melting temperature
TPB	- Tryptose phosphate broth
UTR	- untranslated region
ssRNA	- single-stranded ribonucleic acid
TCID50	- 50 % tissue culture infective dose

List of figures

List of tables

1. Introduction

1.1. Introduction to arthropod-borne viruses

Arthropod-borne viruses (arboviruses) are a taxonomically heterogeneous group of viruses transmitted by arthropod vectors to vertebrate hosts. It is estimated that over 90% of all zoonotic arboviruses are transmitted by mosquitoes (McGraw & O'Neill, 2013). Warmer temperatures, prolonged summers, and altered rainfall patterns have expanded mosquito activity and breeding range in regions of the Northern hemisphere, resulting in increased arboviral derived disease and subsequent public-health burden (Barber et al., 2010; Canali et al., 2017; D'Amore et al., 2022; Erazo et al., 2024; Franklinos et al., 2019; Guzzetta et al., 2017; Parra et al., 2023; Staples et al., 2014; Zohrabian et al., 2004). Indeed, in the last two decades Europe has reported not only travel associated, but recurrent autochthonous human cases of arboviruses such as Dengue virus and Chikungunya virus (Calzolari, 2016; Grandadam et al., 2011; La Ruche et al., 2010; Rezza et al., 2007; Schaffner et al., 2013; Schmidt-Chanasit et al., 2010). These viruses are primarily associated with tropical/subtropical regions, and are mainly correlated to the ecological spread of opportunistic mosquito species, such as *Aedes albopictus* (*Ae. albopictus*) and *Aedes japonicus* (Heitmann, Jansen, Lühken, Helms, et al., 2018; Medlock et al., 2012; Sprenger & Reiter, 1987). However, another substantial number of medically important arboviruses are vectored by mosquitoes of the *Culex* genus, and are far less researched (Haba & McBride, 2022; Jansen et al., 2023; Vogels et al., 2017). In temperate regions, mosquito-borne arboviruses represent a major public health concern, with West Nile virus (WNV) being the predominant *Culex*-borne virus endemically circulating in Europe (De La Calle-Prieto et al., 2024; Giesen et al., 2023). It has been observed that in recent years, climate change has intensified the transmission risks of *Culex*-borne arbovirus across temperate regions (Barrera et al., 2019; Bellini et al., 2014; Caillouët et al., 2008; Caillouët & Robertson, 2020; Jacquot et al., 2017; Madhav et al., 2024; Nash et al., 2001; Paull et al., 2017; Reisen et al., 2006; Russell et al., 2022; Shaman et al., 2005; Wilke et al., 2021).

1.1.2. Important arboviruses transmitted by *Culex* spp. in European context

1.1.2.1. West Nile virus

WNV (family: *Flaviviridae*, genus: *Orthoflavivirus*, species : Orthoflavivirus nilense) is one of the most widely distributed arbovirus worldwide, and the major cause of mosquito-borne encephalitis in temperate regions (Alli et al., 2021; Antón Berenguer et al., 2025; Maayan Eshed

et al., 2024). Most WNV infections in humans and horses are asymptomatic, or result in a self-limiting influenza-like illness characterized by fever, fatigue, and myalgia. Approximately <1% of human infections and up to 10% of equine infections progress to neuroinvasive disease, including meningitis, encephalitis, or acute flaccid paralysis, which can be fatal or result in long-term neurological sequelae. However, these numbers were assessed largely based on outbreaks occurring in the United States of America (USA) (Beck et al., 2013; Bode et al., 2006; Carson et al., 2012; Hayes & O'Leary, 2004; Jean et al., 2007; Lindsey et al., 2009, 2012; Nash et al., 2001; Patnaik et al., 2006). In Europe alone, 989 locally acquired human infections, and 6.4% of fatality, were reported across 13 countries between January and 3th of October, 2025. Exceeding the average of 687 cases reported during the same period over the past decade (Prevention & Control (ECDC), 2025). With no specific prophylaxis or treatment available for humans, WNV represents the number one cause of *Culex*-borne arbovirus infection in Europe (De La Calle-Prieto et al., 2024; Farajollahi et al., 2011; Lustig et al., 2018).

WNV circulates in an enzootic cycle between *Culex* mosquitoes and avian hosts, while humans and horses act as incidental dead-end hosts (Andreadis, 2012; Balenghien et al., 2008; Chancey et al., 2015a; Haba & McBride, 2022; Jansen et al., 2023; Jupp, 1974; Kilpatrick et al., 2005; Muñoz et al., 2012; Vogels et al., 2017). Environmental factors such as temperature, rainfall, and avian host density strongly influence viral amplification and transmission dynamics (Zeller & Schuffenecker, 2004). Outbreaks have been reported across Africa, the Middle East, Europe, and the Americas since its discovery in Uganda in 1937, with its introduction into North America in 1999 marking a major global expansion (Smithburn et al., 1940).

Genomically, WNV possesses a single-stranded positive-sense RNA (+ssRNA) genome of approximately 11 kb. The genome is organized into a single open reading frame (ORF) flanked by structured 5′ and 3′ UTRs. This ORF is translated as a single polyprotein, subsequently cleaved into three structural (capsid (C), premembrane/membrane (prM/M), and envelope (E)) and seven non-structural proteins (NS1 through NS5). The viral RNA contains a type I 5′ cap structure (m^7GpppAm) but lacks a poly(A) tail, and is flanked by highly structured 5′ and 3′ untranslated regions (UTRs) (Brinton, 2009). Replication occurs in association with endoplasmic-reticulum-derived vesicles, where viral non-structural proteins reorganize host membranes to form replication complexes that protect double-stranded RNA (dsRNA) intermediates from antiviral defences (Lwande et al., 2015).

Phylogenetically, at least nine genetic lineages of WNV have been identified on the basis of signature amino acid substitutions and deletions in coding-sequence for the envelope or the non-structural protein 5. Yet only lineages 1 and 2 have been associated with significant human and animal disease (Chancey et al., 2015b; Pauli et al., 2013; Pesko et al., 2012). Lineage 1 exhibits the broadest geographic distribution, encompassing Africa, Europe, Asia, and the Americas. Within lineage 1, clade 1a includes the strains responsible for the North American epidemics following the 1999 New York introduction, while clade 1b corresponds to Kunjin virus, endemic to Australia.

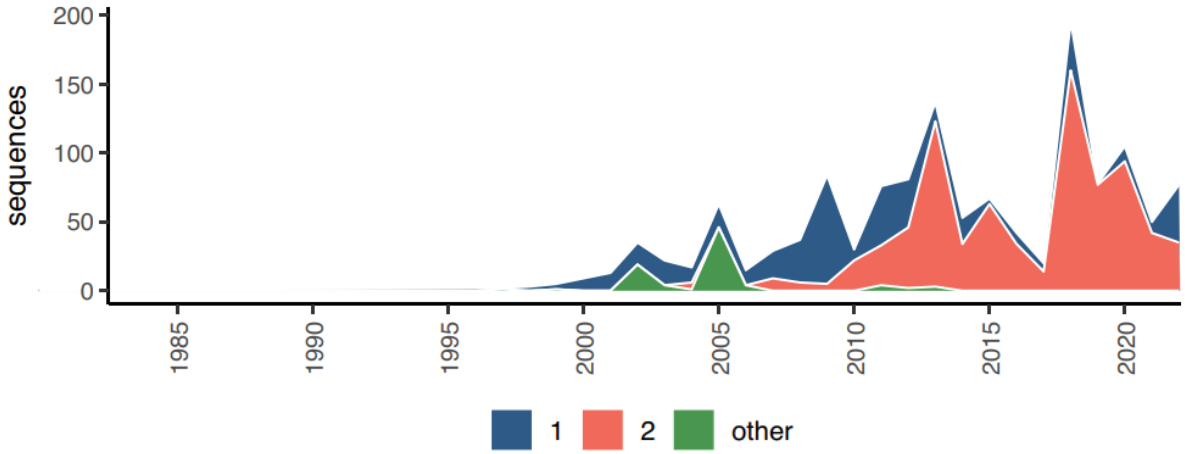

Figure 1. Report of West Nile virus lineage occurrence in Europe over time. Time series of reported European WNV lineage 1 (blue), 2 (red), and others (green) partial and whole-genome sequences to NCBI GenBank **(**Retrieved from (Koch et al., 2024)).

Since the European introduction of lineage 2 in the early 2000s, a notable epidemiological shift has occurred, marked by the dominance of lineage 2 over lineage 1a as the predominant circulating lineage (Figure 1). Since then, WNV has progressively expanded into more northern regions, and the number of reported cases has steadily increased across the continent, albeit with substantial interannual fluctuations. While improved diagnostic capacity and mandatory reporting have contributed to this trend, the broader emergence of lineage 2, together with the climate-driven expansion of vector habitats, has likely facilitated enhanced transmission in temperate regions (Cardoen et al., 2017; Koch et al., 2024). Although most human cases are not routinely characterized at the lineage level, sequencing of mosquito and avian samples during outbreaks strongly suggests that lineage 2 has been responsible for the majority of European WNV outbreaks between 2010 and 2020 (Koch et al., 2024).

1.1.2.2. Usutu virus

USUV (family *Flaviviridae*, genus: *Orthoflavivirus*, species: Orthoflavivirus usutuense) is an emerging arbovirus, taxonomically close to WNV within the Japanese encephalitis virus (JEV) serocomplex (Kuno et al., 1998). First isolated in 1959 from *Culex neavei* in Swaziland, USUV has expanded its range from Africa into Europe, where it now circulates endemically alongside WNV (McIntosh, 1985; Schopf et al., 2025a, 2025b; Simonin, 2024; Williams et al., 1964). Transmission cycles primarily involve *Culex pipiens* mosquitoes and several avian hosts, which serve as key amplifying hosts (Becker et al., 2012; Münger et al., 2025; Roiz et al., 2019; Vilibic-Cavlek et al., 2020). USUV infection in birds often causes high mortality, especially among Eurasian blackbirds, great grey owls, and house sparrows, and large-scale die-offs have been recorded during major epizootics in 2016 and 2018 across Central and Western Europe (Clé et al., 2019; Folly et al., 2020; Lühken et al., 2017; Simonin, 2024). In humans, most infections are asymptomatic or result in mild febrile illness; however, rare cases of neuroinvasive disease have been reported, primarily in immunocompromised patients (Cadar & Simonin, 2023; Cavrini et al., 2009).

Similar to other members of the *Flaviviridae* family, USUV possesses an +ssRNA genome of approximately 11 kb. This genome is organized into a single ORF flanked by structured 5′ and 3′ UTRs. The ORF encodes a polyprotein for three structural proteins (C, prM/M, E), and seven non-structural proteins, NS1 through NS5. The viral RNA features a type I 5′ cap but lacks a polyadenylated tail, consistent with the canonical organization observed in flaviviruses (Zhou et al., 2023).

Eight genetic lineages of USUV have been delineated, three African and five European, suggesting repeated introductions from Africa followed by local diversification (Bakonyi et al., 2014). USUV is reported to now co-circulate with WNV across overlapping ecological niches in *Culex* mosquitoes and avian hosts presenting a growing challenge for both surveillance and vector control. Sequential or simultaneous infections in shared vectors and reservoirs may result in competitive or synergistic interactions that alter vector competence and consequently influence viral transmission, persistence, and spillover risk. As both viruses continue to expand northward and establish endemic transmission in Europe, the design of more effective public health and ecological control strategies is required (Simonin, 2024).

1.2. *Culex* mosquitoes as vectors

1.2.1. Ecological and taxonomic diversity: *Culex pipiens* complex, and *Culex torrentium*

Mosquitoes of the genus *Culex* comprise roughly 770 species across 26 subgenera. The subgenus *Cx. pipiens* Linnaeus 1758 contains the medically important complex of *Cx. pipiens* (Wilkerson et al., 2021). The *Cx. pipiens* complex is composed by many species with panmictic, essentially global distribution except for regions with extreme climate condition: *Cx. pipiens pallens*, *Cx. quinquefasciatus*, *Cx. australicus*, *Cx. globocoxitus, and finally the Cx. pipiens pipiens senso stricto* (*Cx. pipiens ss*) biotypes *pipiens* (*Cx. p.* biotype *pipiens*) and *molestus* (*Cx. p.* biotype *molestus*). *Cx. pipiens ss* represents the mosquito species with the most relevance for human and animal health in Europe within the *Cx. pipiens* complex, associated with the transmissions of several pathogens (Farajollahi et al., 2011; Harbach, 2011; Laurito et al., 2017).

Cx. pipiens ss mosquitoes serve as vectors for several medically important arboviruses, including WNV, USUV, Japanese encephalitis virus, and St. Louis encephalitis virus, as well as human lymphatic filariasis (Harbach, 2011; Nikolay et al., 2012; Talla et al., 2016; Tandina et al., 2016). The biotypes (*pipiens* or *molestus*) mainly differ in their habitat and host preference. Biotype *molestus* is found in urban settings and is thought to have an anthropophilic/-phagic feeding pattern. Mosquitoes of the biotype *pipiens* are associated with rural/suburban areas, and described as ornithophilic/-phagic. However, a recent study conducted by Wehmeyer et al, challenges this concept by presenting that *Cx. pipiens ss* display similar host feeding preference, being all capable of acting as bridge vectors for arbovirus such as WNV (Wehmeyer et al., 2024). Besides their ecological versatility and host feeding preference they exhibit seasonal activity and overwintering strategies that enhance survival and transmission potential as it has been described for WNV overwintering in *Cx p.* biotype *pipiens* (Kampen et al., 2021).

Mosquitoes of the *Culex torrentium* species, closely related to *Cx. pipiens ss*, are widespread in Europe and often co-occur with *Cx. pipiens ss* (Hesson et al., 2014). Its potential as vector of WNV is increasingly recognized (Höller et al., 2026; Jansen et al., 2019, 2019; Leggewie et al., 2016). *Culex torrentium* feeding patterns present host feeding preferences with great similarity to *Cx. pipiens ss* where specimens were found to feed on birds, humans, or non-human mammals, showing potential to also act as a bridge vector for WNV or other arbovirus, and are therefore considered as a mosquito species of public health concern (Wehmeyer et al., 2024).

However, little is known about the vector dynamics of *Cx. torrentium* and *Cx. pipiens ss*, or whether these dynamics could be manipulated to mitigate arbovirus transmission by the mosquito.

1.3. Vector competence

"Vector competence" refers to the intrinsic ability of an arthropod vector, in this case the mosquito, to acquire, support replication of, and transmit a pathogen to a new host (Bellone & Failloux, 2020; Tabachnick, 1994; V. Y. Wu et al., 2022). However, this process is influenced by a complex multivariable setting, which can result in different outcomes on the success of a pathogen transmission. Factors influencing include the mosquito population, same mosquito species but with different geographic distribution, same virus family but different lineages/strains, and between virus families (Blair, 2022; Sanchez-Vargas et al., 2004; Souza-Neto et al., 2019; Wei et al., 2020).

The inner drivers of vector competence are characterised by the species genetic background (Beerntsen et al., 2000), physical barriers (Franz et al., 2015; Hardy et al., 1983), mosquito immune capacity (Bartholomay & Michel, 2018; Kumar et al., 2018; W.-S. Lee et al., 2019; Tikhe & Dimopoulos, 2021), microbiota (Cansado-Utrilla et al., 2021), and feeding behaviour (Bellone & Failloux, 2020; Jupatanakul et al., 2014; Kent et al., 2010; Lambrechts et al., 2009, 2013; Tabachnick, 2013). Despite mosquitoes' major role as disease vector, very little is known about the specific factors and mechanisms that determine the different components of vector competence. Most of the knowledge comes from studies on *Ae. aegypti* or is extrapolated from studies on model organisms such as *Drosophila*, particularly regarding the mosquito immune response and microbiota. Understanding these factors has recently gained significant attention in arboviral research, especially in the context of developing novel vector control strategies (Galiana-Arnoux et al., 2006; Rij et al., 2006; X.-H. Wang et al., 2006). However, *Culex* mosquitoes despite their central role as vectors of zoonotic flaviviruses such as WNV and USUV remain comparatively understudied.

Molecular, immunological, and microbiota-related determinants of vector competence in *Culex* species are still poorly resolved, and current control strategies for *Culex*-borne diseases rely heavily on chemical adulticides and larvicides (Belinato et al., 2013; Bellini et al., 2014; Chaskopoulou et al., 2011; Elnaiem et al., 2008; Macedo et al., 2010; Mwangangi et al., 2011; Rubio et al., 2018; Seccacini et al., 2008; Su et al., 2023; Trout et al., 2007). Yet, increasing

insecticide resistance and environmental safety concerns highlight the need for innovative approaches that target mosquito-virus interactions rather than mosquito populations alone (Arich et al., 2024; Hafez & Abbas, 2021; Lopes et al., 2019; Scott et al., 2015; Xu et al., 2005). These challenges underscore the importance of understanding how arboviruses establish infection within the mosquito host. How intrinsic antiviral mechanisms, particularly RNA interference (RNAi) pathways and its interactions with the mosquito microbiota, shape the outcome of infection and ultimately determine vector competence (Madhav et al., 2024).

1.3.1. Mosquito antiviral responses

The mosquito antiviral immune response plays a central role in limiting viral replication and shaping the outcome of infection. Following ingestion of a viremic blood meal, an arbovirus first enters the midgut lumen, where it must attach to and infect the midgut epithelial cells (Figure 2) (M. Liu et al., 2008; Neelakanta & Sultana, 2016; Smartt et al., 2009; Yun & Lee, 2018). This initial step constitutes the *midgut infection barrier*. At this early stage of infection, epithelial integrity, antimicrobial peptides and interactions with the resident microbiota can restrict viral replication before becoming a systemic infection (W.-S. Lee et al., 2019; Medigeshi, 2011).

If the virus successfully replicates in the midgut epithelium, it must then cross the basal lamina, the *midgut escape barrier*, to enter the haemocoel (Figure 2). There, the virus is exposed to circulating immune factors, including haemocytes that mediate phagocytosis and melanisation, as well as humoral effectors such as antimicrobial peptides and reactive oxygen and nitrogen species. Enduring viruses can then disseminate to secondary tissues and, critically, the salivary glands (Richards et al., 2012).

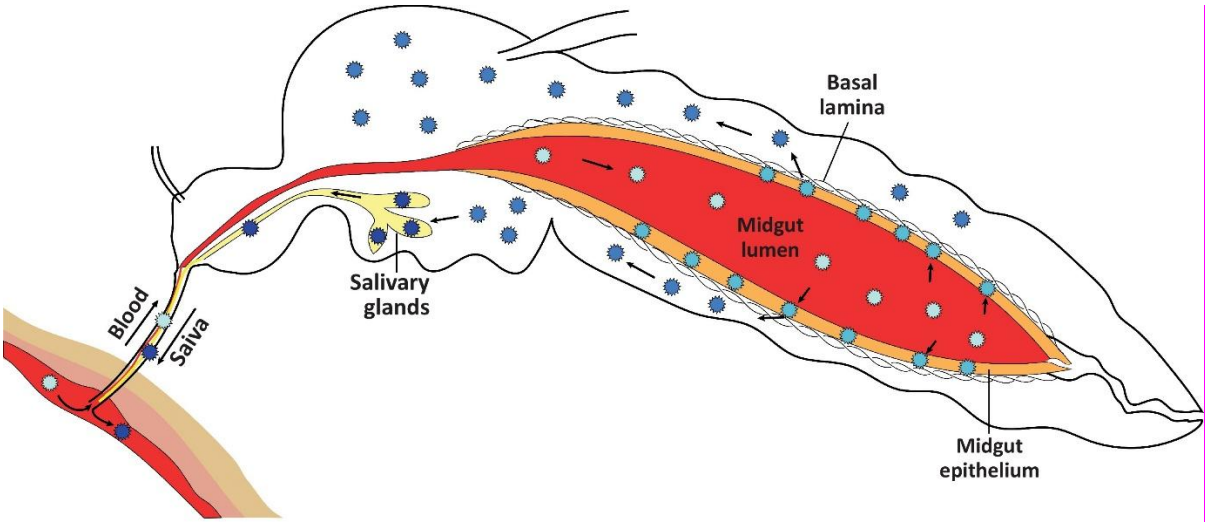

Figure 2. Arbovirus infection cycle within a mosquito vector (Rückert & Ebel, 2018). After a mosquito takes an infectious blood meal, the virus initially infects and replicates in the epithelial cells lining the midgut. Viral particles then need to cross the midgut's basal lamina to enter the hemocoel and spread throughout the body, reaching additional tissues. A systemic infection becomes established once the virus disseminates to multiple organs, eventually reaching the salivary glands. Infection of the salivary glands enables the virus to be transmitted to a new vertebrate host during the mosquito's next blood feeding. Several intrinsic anatomical and physiological barriers, such as midgut and salivary gland infection and escape barriers, play key roles in controlling this dissemination process (Rückert et al., 2017).

Infection of the salivary glands represents the final and decisive step, as only viruses that successfully invade this tissue can be released in the saliva during subsequent blood feeding and be transmitted into a new vertebrate host. Even at this stage, local immune mechanisms, such as apoptosis, autophagy and innate signalling pathways, can limit viral replication. Across all compartments, however, intracellular antiviral mechanisms, in particular RNA interference (RNAi), act as a central line of defence by recognising and degrading viral RNA (Ahlers et al., 2019; Altinli et al., 2023; Blair, 2011; Donald et al., 2012; Göertz et al., 2019; Madhav et al., 2024; Paradkar et al., 2012, 2014; Rückert et al., 2019; Walsh et al., 2022).

1.3.1.1. RNA-interference pathways

RNAi is a sequence-specific posttranslational silencing process of gene expression induced by dsRNA. In eukaryotes, gene regulation can occur by a variety of small RNA pathways, which can be involved in transposon or endogenous gene expression regulation and also act against virus infections. All described endogenous RNAi pathways share a conserved effector complex, which contains at least a protein from the Argonaute (AGO) family and a short single-stranded RNA (ssRNA) molecule. Such AGO-RNA complexes can repress the transcription of genes,

target messenger RNA (mRNA) for site-specific cleavage, or block mRNA translation into proteins (Sioud, 2021).

In mosquitoes, RNAi is described as the main antiviral defence response and comprises three major classes of small RNAs (sRNA): (i) small interfering RNAs (siRNAs), (ii) microRNAs (miRNAs), (iii) and PIWI-interacting RNAs (piRNAs) (Figure 3). While these pathways have distinct biological functions, they can act to shape cellular responses to viral infection and influence vector competence (Altinli et al., 2023; Bivalkar-Mehla et al., 2011; Blair, 2011; Blair & Olson, 2015; Brackney et al., 2009; Dietrich et al., 2017; Leggewie & Schnettler, 2018; Scherer, 2021).

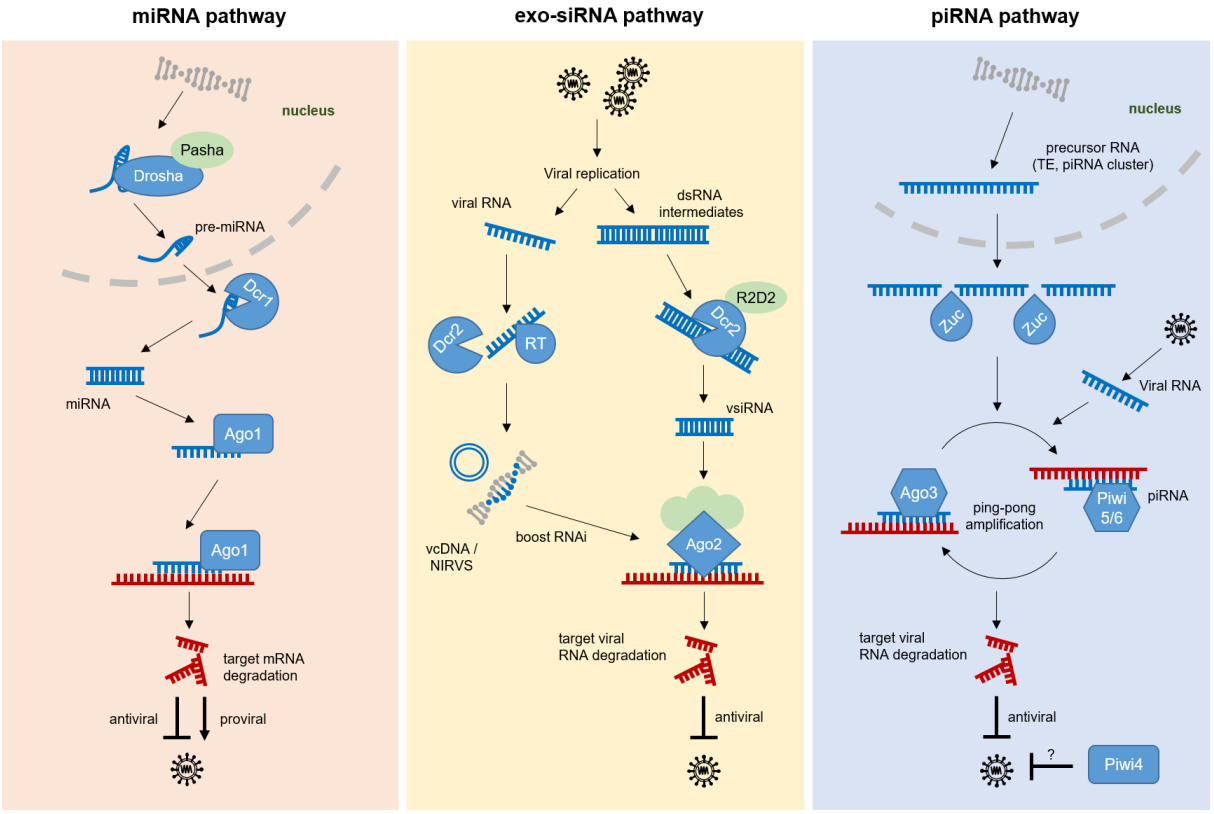

Figure 3. Overview of mosquito RNAi pathways involved in antiviral defence. Mosquitoes possess three main RNAi pathways to modulate viral replication. The microRNA (miRNA) pathway (left) acts on precursor miRNAs of host or viral origin, which are processed by Dicer-1 into ~22–23 nt duplexes. These duplexes load into Argonaute-1 (AGO1) and guide the complex to regulate complementary RNA transcripts. The small interfering RNA (siRNA) pathway (middle) responds to exogenous double-stranded RNA. Dicer-2 cuts this dsRNA into ~21 nt siRNA pairs, and one strand is incorporated into an AGO2-based effector complex that targets and cleaves viral RNA. The PIWI-interacting RNA (piRNA) pathway (right) derives from single-stranded precursors that bind PIWI proteins, including AGO3. Through the ping-pong amplification mechanism, this pathway generates ~24–30 nt piRNAs, showing the characteristic U1/A10 sequence bias and 10-nt overlap pattern. Retrieved from (Scherer, 2021).

The miRNA pathway primarily regulates host gene expression, but can influence antiviral defence indirectly or directly. Endogenous miRNAs (20–25 nucleotides) are derived from host hairpin transcripts processed by Drosha in the nucleus and Dicer-1 in the cytoplasm, before being loaded into the RNA induced silencing complex (RISC) with Argonaute-1 as catalytic factor. By targeting host mRNAs involved in immunity, metabolism, or cellular stress responses, miRNAs modulate the cellular environment in ways that may restrict or facilitate viral replication (Bavia et al., 2016).

The exogenous (exo-)siRNA pathway constitutes the primary antiviral RNAi response in mosquitoes. During viral replication, dsRNA intermediates are generated and recognised by the RNase III enzyme Dicer-2 (Dcr-2), in association with its co-factor R2D2, Dcr-2 cleaves these intermediates into 21-nucleotide virus-derived (vsiRNA). These 21-nucleotide vsiRNAs are subsequently loaded into RISC, which in association with the catalytic enzyme Argonaute-2 (AGO2). AGO2 uses the guide strand to identify complementary viral RNA molecules, which are then cleaved and degraded, providing a potent, sequence-specific suppression of viruses including flaviviruses, alphaviruses, and bunyaviruses (Blair & Olson, 2015; Campbell et al., 2008; Cirimotich et al., 2009; Kemp et al., 2013; Khoo et al., 2010; Myles et al., 2008; Schnettler et al., 2013; Schulz & Becker, 2018).

The piRNA pathway is a Dicer-independent RNAi mechanism primarily associated with transposon silencing in the germline, but in mosquitoes it is hypothesized to contributes to antiviral defence in somatic tissues (Ku & Lin, 2014; Varjak et al., 2018). Virus-derived piRNAs (vpiRNAs), typically 24–30 nt long, are generated from ssRNA precursors originating from viral genomes, endogenous viral elements (EVEs), or piRNA clusters. These are processed by a combination of helicases and endonucleases before loading onto PIWI proteins such as Piwi1-7 and AGO3 (Girardi et al., 2017; P. Liu et al., 2016; Tassetto et al., 2019). In mosquitoes, primary piRNAs are typically antisense to the target sequence and exhibit a 5′ uridine (U1) bias, while secondary piRNAs arise through the ping-pong amplification cycle, producing a complementary sense piRNA with an adenine at position 10 (A10) (Aravin et al., 2007; Czech & Hannon, 2016; Han et al., 2015; Huang et al., 2017; Miesen et al., 2016; Mohn et al., 2015; Senti & Brennecke, 2010; Yamanaka et al., 2014). This cycle allows amplification of specific piRNAs and generates a characteristic U1/A10 nucleotide signature and a 10 nt 5´overlap of the primary and secondary piRNAs; reflecting reciprocal cleavage between Piwi- and AGO3-bound piRNAs.

1.3.1.2. RNAi-virus interactions in *Culex* spp.

Arbovirus replication in *Culex* mosquitoes triggers characteristic RNAi responses, most prominently the production of vsiRNAs (Brackney et al., 2010). The production of vsiRNAs has been repeatedly observed during WNV infection in *Culex tarsalis* (*Cx. tarsalis*) and *Culex quinquefasciatus* (*Cx. quinquefasciatus*)-derived cell lines, demonstrating that the siRNA pathway is active against flaviviruses in *Culex* spp. (Fros et al., 2015; Göertz et al., 2019; Rückert et al., 2019). Early and persistent production of vsiRNAs has also been reported in *Cx. quinquefasciatus* Hsu cells and *Cx. tarsalis* CT cells infected with WNV, including during long-term persistent infection (Göertz et al., 2019; Rückert et al., 2019). Similar vsiRNA profiles have been described in *Cx. pipiens sl* following infection with both WNV and USUV, supporting the view that the siRNA pathway constitutes a major antiviral response across *Culex* species.

In contrast, the piRNA pathway shows more variable involvement. Infection of *Cx. pipiens sl* and *Cx. tarsalis* cell lines with WNV and SINV produce piRNA-sized small RNAs, but these typically lack the canonical ping-pong amplification signature, suggesting limited or non-canonical piRNA activity against these viruses in *Culex*, unlike what is described for SINV in *Aedes* cells (Fros et al., 2015; Göertz et al., 2019; Miesen et al., 2016; Rückert et al., 2019; Tassetto et al., 2019; Vodovar et al., 2012, 2012). Notably, piRNA responses appear species- and virus-specific: for example, piRNA-sized reads are detectable in WNV-infected *Cx. pipiens*, but not consistently in *Cx. quinquefasciatus* (Fros et al., 2015; Rückert et al., 2019). In contrast to flaviviruses and alphaviruses, bunyaviruses such as Orthobunyavirus bunyamweraense virus (BUNV) induce robust vpiRNA production with clear ping-pong signatures in *Cx. quinquefasciatus* Hsu cells and *Aedes* cell lines, consistent with activation of the canonical antiviral piRNA pathway (Altinli et al., 2023; Scherer, 2021; Varjak et al., 2018; Walsh et al., 2022).

1.3.2. Microbiota and vector competence

The mosquito endogenous microbiota is primarily located in the midgut but can also be found in other tissues, such as the gonads, and includes viruses, bacteria and eukaryotes (J. Wang et al., 2023). Following ingestion of an infectious blood meal, virions enter the mosquito midgut and therefore come into direct contact with the resident microbiota and their metabolic products. Increasing evidence indicates that the mosquito microbiota plays an important role in shaping vector development, reproduction, metabolism, immunity, and vector competence. Therefore it

has been explored to develop non-chemical-based alternative control methods that aim to reduce vector populations, or vector competence (Cirimotich et al., 2011; J. Wang et al., 2023).

Most work to date has focused on bacterial symbionts particularly *Wolbachia* which can enhance basal immunity, stimulate antiviral RNAi, and interfere with arbovirus replication, thereby reducing transmission in several mosquito species (Caragata et al., 2014; Howard et al., 2011; Hussain et al., 2011; Pan et al., 2012; Ramirez et al., 2014; S. Wang et al., 2012; Woolfit et al., 2015; Zhang et al., 2013). Such findings have led to the development of microbiota-based vector control strategies, including *Wolbachia* releases and paratransgenic approaches that engineer bacterial symbionts to deliver antiviral effector molecules (Glaser & Meola, 2010; Hoffmann et al., 2011; Moreira et al., 2009).

Recently, attention has been made to insect-specific viruses (ISVs) as another potential tool for the development of microbiota-based vector control strategies. ISVs are a major but historically overlooked component of the mosquito microbiota with growing evidence for their capacity to modulate vector competence (Agboli et al., 2019).

ISVs were first described in the mid-1970s with the detection of the cell fusing agent virus (CFAV) in *Aedes aegypti* (*Ae*. *aegypti*)-derived cell lines, where viral replication was restricted to insect cells and absent in vertebrate systems (Stollar & Thomas, 1975). Advances in sequencing technologies, together with improved bioinformatic tools and reduced costs, have markedly accelerated ISVs discovery over the past decade across diverse insect hosts (Calisher & Higgs, 2018). ISVs are now recognised as components of the insect microbiome and are defined by their inability to replicate in vertebrate hosts (B. Bolling et al., 2015). In contrast to arboviruses, which can alternate between vertebrate hosts and mosquito vectors, ISVs are thought to be maintained primarily through vertical and horizontal transmission between mosquitoes (Blitvich & Firth, 2015; B. G. Bolling et al., 2011; Elrefaey et al., 2020; Haddow et al., 2013; Hermanns et al., 2020; Junglen et al., 2017; Nasar et al., 2012; Saiyasombat et al., 2011; Stollar & Thomas, 1975). Notably, many ISVs cluster within viral families that include medically and veterinary important arboviruses, such as *Flaviviridae*, *Togaviridae*, and *Peribunyaviridae* (Marklewitz et al., 2013; Öhlund et al., 2019), and phylogenetic analyses suggest that ISVs may represent ancestral lineages of contemporary dual-host arboviruses (Marklewitz et al., 2015). This close evolutionary relationship has positioned ISVs as valuable models for investigating virus evolution, host restriction, and the molecular determinants underlying vector competence.

A growing body of experimental evidence indicates that prior infection with certain ISVs can interfere with subsequent arbovirus infection, a phenomenon attributed to mechanisms such as superinfection exclusion or priming of antiviral immune responses. This interference is highly virus-specific, with some ISVs significantly reducing arbovirus replication while others exhibit little or no effect. For example, *Cx. annulirostris* mosquitoes persistently infected with the insect-specific Palm Creek virus showed reduced susceptibility to WNV following oral exposure (Hall-Mendelin et al., 2016). Similar inhibitory effects have been reported in mosquito-derived cell lines infected with Nhumirim virus or co-infected with CFAV and Phasi Charoen-like virus (Kenney et al., 2014; Schultz et al., 2018). However, CFAV and other ISVs, including Culex flavivirus, have failed to consistently suppress pathogenic flavivirus replication (Kent et al., 2010; Kuwata et al., 2015; Schultz et al., 2018). Underscoring the importance of careful investigation when it comes to ISV-arbovirus interaction.

The molecular basis of ISV-mediated interference remains poorly understood. Still, it is hypothesised to involve competition for host cellular resources required for viral entry, genome replication, translation, or particle assembly, as well as modulation of antiviral immune pathways (Y.-M. Lee et al., 2005). Importantly, viral phylogenetic proximity may influence the likelihood and magnitude of interference, with closely related viruses more frequently associated with suppressive effects (Barbanti-Brodano et al., 1970; Blitvich & Firth, 2015; Burivong et al., 2004; Nasar et al., 2015; Pepin et al., 2008; Sundin & Beaty, 1988; Tscherne et al., 2007). Building on their stable association with mosquito hosts, and phylogenetic proximity with arbovirus, ISVs have emerged as promising candidates for novel vector control strategies aimed at reducing arbovirus transmission. Similar to bacterial symbionts such as *Wolbachia*, the predominantly vertical transmission of ISVs offers the potential for long-term persistence within mosquito populations, while their insect host restriction supports their safety as biocontrol agents. Despite their potential, the application of ISVs as biocontrol agents remains at an early stage. Many ISVs have yet to be evaluated for their ability to establish stable, persistent infections in mosquito populations or to consistently block transmission of medically relevant arboviruses. Further research is therefore required to identify suitable ISV candidates, elucidate their mechanisms of action, and assess their ecological stability and effectiveness under natural conditions.

1.3.2.1. Insect-specific viruses of interest for the current study

The current work focuses on three ISVs selected for their phylogenetic diversity: Eilat virus (EILV), Agua Salud virus (ASALV), and Niénokoué virus (NIEV). Most ISV-arbovirus interaction studies to date have considered viruses within the same family, particularly insect-specific flaviviruses with flavi-arboviruses, and the few insect-specific alphaviruses with alpha-arboviruses. In contrast, cross-family interactions, such as alphavirus ISVs with flavivirus arboviruses, as well as ISV-arbovirus interactions studies in *Culex* systems, remain largely unexplored.

EILV is an alphavirus (family *Togaviridae*) originally isolated from *Anopheles coustani* mosquitoes in Israel (Nasar et al., 2012; Samina et al., 1986). While related to dual-host alphaviruses such as Sindbis and Semliki Forest viruses, EILV replication is restricted to the insect host due to entry and genomic replication barriers in the vertebrate host (Nasar, Gorchakov, et al., 2014). EILV efficiently infects a broad range of mosquito species, belonging to both the *Aedes* and *Culex* genus. Furthermore, EILV has been shown to interfere with the replication of several pathogenic alphaviruses, including Eastern, Western, and Venezuelan equine encephalitis viruses, Chikungunya virus, and Sindbis virus, as well as flaviviruses such as WNV and Bagaza virus, in mosquito cells (Guggemos et al., 2024; Joseph et al., 2024; Nasar et al., 2015). These properties make EILV a valuable tool for studying ISV-arbovirus interactions (Nasar, Gorchakov, et al., 2014; Nasar et al., 2015).

ASALV, also an alphavirus (family *Togaviridae*) is an ISV isolated from *Cx. declarator* mosquitoes in Panama (Hermanns et al., 2020). Compared with EILV, ASALV is far less studied, and to date, its potential interactions with arboviruses have not been explored or documented. Importantly, ASALV can also infect multiple mosquito genera, including *Culex*, supporting its suitability for experimental studies of vector competence (Altinli et al., 2022; Hermanns et al., 2020; Ramos et al., 2024). Phylogenetically, ASALV and EILV are distinct within the alphavirus clade, incising even more the interest of exploring their ISV-arbovirus interactions outcome (Hermanns et al., 2020).

NIEV is an insect-specific flavivirus (ISF) isolated from *Culex* mosquitoes in Côte d'Ivoire, clustering with other *Culex*-associated ISFs (Halabi & Mayrose, 2021; Junglen et al., 2009). Its host restriction appears to result from limitations in viral attachment/entry, replication, and assembly/release (Junglen et al., 2017). Even though NIEV belong to the ISF group, very few

studies have explored NIEV's impact on arbovirus replication, with only one report suggesting a reduction of WNV, though statistical support was limited (Schulze, 2023). Together, these three ISVs (EILV, ASALV and NIEV) provide a diverse framework allowing investigation of both within-family (ISV-arbovirus) and cross-family interactions on the mostly neglected *Culex* spp. mosquitoes.

1.4. *Culex* research gap

Research on mosquito-arbovirus interactions has historically focused on *Aedes* mosquitoes, particularly *Ae. aegypti* and *Ae. albopictus*, due to their role in transmitting Dengue, Zika, and Chikungunya viruses. Well-characterized cell lines such as Aag2 and C6/36 have become standard tools for studying virus-host interactions, RNAi, and antiviral immunity (Brackney et al., 2010; Léger et al., 2013). By contrast, research on *Culex* systems remains limited.

Cell culture systems play a pivotal role in advancing our understanding of virus-mosquito interactions. They serve as essential tools to study, among others, viral replication, identify pro- and antiviral host factors, and test genetic or molecular hypotheses that are difficult to address *in vivo*. In particular, mosquito-derived cell lines enable detailed investigation of vector-virus molecular interactions, functional characterization of immune pathways, and evaluation of promoter activity. These tools are especially valuable for non-model organisms such as *Culex* mosquitoes, where *in vivo* experimentation can be challenging due to ecological, biosafety, or technical constraints (Rückert, 2026). Until recently, only a few *Culex* cell lines were available, such as those derived from *Cx. tarsalis* (CT), *Cx. quinquefasciatus* (HSU), and *Cx. p.* biotype *pipiens* (CpE3) (Fallon et al., 2023). These lines do not fully represent European species, and even closely related species within the *Cx. pipiens* complex may differ in vector competence, distribution, and virus-host interactions (Dietrich et al., 2017; Jansen et al., 2023; Shocket et al., 2020).

The asymmetry in available research tools between *Aedes* and *Culex* mosquitoes limits our understanding of arbovirus persistence in temperate regions and constrains investigation of how ISVs influence arboviral replication and transmission in *Culex* vectors. Evidence from studies in *Aedes* systems suggests that ISVs can modulate innate immunity, or competitively interfere with secondary infections (Nasar et al., 2015; Patterson et al., 2020). However, equivalent data for *Culex* species are scarce, and the molecular mechanisms remain largely unknown.

Addressing this gap is crucial for contextualizing *Culex* vector competence and understanding how intrinsic antiviral mechanisms and ISVs shape arbovirus maintenance in nature. This thesis therefore focuses on *Culex*-borne arboviruses, like WNV and USUV, and their interactions with representative ISVs (EILV, NIEV, and ASALV) using *in vitro* and *in vivo Culex* systems.

1.5. Dissertation aims

Although ISVs are increasingly recognized as modulators of arbovirus replication and potential tools for vector control strategies (B. Bolling et al., 2015; Hobson-Peters et al., 2013, 2019; Nouri et al., 2018; Öhlund et al., 2019; Patterson et al., 2020), most studies have focused on within-family viral interactions and mosquito systems other than *Culex*.

Focussing on the understudied *Culex* species, the aim of this thesis was to investigate the ability of different ISVs, belonging to two distinct virus families, to interact with and potentially interfere with arbovirus infection in mosquitoes and mosquito-derived cell cultures.

Specifically, this thesis aimed to:

- Assess ISV-arbovirus interactions *in vitro*, evaluating whether EILV or NIEV can interfere with WNV and USUV replication in *Cx. tarsalis*-derived CT cells (Chapter 3.1: *ISV interference in Culex tarsalis-derived CT cells*).
- Examine ISV-arbovirus interactions *in vivo*, determining the effect of ASALV on WNV replication in *Cx. p.* biotype *pipiens* and *Cx. torrentium* mosquitoes using interference assays (Chapter 3.3: *In vivo ISV-WNV co-infections*).
- Characterize novel mosquito cell lines as experimental systems, including the newly established *Cx. pipiens ss*-derived cell lines CPE/LULS50 and CPL/LULS56 (Bell-Sakyi et al., 2021), and evaluate their susceptibility to ISVs and arboviruses from belonging to the *Flaviviridae*, *Togaviridae*, and *Peribunyaviridae* families (Chapter 3.2: *Characterization of Cx. pipiens ss-derived cell lines*). Viral-induced RNAi responses and the presence of basal ISV infections were additionally assessed using small RNA sequencing.

Importantly, most published interference studies rely on simultaneous co-infection or short sequential infections. These experimental setups do not fully reflect natural infection dynamics or the conditions under which ISVs might be applied as biological control tools. By incorporating both acute and persistent infection models, this work aims to provide further

insight into how ISV-arbovirus interactions may occur under more biologically relevant conditions.

2. Material and methods

Table 1. List of consumables.

Consumable	Manufacturer / Supplier
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	Basel, Switzerland
Biosphere Filter Tips 10 µL	1000 µL Sarstedt AG & Co. KG, N¨umbrecht, Germany
Cell culture flask T25, T75	Greiner Bio-One GmbH, Frickenhausen, Germany
Cell scraper 25 cm	Sarstedt AG & Co. KG, N¨umbrecht, Germany
Conical Centrifugation tube 15 mL, 25 mL	Sarstedt AG & Co. KG, N¨umbrecht, 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, N¨umbrecht, Germany
Parafilm	Bemis, Oshkosh, NE, USA
Serological pipets 5, 10, 25 ml	Corning Inc., Glendale, AZ, USA

2.1. Chemical Materials

Table 2. Utilized chemicals for experiments.

Chemical	Manufacturer / Supplier
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
Dulbecco's Modified Eagle Medium (DMEM)	PAN-Biotech GmbH, Aidenbach, 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
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
Glasgow's Minimal Essential Medium (GMEM)	Gibco / Thermo Fisher Scientific Inc., Waltham, MA, USA
Isopropanol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Leibovitz's L-15 medium	Gibco / Thermo Fisher Scientific Inc., Waltham, MA, USA
Nuclease-free water	Ambion / Thermo Fisher Scientific Inc., Waltham, MA, USA
Phosphate buffered saline (PBS)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Random hexamer primer	Thermo Fisher Scientific Inc., Waltham, MA, USA
RNAse inhibitor	Promega Corp., Fitchburg, WI, USA
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH + Co. KG, Karlsruhe, 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

Table 3. Primary and secondary antibodies conjugated to fluorescence dyes used for immunostaining.

Antibody	**Dilution**	**Specificity**	**Species**	**Reference / Manufacturer**
3G1.1 dsRNA Hybridoma 3G1.1 supernatant	1:100	anti-dsRNA	mouse	(O'Brien et al., 2015)
DAPI	1:2000	DNA	rabbit	Thermo Fisher Scientific Inc., Waltham, MA, USA
Alexa FluorTM 488 goat anti-mouse IgG (H+L) 2 mg/ml	1:1000	goat	Invitrogen / Thermo Fisher Scientific Inc.,	Alexa FluorTM 488 goat anti-mouse IgG (H+L) 2 mg/ml

	Waltham, MA, USA

Table 4. List of buffers and stock solutions.

Buffer	Amount	Reagent
TAE (10x) buffer	242 g 57.1 ml 100 ml ad 1000 ml	TRIS 5.7 % acetic acid EDTA (pH 8.0) / 0.5 M stock solution dH_2O
Formaldehyde (8 %)	10 ml 40 ml	Formaldehyde (37 %) 1x PBS
PBS (10x)	8 g 0.2 g 1.44 g 0.24 g ad 1000 ml	NaCl KCl Na_2HPO_4 KH_2PO_4 dH_2O
PBS	80 ml 720 ml	10x PBS dH_2O
0.1 % SDS/PBS	0.1 ml 99.9 ml	SDS PBS
PBS-T	3 ml 597 ml	Triton X-100 PBS
Blocking solution	1 ml 9 ml	FCS PBT
Crystal violet (10x)	10 g 50 ml 100 ml ad 340 ml	Crystal violet Formaldehyde (37 %) Ethanol (96 %) dH_2O
Crystal violet (1x)	100 ml 100 ml ad 800 ml	Crystal violet (10x) stock solution Formaldehyde (37 %) dH_2O

Table 5. Kits listed with manufacturer or supplier.

Kit	Manufacturer / Supplier
GoTaq DNA polymerase	Promega Corp., Fitchburg, WI, USA M-MLV
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, D¨uren, Germany
QuantiTect SYBR Green PCR kit	Qiagen, Hilden, Germany
QuantiTect Probe PCR Master Mix	Qiagen, Hilden, Germany

KOD DNA polymerase	Merck KGaA, Darmstadt, Germany
QIAamp DNA Mini Kit	Qiagen, Hilden, Germany

Table 6. List of primers sequence, melting temperature and corresponding enzyme.

ID	Sequence (5'-3')	Tm	Enzyme
EILV	fw TCCAGTGACAGACAATCCGC rv GAAGGACGAGCAGACTGTGA	55	GOtaq/ SYBR green
NIEV	fw CATGTGGAGTGGGCGGAATA rv TGGGCCAGCTCTAACAGGAA	60	GOtaq/ SYBR green
NIEV-Q	fw ATGTGCGTCGGGACTTGATT rv CGTTCCCGTCATAGAGAGGC	60	SYBR green
USUV	fw CTGAGAAGGGAGGAAAAG rv GCCACAATGAGTGTTATG	60	GOtaq/ SYBR green
WNV	fw AGTAGTTCGCCTGTGTGAGC rv GCCCTCCTGGTTTCTTAGA	56	GOtaq/ SYBR green
Cx-GAPDH	fw TCAAGCAGAAGGTCAAGGAAG rv GTTGTCGTACCAGGAGATGAG	55	GOtaq/ SYBR green
ASALV	fw CATCTGGACGACCTCGCTAC rv CTGGCCCGTTAATCGCAATG	56	GOtaq/ SYBR green
BUNV	fw ACTCCACACTACAAACTTGC rv ACTGGGTTGTTCCGGTTG	58	KOD
SINV	fw CACACCAAATGACCATGC rv TGTGCTCGGAATACATTC	56	GOtaq

2.2 Biological Materials

2.2.1 Cells

Mosquito cell lines

Mosquito cells *Cx. tarsalis*-derived CT kindly provided by Nijmegen (Radboud University) (J. & G.H., 1976), CPE/LULS50 cell line consists of a mixture of *Cx*. *p*. biotype *pipiens* and biotype *molestus* embryo-derived cells, and the CPL/LULS56 cell line consists only of *Cx*. *p*. biotype *molestus* larvae-derived cells (Bell-Sakyi et al., 2021). The *Ae. albopictus* cell line C6/36, derived from a pool of larvae and presents a deficient siRNA pathway due to a mutation in the Dcr2 gene caused by a homozygous frameshift mutation (Igarashi, 1978; Morazzani et al., 2012a; Singh, 1967). C6/36 cell line was kindly provided by A. Kohl (University of Glasgow Centre for Virus Research [CVR], Glasgow, UK).

All mosquito cell lines were maintained at 28°C in L-15 (Leibovitz) medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% tryptose phosphate broth (TPB;

Gibco Life Technologies), 100 units/ml penicillin, 100 µg/ml streptomycin; ThermoFisher Scientific) and either 10% foetal bovine serum (FBS; ThermoFisher Scientific) for C6/36 cells or 20% FBS for CPE/LULS50 and CPL/LULS56 cells.

Mammalian cell lines

The human hepatoma cell line, HuH-7, derived from a well-differentiated hepatocellular carcinoma (Nakabayashi et al., 1984), and the baby hamster kidney cell line, BHK-21, derived from the parental cell line C-13 from baby hamster kidneys of five unsexed 1-day old hamsters (Macpherson & Stoker, 1962), were maintained at 37ºC with 5% CO_2 in Glasgow Minimum Essential Medium (GMEM; ThermoFisher Scientific) supplemented with 5% FBS, 10% TPB (Gibco Life Technologies), 100 units/ml penicillin, and 100 µg/ml streptomycin.

Additionally, the African green monkey (*Chlorocebus aethiops*) kidney epithelia cell line, Vero (Vero CCL-81; ATCC, Manassas, VA, USA), was maintained at 37ºC with 5% CO_2 in DMEM medium (PAN-Biotech GmbH) supplemented with 10% FBS, 10% TPB, 100 units/ml penicillin, and 100 µg/ml streptomycin.

2.2.2. Virus stocks and titration

Arboviruses stocks and titration

Orthobunyavirus bunyamweraense (BUNV; segment S accession number NC_001927, segment M accession number NC_001926, segment L accession number NC_001925), and Orthobunyavirus bunyamweraense expressing Nano luciferase (BUNV-Nluc) stocks were produced as described elsewhere (Dietrich et al., 2017). Briefly, BUNV or BUNV-Nluc stock was produced in BHK-21 cells, the supernatant was harvested upon the detection of cytopathic effect (CPE), and cell debris was removed by centrifugation. Virus titer was determined via plaque assay on BHK-21 cells with a 1:1 mixture of 1.2 % Avicell in water and 2xMEM with 4% FBS as overlay (Varela et al., 2013).

Usutu virus (USUV 491; accession number KY426758) (Cadar et al., 2017), and West Nile virus lineage 2 (WNV ED-I-33/18-UM; accession number MH924836.1) (Ziegler et al., 2019), stocks were produced in Vero CCL-81 cells. Virus titer was determined via $TCID_{50}$ assay on Vero CCL-81 cells for USUV and HuH-7 cells for WNV2.

Sindbis virus (SINV, TOTO1101 strain, accession number NC_001547) was kindly provided by Dr Andres Merits (University of Tartu, Estonia). The virus was recovered from infectious cDNA clones, as previously described (Rice et al., 1987). Briefly, the plasmid was linearized

with XhoI, purified, and *in vitro* transcribed using the SP6 RNA Polymerase (Ambion, Invitrogen) in the presence of a cap analogue. The resulting RNA was transfected into BHK-21 cells, and upon observation of CPE, the supernatant was collected and used to infect fresh BHK-21 cells in a T75 flask. Upon CPE observation, the supernatant was harvested, clarified by centrifugation, and virus titer was determined by $TCID_{50}$ assay on BHK-21 cells.

Semliki Forest Virus expressing Nano luciferase, plasmid pCMV-SFV6-2SG-Nluc (SFV-Nluc, accession number KT009012.1) (Ferguson et al., 2015; Saul et al., 2015; Scherer et al., 2021), stock was produced in BHK-21 cells, the supernatant was harvested upon the detection of CPE, and cell debris was removed by centrifugation (10 min, 1000 xg, room temperature), and stored at -80°C. Virus titer was determined via plaque assay on BHK-21 cells with a 1:1 mixture of 1.2 % Avicell in water and 2xMEM with 4% FBS as overlay (Varela et al., 2013).

Insect-specific viruses stock and titration

Eilat virus (EILV, accession number JX678730.1) (Nasar et al., 2012), Agua Salud alphavirus (ASALV, strain PA-2013-MP416, accession number MK959114) (Hermanns et al., 2020), Niénokoué virus (NIEV; accession number NC_024299.2) (Junglen et al., 2017), and Herbevirus herberti (HERBV, Herbert virus strain F23/CI/2004, accession number: NC_038714; NC_038713; NC_038712) (Marklewitz et al., 2013) were used in this study. For virus stock production, a flask (T25) 70% confluent with C6/36 cells with cell medium (Leibovitz's L15 medium Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% foetal calf Serum (Thermo Fisher Scientific Inc., Waltham, MA USA), 1x penicillin/streptomycin (Thermo Fisher Scientific Inc.,Waltham, MA, USA), and 10% tryptose phosphate broth (Gibco Life Technologies, Paisley, UK), was inoculated with the respective virus, and incubated at 28°C. Three days after inoculation, the cleared infectious supernatant was collected and stored at -80°C.

ISVs titrations were performed by $TCID_{50}$ assay with C6/36 cells. To this end, 4.10^4 cells/well were seeded in a 96-well plate in Leibovitz's L15 cell medium (supplemented as described above). After a three-day incubation period at 28°C, the cells were first checked for morphological changes, since ISVs can cause morphological changes without causing a clear CPE. At that point, cells were fixed for 30 min at 4% formaldehyde and EILV and NIEV virus titer was assessed via $TCID_{50}$ assay in C6/36 cells by immunofluorescence detection of dsRNA using the monoclonal antibody 3G1 (O'Brien et al., 2015), labelled with goat anti-mouse IgG (H+L) Alexa Fluor 488 (Invitrogen). HERBV virus titer was assessed via $TCID_{50}$ assay in C6/36 cells by immunofluorescence detection of anti-HERBV-N (#284, rabbit polyclonal),

labelled with donkey anti-rabbit IgG (H+L) Alexa Flour 594 (Invitrogen). DNA were labelled with DAPI stain (4′,6-diamidino-2-phenylindole). Finally, for ASALV, cells were fixed in 8% formaldehyde for 30 min and stained with crystal violet. The virus titer was calculated according to the Spearman–Kaeber algorithm (Hierholzer & Killington, 1996).

2.3. Molecular methods

2.3.1. RNA isolation

Sample preparation for RNA extraction was performed depending on biological origin and experimental condition. To extract approximately 10^7 cells for BSL2 experiments (6 well plate, 24 well plate, or over all culture suspension) cells suspension were centrifuged 5 min at 6000 rpm, room temperature (RT) down to a pellet. To extract approximately 10^7 cells for BSL3 experiments (6 well plate, 24 well plate) cells supernatant was discarded, and cells were carefully suspended in PBS followed by 10 min centrifugation at 5000 rpm, and cell pellet supernatant was discarded. For mosquito samples, individuals were homogenised in 500 µLof GMEM (Thermo Fisher Scientific, Waltham, MA, USA) without supplements. The homogenate was clarified by centrifugation during 5 min at 1000 rpm at 4°C. 200 µLof the supernatant was used for RNA extraction.

For RNA extraction, TRIzol Reagent (Invitrogen/Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for cell pellet samples, while TRIzol LS Reagent (Invitrogen, Carlsbad CA, USA) was used for mosquito samples, which were in liquid suspension. Both reagents are monophasic solutions of phenol and guanidinium isothiocyanate designed for the isolation of total RNA, DNA, and proteins; however, they differ slightly in formulation to optimize performance for different sample types. TRIzol Reagent is formulated for solid or dense samples such as cell pellets or tissues and requires a reagent-to-sample ratio of approximately 10:1. In contrast, TRIzol LS Reagent is slightly more concentrated and specifically designed for liquid or suspended samples, allowing efficient extraction with a lower reagent-to-sample ratio of 3:1. This distinction ensures optimal phase separation and nucleic acid yield for each sample type, hence the use of TRIzol for cell pellets and TRIzol LS for mosquito suspensions.

After TRIzol or TRIzol LS addition all samples types were treated the same. Phase separation was induced with chloroform (100 µL / 1 ml TRIzol) by centrifugation for 15 min at 10500 rpm and 4°C. Afterwards, the upper aqueous phase was transferred to a new tube adding 10 mg/ml glycogen as carrier and isopropanol (250 µL / 1ml TRIzol) to the sample to promote precipitation. After 10 min of incubation at room temperature, samples were again centrifuged

for 15 min at 12000 rpm and 4°C. Supernatant was discarded and the remaining pellet was washed twice with 70 % ethanol, centrifuged for 10 min at 12000 rpm and 4 °C after each washing step. Pellet was air-dried and resuspended in 20 µL RNase-free water. The concentration and quality of RNA was measured and calculated by photospectrometry by means of 'NanoDrop' (Thermo).

2.3.2. Nucleic synthesis

cDNA Synthesis

Synthesis of cDNA from total RNA was performed using M-MLV reverse transcriptase (Promega, Fitchburg, WI, USA) according to the manufacturer's specifications. In short, 1 µg of RNA sample was mixed with 1 µL random hexamer solution and water to 15 µL. The solution was incubated at 70°C for 5 min and subsequently cooled to 4°C for 1 min and transferred to 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 were added. The solution was incubated for 1 h at 37°C and subsequently heat inactivated for 10 min at 70°C.

Polymerase chain reaction (PCR)

PCR is used for the amplification of specific DNA sequences. GoTaq DNA polymerase (Promega, Madison, WI, USA) or KOD Hot Start DNA polymerase (Promega, Madison, WI, USA) with proof-reading ability were used for preparative PCRs for infection control according to the manufacturer's protocol. Oligonucleotide primers and melting temperatures are listed in Table 6. Reagents for a single sample and protocol are listed below (GoTaq: Table 7, Table 9; KOD: Table 8, Table 10).

Table 7. GoTaq polymerase RT-PCR reaction mix.

Amount	Reagent
10 µL	5x GoTaq buffer
0.25 µL	Primer forward (100 pmol/µL)
0.25 µL	Primer reverse (100 pmol/µL)
1 µL	dNTPs (10 mM)
0.5 µL	GoTaq polymerase
xx µL	DNA (< 500 ng)
ad 50 µL	ddH_2O

Table 8. KOD polymerase RT-PCR reaction mix.

Amount	Reagent
5 µL	10x KOD buffer
1.5 µL	Primer forward (10 pmol/µL)
1.5 µL	Primer reverse (10 pmol/µL)
5 µL	dNTPs (2 mM)
1 µL	KOD polymerase
3 µL	$MgCl_2$
xx µL	DNA (1 pg - 10 ng)
ad 50 µL	ddH_2O

Table 9. GoTaq polymerase protocol.

Temperature	Time	Repeat
95 °C	2 min	1x
95 °C	30 sec	30 - 35x
xx °C	30 sec	30 - 35x
72 °C	(60 sec for 1 kb)	30 - 35x
72 °C	7 min	1x
4ºC	∞	

Table 10. KOD polymerase protocol.

Temperature	Time	Repeat
95 °C	2 min	1x
95 °C	20 sec	20 - 40x
xx °C	10 sec	20 - 40x
70 °C	(15 sec for 1 kb)	20 - 40x
70 °C	7 min	1x
4ºC	∞	

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 analysed using the GelDoc Imaging System (BioRad).

Quantitative Real-Time PCR (qPCR)

qPCR was used to determine expression levels of several transcripts and viral RNA levels. Previously reverse transcribed cDNA was used (QuantiTect SYBR Green PCR kit, Qiagen, Germany), and qPCR was performed using specific primers (Table 1) and a LightCycler 480 (Roche, Basel, Switzerland) according to manufacturer's instructions. All PCR reactions were performed in three technical replicates. The data were analysed using a LightCycler 480 II Instrument (Roche) and the LightCycler 480 software (version 1.5.0 SP4), either using (i) absolute quantification for virus copy numbers quantification of single infections, or (ii) advanced relative quantification (delta-delta CT method) for virus copy numbers originated from two different experimental condition using a housekeeping gene (Cx-GAPDH) for normalization of data resulting from ASALV-WNV co-infection in mosquitoes (Livak & Schmittgen, 2001). Standard curves for each gene were created using dilutions of the specific PCR products, and genome copy numbers were calculated and used for both absolute and relative quantification assays.

Table 11. SYBR Green qPCR reaction mix.

Amount	Reagent
5 µl	2x QuantiTect Master Mix
0.3 µl	Primer forward (10 µM)
0.3 µl	Primer reverse (10 µM)
3.4 µl	ddH_2O

Small RNA sequencing and analysis

For small RNA sequencing, a total of $6.5x10^5$ of CPE/LULS50 or CPL/LULS56 cells per well were seeded in a 6-well plate in duplicate (replicate A and B), infected with SINV, BUNV, and WNV (MOI 10) and incubated at 28 °C for 48 h for SINV and BUNV, and 96 h for WNV. Total RNA was isolated using TRIzol (Thermo Fisher Scientific Inc., Waltham, MA, USA) with glycogen as a carrier, according to the manufacturer's instructions.

Library preparation and Illumina-based small RNA sequencing were performed at BMKGene (Münster, Germany), as previously described (Scherer, 2021). In short, small RNA sequencing was performed on an Illumina NovaSeq SP (Illumina, San Diego, CA, USA), with single-end 50 bp reads (SE50), generating final libraries comprising between 12 and 17 million clean reads per sample.

Resulting sequence reads were aligned to the respective virus reference genome (SINV GenBank accession number: NC_001547, BUNV GenBank accession number: NC_001927.1; NC_001926.1; NC_001925.1, WNV GenBank accession number: MH924836.1) as previously described (Varjak, Donald, et al., 2017). For virus discovery, small RNA reads were aligned to a curated database of all viral genomes, including reference flavivirus sequences from the NCBI Taxonomy Browser, the insect-associated viruses compiled from previously published research (Agboli et al., 2019; B. Bolling et al., 2015; Moonen et al., 2023; Patterson et al., 2020). Following established pipelines (Franzke et al., 2018).

Luciferase assays

Relative luciferase activity was determined by Nano-Glo Luciferase Assay System (Promega Corp., Fitchburg, WI, USA) in a GloMax Navigator multi-luminometer following cell lysis in Passive Lysis buffer (Promega Corp., Fitchburg, WI, USA) according to manufacturer's protocols. 10 µL of lysed cell solution was used for the luciferase measurement.

2.4. Experimental Methods

2.4.1. Establishment of mosquito cell lines with persistent infection of ISVs.

To achieve a persistent infection with EILV or NIEV on CT cell line, and NIEV or ASALV on CPELUL50 cell line, 10^6 cells were seeded in 2 mL L-15 medium into a single well of a 6-well plate. After a 24h incubation period, the cell SN was replaced with 200 µL medium containing the desired ISV at the MOI 0.1 and incubated for 1h, followed by SN replacement with 2 mL of fresh complete L-15 medium. Cells were kept in a 6-well plate until confluency and then transferred to a 25T flask. They were kept and passaged at least 7 times, after which the cells were checked for viral RNA. To this end, total RNA was extracted, reverse-transcribed and used as template for endpoint PCR with the respective virus-specific primer pairs to confirm ISV presence, and GAPDH primers were used as housekeeping gene. 1 kb DNA Ladder used as a marker. If positive ISV persistent infection were considered established and three cell cultures (A, B and C) were kept running in parallel and used as replicates for further experiments.

Additionally, ISV persistent infection in cells were further confirmed after the experiment conclusion to ensure ISV presence throughout the experiments.

2.4.2. CT cell line ISV co-infections with Arbovirus

$2x10^5$ of CT cells persistently infected with EILV or NIEV were seeded along with naive CT cells in 1 mL L-15 medium on 24 well plates for co-infection assays with WNV or USUV. For BUNV and SFV, $6x10^4$ of CT cells persistently infected with EILV or NIEV were seeded along with naive CT cells in 200 µL L-15 medium on 96 well plates. From this point all non-infected CT cells were inoculated with either MOI 0.1 of the arbovirus alone, or both arbovirus MOI 0.1 and NIEV or EILV, respectively, with a MOI 1. EILV or NIEV persistently infected CT cells were likewise inoculated with arbovirus MOI 0.1.

For the co-infection experiment for EILV, cell SN was sampled at 0-, 1-, 2-, 3-, and 4- days post-infection (dpi) for WNV or USUV. For the co-infection experiment for NIEV, cell SN was sampled at 0-, 1-, 2-, 3-, and 4-dpi for WNV, and 0-, 1-, 2-, 3-, and 6-dpi for USUV. For BUNV-Nluc and SFV-Nluc cells were harvested at 0-, 1-, 2-, 3-, 7-, 9-dpi, and 6-, 18-, 24-, 48-, 72-hpi, respectively for both EILV or NIEV co-infection experiments.

WNV and USUV titer were estimated via $TCID_{50}$ on HuH7 cells. For BUNV-Nluc and SFV-Nluc luciferase activity was measured. Experiments were performed in technical duplicates and biological triplicates.

2.4.3. Virus growth kinetics on CPE/LULS50 or CPL/LULS56

For virus growth kinetics on CPE/LULS50 or CPL/LULS56 cells, a total of 6.5×10^5 of cells were seeded per well in a 6-well plate and incubated overnight at 28ºC. Approximately 24 h post seeding, the cells were infected with either BUNV, SINV, WNV or USUV at a MOI 10, in duplicate. For the ISVs (EILV, NIEV, and Herbert virus; HERBV), a MOI of 1 was used. The supernatant was sampled at 0-, 1-, 2-, and 3-dpi for all viruses. Additional samples were collected on day 6 post infection for all arboviruses, and day 8 post infection for USUV and WNV. For all samples, a $TCID_{50}$ was performed to assess virus titer in three independent biological replicates.

2.4.4. Mosquitoes species and rearing

2.4.4.1. Mosquito strains and field collected mosquito samples

Culex torrentium mosquitoes were obtained from egg raft collections carried out in Hamburg area/Germany [Langenlehsten 53°30′N 10°44′E]. Specimens for infection experiments were collected from May to September in 2023 and 2024. The egg collection was carried out using gravid traps filled with hay infusion placed in proximity to natural breeding sites of *Culex* mosquitoes, i.e. water bodies to attract gravid females and stimulate egg deposition. Traps were checked twice a day for freshly deposited egg rafts, which were retrieved from the water surface using a wooden spatula and placed in individual plastic cups for transportation to the laboratory (Leggewie et al., 2016). *Cx. p.* biotype *pipiens* lab colony started in 2020 (Wageningen University, Netherlands), and stablished in 2024 at Bernhard Nocht Institute for Tropical Medicine.

2.4.4.2. Rearing of larvae and adult mosquitoes

Field-collected egg rafts were flooded separately in dechlorinated water, and hatched larvae were fed on TetraMin flaked fish food (Tetra GmbH, Melle, Germany). For *Culex* taxonomy differentiation, DNA was extracted from 3-4 larvae from each egg raft (DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany), followed by molecular identification through multiplex quantitative real-time PCR (qRT-PCR). Once identified, larvae were pooled according to species or biotype and emerging females (4–14 days of age) were distributed into plastic vials at 10 females each.

Adult *Cx. torremtium* mosquitoes and adult *Cx. p*. biotype *pipiens* lab colony mosquitoes were kept at 25°C with a relative humidity of 80% and a 16 h:8 h light: dark photoperiod and fed on fructose pads (8% D(−)-Fructose, Carl Roth GmbH, Karlsruhe, Germany; 0.02% 4-Aminobenzoic acid, Sigma Aldrich, Seelze, Germany) for maintenance and starved 48h prior to infection. To facilitate egg production, a blood meal consisting of human erythrocyte concentrate (Blood group 0, Blood bank, University Hospital Hamburg)/50% FCS (PAA/GE Healthcare Life Sciences, Germany)/0.5% fructose (Carl Roth GmbH, Karlsruhe, Germany) was provided weekly.

2.4.5. Mosquitoes susceptibility to ISV and WNV

2.4.5.1. Oral administration of ISV and WNV

For oral administration of ISV at $1x10^7$ $TCID_{50}$/mL, *Cx. torrentium* mosquitoes were starved of fructose for 48h prior to infection. For the respective ISV: EILV, or ASALV, an ISV-PBS solution ($1x10^7$ $TCID_{50}$/mL, PBS, and blue food dye) embedded cotton sticks were inserted into the vessel containing 10 female mosquitoes (4-14 days of age). After one to two hours at room temperature, individuals were anesthetized with CO_2 and engorged mosquitoes were kept, while non-fed ones were sacrificed.

As for WNV oral administration, this was performed via an artificial blood meal. Two embedded cotton sticks, and a 50µLdrop containing WNV at $1x10^7$ $TCID_{50}$/mL blood solution (human erythrocyte concentrate as described above in the section "Rearing of larvae and adult mosquitoes"), were inserted into the vessel containing 10 female mosquitoes (4-14 days of age). After one to two hours at room temperature, individuals were anaesthetized with CO_2 and engorged mosquitoes were kept, while non-fed ones were sacrificed.

After infection, mosquitoes were provided with fructose feeding solution on cotton pads *ad libitum*, and incubated in climate chambers with either 25°C (humidity of 80% and a 12h:12h light:dark photoperiod) for the ISV, or 27°C +/-5°C (humidity of 80% and a 12h:12h light:dark photoperiod) for WNV.

Inoculated mosquitoes were either harvested at 0, 3, 7, or 14 dpi for the ISV, or 0, 7, 14, 21 dpi and used in further experiments (salivation assays) for WNV.

2.4.5.2. Intrathoracic injection of ISV

Intrathoracic injection, for direct ISV inoculation of female mosquitoes (4-14 days of age), was performed with individuals anesthetize under concurrent CO_2. Mosquitoes were then injected with a dilution of 10^3 PFU in PBS of the respective virus (EILV, ASALV or NIEV) into the dorsolateral region of the thorax. Injections were performed using the Drummond Nanoject II (Drummond Scientific Company, USA) with self-crafted glass capillaries. After infection, mosquitoes were provided with fructose feeding solution on cotton pads *ad libitum*, and kept in climate chambers at 25°C (humidity of 80% and a 12h:12h light:dark photoperiod). Inoculated mosquitoes were harvested at 0, 3, 7, or 14 dpi.

2.4.6. *In vivo* ASALV-WNV co-infection experiments

Female mosquitoes (4–14 days of age) were first intrathoracically injected with the ISV ASALV (see section 2.4.5.2. “Intrathoracic injection of ISV”). After 3 dpi, the ISV mosquitoes were exposed to a WNV artificial blood meal (see section 2.4.5.1. “Oral administration of ISV and WNV”; page 31). After infection, mosquitoes were provided with fructose feeding solution on cotton pads ad libitum. Incubation was performed in climate chambers with 27°C +/-5°C (humidity of 80% and a 12h:12h light:dark photoperiod). Inoculated mosquitoes were harvested at 7, 14, 21 dpi, and submitted for salivation assays at 21 dpi.

2.4.7. Mosquitoes processing

2.4.7.1. Salivation Assay

A salivation assay was performed using ASALV-injected and WNV-exposed female mosquitoes 21 dpi. Mosquitoes were anaesthetised with CO_2, legs and wings were removed, and individuals were forced to salivate in 10 µL of PBS in a cut filter tip, as previously described (Heitmann, Jansen, Lühken, Leggewie, et al., 2018). After 30 minutes of forced salivation, the tip containing the PBS-saliva mixture was removed and placed into a reaction tube containing 10 µL of PBS. Finally, 20 µL saliva + PBS was added to HuH7 cells seeded in 96-well plates. Cell morphological changes or CPE were registered. All bodies were homogenized and total RNA was extracted from individual mosquitoes and WNV copies numbers were determined by virus-specific real-time PCR.

2.4.7.2. Homogenate Preparation

To obtain samples from whole mosquitoes or mosquito’s bodies, individuals were anesthetized with CO_2, transferred to individual vessels and frozen at -80°C at least overnight. Afterwards homogenization per mosquito was performed in 500 µL GMEM (Thermo Fisher Scientific, USA) without supplements, by means of an electric pestle, RNA was isolated with Trizol LS (Invitrogen, Carlsbad CA, USA). Total RNA was extracted from individual homogenized mosquitoes and virus copy numbers were determined by virus-specific real-time PCR.

3. Results

3.1 ISV interference in *Culex tarsalis*- derived CT cells

3.1.1 Establishment of EILV and NIEV Persistently Infected Cell Lines

As previously shown (Agboli et al., 2023), ISVs can infect and interfere with the replication of arboviruses in *Cx. tarsalis*-derived CT cells. In order to study the effect of both acute and persistent EILV or NIEV co-infections on arboviruses, the capability of these viruses to establish a persistent infection was investigated. Furthermore, as both viruses belong to different families/genera, EILV to the alphaviruses, and NIEV to the flaviviruses, it would allow a direct comparison of the interactions of ISV that are either genetically closer or divergent from flavi-arboviruses.

To this purpose, CT cells were inoculated with either EILV or NIEV and passaged at least 7 times. No CPE was observed through this period, and at passage 7 total RNA was extracted, reverse transcribed and analysed by end-point PCR using EILV or NIEV specific primers.

Both EILV and NIEV exhibited a positive PCR amplification product (Figure 4), indicating that both EILV and NIEV infect CT cells in a persistent manner.

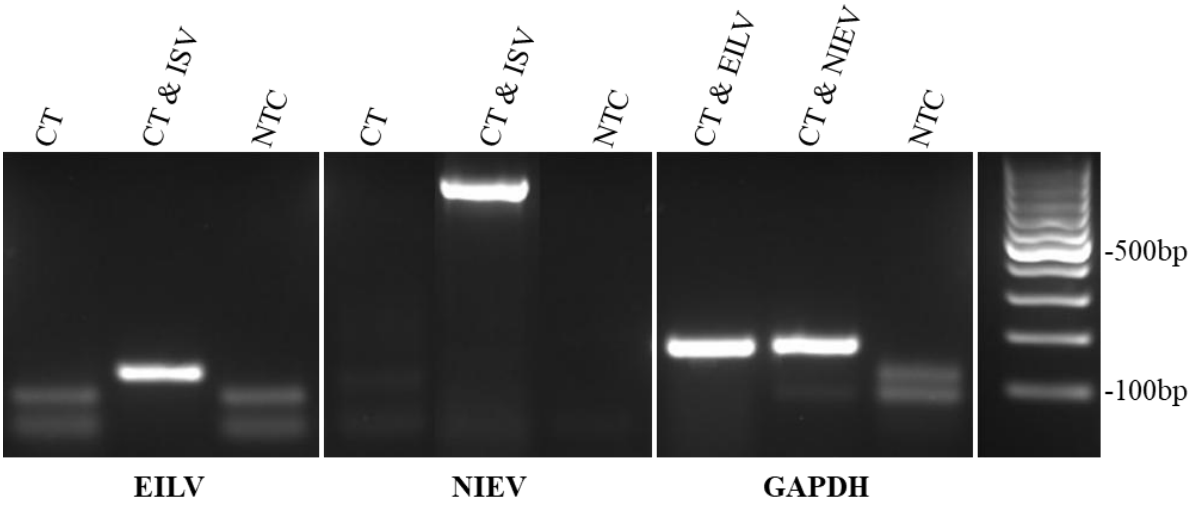

Figure 4. Persistently infected CT cells with either EILV or NIEV. CT cells were inoculated with either EILV or NIEV with a MOI 0.1 and passaged 7 times. Total RNA was extracted, reverse-transcribed and used as template for endpoint PCR with the respective virus specific primer pairs to confirm ISV presence. CT: non-infected; NTC: non-template control; CT & ISV: EILV or NIEV infected cells after the 7th passage. GAPDH was used as a housekeeping gene. 1 kb DNA Ladder used as a marker. Same gel results and rearranged for visual purposes.

3.1.2. *In vitro* co-infection experiments of ISV and arbovirus

In order to elucidate the interactions of NIEV and EILV in both acute and persistent co-infections, persistently infected cell lines were used along with naive CT cells for co-infection assays with WNV or USUV. All non-infected CT cells were inoculated with either MOI 0.1 of the arbovirus alone, or both arbovirus MOI 0.1 and NIEV or EILV with a MOI 1. EILV or NIEV persistently infected CT cells were likewise inoculated with arbovirus MOI 0.1. At the indicated time points, cell culture supernatant was collected, and arbovirus titration was determined via $TCID_{50}$ on HuH7 cells.

In addition to WNV and USUV, the same experiment was performed for BUNV-Nluc and SFV-Nluc, where cells were harvested and luciferase activity was measured on the respective time points. Both BUNV-Nluc (*Peribunyaviridae*) and SFV-Nluc (*Togaviridae*) were selected as representatives of a virus family other than *Flaviviridae*, to evaluate if the results can be generalised across virus families.

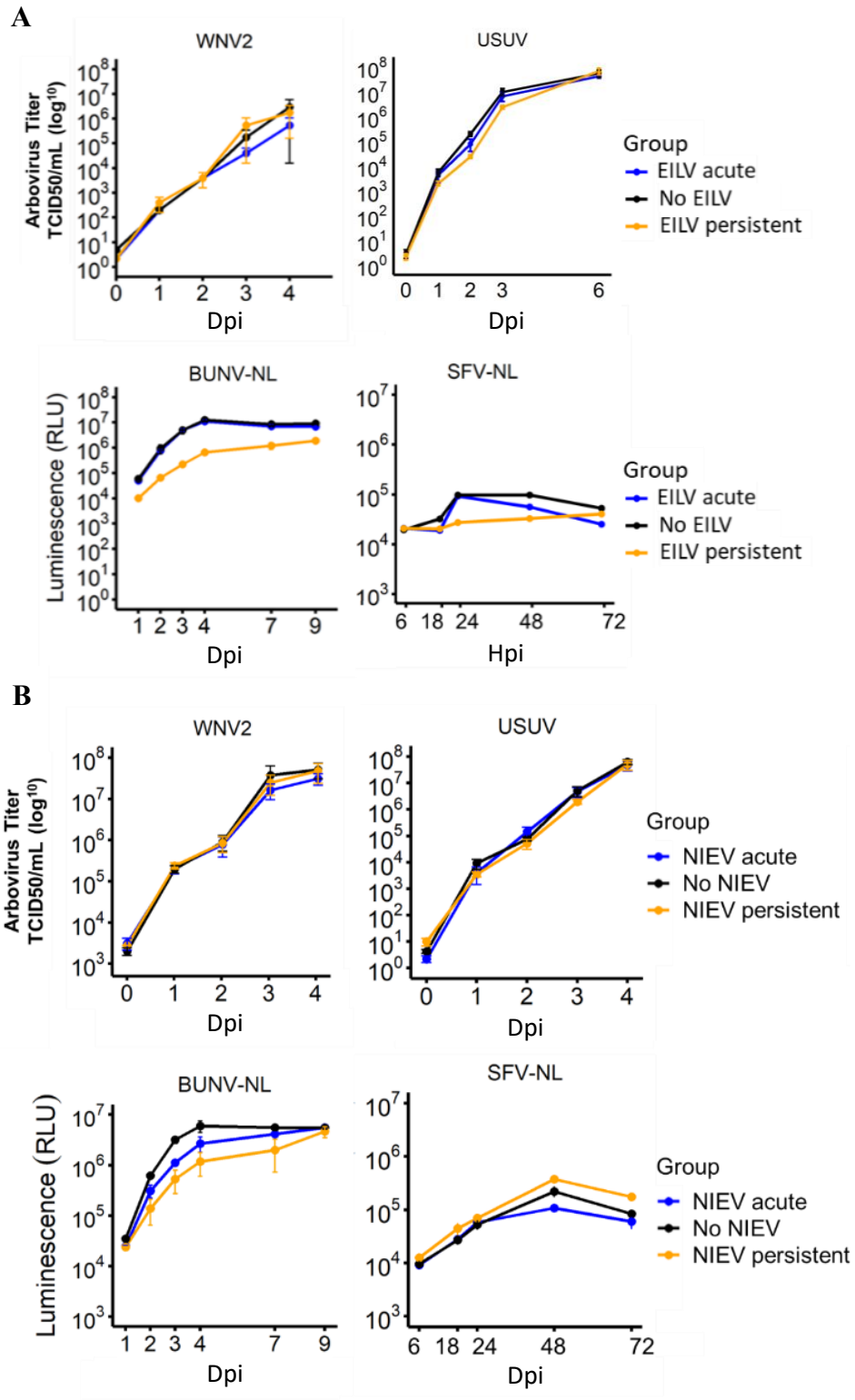

Figure 5. Growth kinetics of co-infections of insect specific viruses acute or persistent infection with different arbovirus. CT cells with a persistent (yellow), acute (blue) or no infection (black) of ISV (MOI 1): EILV (A) or NIEV (B) were infected with WNV, USUV, BUNV-Nluc, or SFV-Nluc with a MOI 0.1. Cell culture supernatants were collected at indicated time points, and titer determined via $TCID_{50}$/mL for WNV and USUV. As of BUNV-Nluc and SFV-Nluc, cells were collected at indicated time points, and Luminescence (RLU) was measured. The data points and error bars represent the means of three independent biological

experiments and the standard error of the mean. The data was log-transformed and the mean and standard error of the mean are represented.

For both EILV (Figure 5A) and NIEV (Figure 5B) co-infections, WNV or USUV titer in acute and persistent co-infected cells were similar to the control, with no interference observed between the experimental conditions tested.

As of BUNV-Nluc interference experiment, an effect on BUNV-Nluc replication was observed on EILV and NIEV persistent infected cells, but only at 3 dpi. For SFV-Nluc, a reduction was observed at 24hpi only for EILV persistent infected cells. However, the opposite was observed for NIEV persistent infection, where SFV-Nluc luminescence resulted in higher RLU when compared with the control group and the NIEV acute infected group.

Both EILV and NIEV were able to persistently infect CT cells without inducing cytopathic effects. Subsequent co-infection experiments revealed no significant interference with WNV or USUV replication, whereas virus-specific effects were observed for BUNV-Nluc and SFV-Nluc depending on the ISV infection. However, CT is a *Cx. tarsalis* derived cell line, which doesn't represent a viable vector of arbovirus transmission in the European region.

3.2. Characterization of *Cx. pipiens ss*-derived cell lines

Cx. pipiens sl mosquitoes are major vectors of WNV and USUV, two flaviviruses of increasing concern in Europe and parts of the Northern Hemisphere (Agboli et al., 2023; Bakonyi & Haussig, 2020; Jansen et al., 2023; Martinet et al., 2019; Walker et al., 2014). Up until recently only a few of *Culex* spp. cell lines existed, such as those derived from *Cx. tarsalis* (CT) and *Cx. quinquefasciatus* (HSU); however, these do not represent the mosquito species present in Europe (Dietrich et al., 2017; Jansen et al., 2023; Shocket et al., 2020). Recently, *Cx. pipiens ss* cell lines have been established: CPE/LULS50 (from *Cx. p.* biotype *pipiens* and biotype *molestus*), CPL/LULS56 (biotype *molestus*) (Bell-Sakyi et al., 2021), and CpE3 (*Cx. p.* biotype *pipiens*) (Fallon et al., 2023). However, these lines have not yet been fully characterized regarding their virus susceptibility, immune responses like RNAi competency and presence of persistent ISVs.

3.2.1 Characterization of viral permissiveness in *Cx. pipiens ss*-derived cell lines

The potential of CPE/LULS50 and CPL/LULS56 cell lines as *in vitro* systems for virus-vector interactions studies was evaluated by first looking at their virus susceptibility (via virus replication kinetics) of clinically relevant arboviruses and representative ISVs, including the

flaviviruses (WNV, USUV, NIEV), alphaviruses (SINV, EILV, ASALV), and peribunyaviruses (BUNV, HERBV).

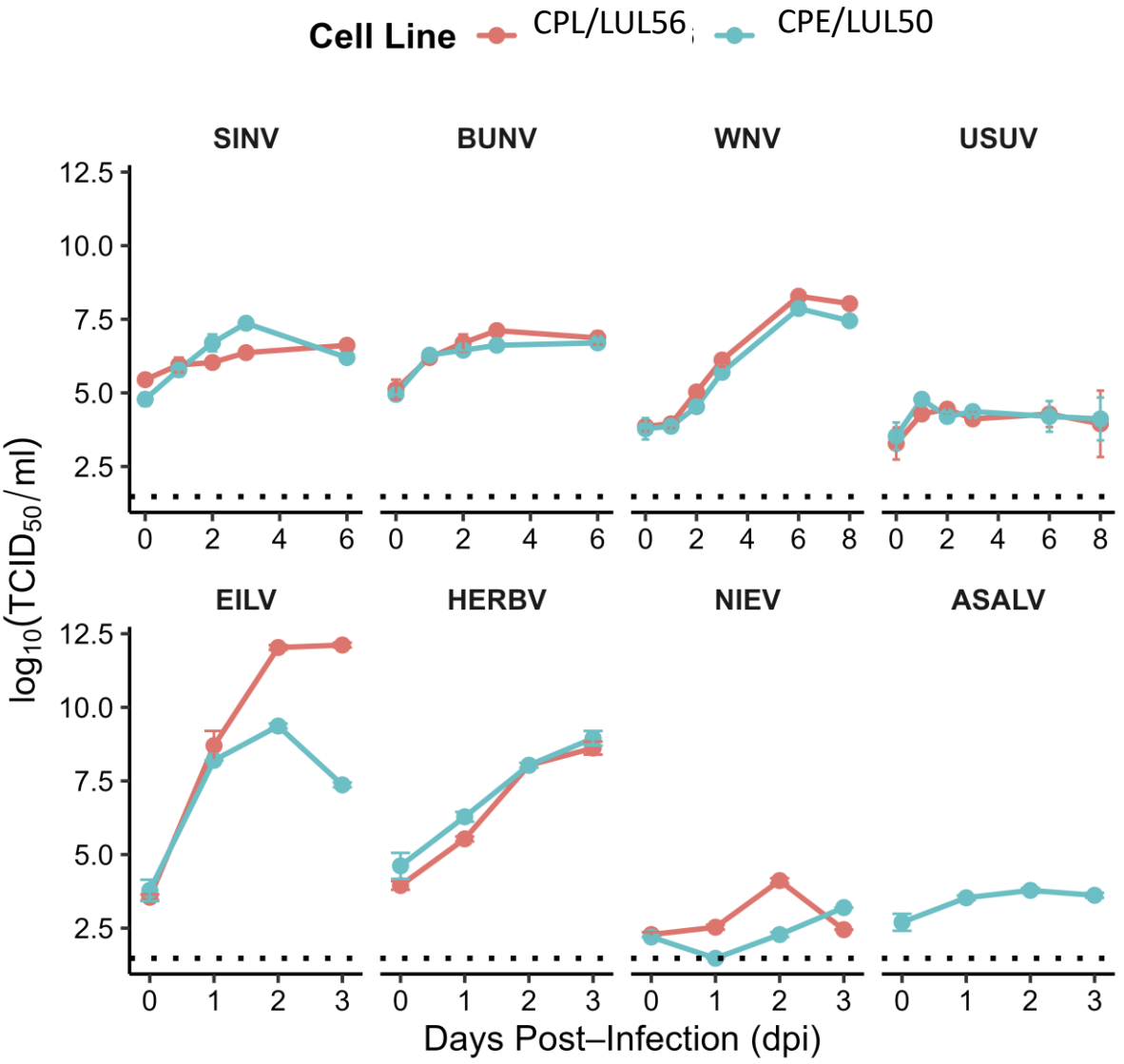

Figure 6. Virus replication kinetics in *Cx. pipiens ss* cell lines CPE/LULS50 and CPL/LULS56. Cells were inoculated with seven different viruses: two alphaviruses (SINV, EILV) and ASALV only for CPE/LULS50, three flaviviruses (WNV, USUV, NIEV) or two peribunyaviruses (BUNV, HERBV), using an MOI 10 for the arboviruses (WNV, USUV, SINV and BUNV) and an MOI 1 for the ISVs (NIEV, EILV, HERBV, and ASALV). The data points and error bars represent the means of three independent biological experiments and the standard error of the mean, respectively. The data was log-transformed and the mean and SEM are represented. The dotted line represents the limit of detection. Figure adapted from (Gothe et al., 2026).

Within the peribunyavirus group, both the arthropod-borne BUNV and the insect-specific HERBV exhibited similar replication kinetics across the two cell lines, with no notable differences observed (Figure 6). In the flavivirus group, replication was detected for WNV and

USUV, showing similar kinetics in both cell lines. In contrast, NIEV, an insect-specific flavivirus, displayed a marked delay in replication in CPE/LULS50 cells compared with CPL/LULS56 (Figure 6).

Among the tested alphaviruses, both the arthropod-borne SINV and the insect-specific EILV replicated robustly, although differences between the cell lines were observed. In CPE/LULS50 cells, post-peak titers of both SINV and EILV declined, whereas CPL/LULS56 cells maintained a plateau in viral titers (Figure 6).

As EILV titers declined relatively rapidly in CPE/LULS50 cells, its suitability for establishing persistent infections for subsequent co-infection experiments appeared limited. Therefore, ASALV was evaluated as a potential alternative candidate for *in vitro* co-infection experiments in this cell line. However, ASALV replication was poorly sustained, showing only a minor increase in viral titers over the sampled time points, and no evidence of persistent infection was detected (data not shown). Consequently, ASALV was not considered suitable for further experiments in this cell line.

Overall, viruses from the *Flaviviridae*, *Togaviridae*, and *Peribunyaviridae* families successfully replicated in both cell lines (Figure 6). Although replication kinetics varied between viruses and cell lines, both CPE/LULS50 and CPL/LULS56 supported infection by a broad range of arboviruses and insect-specific viruses.

3.2.2. Characterization of the *Cx. pipiens ss*-derived cell lines RNAi response during acute infection

To assess RNAi activity in *Cx. pipiens ss*-derived CPE/LULS50 and CPL/LULS56 cell lines, small RNA (sRNA) profiles were analysed following infection with representative arboviruses from three major arboviral families: WNV (*Flaviviridae*), SINV (*Togaviridae*), and BUNV (*Peribunyaviridae*). Cells were infected, total RNA was isolated at 48 hpi (BUNV, SINV) or 96 hpi (WNV), and sRNA libraries were generated and sequenced. Virus-derived small RNAs were analysed to determine the presence and characteristics of siRNA- and piRNA-sized populations.

3.2.2.1. General analysis of sRNA sequencing reads

Small RNA sequencing was performed from CPE/LULS50 and CPL/LULS56 cells in two biological replicates (replicate A and B) infected with WNV, SINV or BUNV. Small RNA sequencing libraries were mapped to WNV, SINV and BUNV (L= large, M= medium, and S=

small segment) genomes (for accession numbers see section “2.2.2. Virus Stocks and Titration”; page:22).

Virus-derived small RNAs were detected in both CPE/LULS50 and CPL/LULS56 cells following infection with WNV, SINV, and BUNV in both technical replicates (Table 12; Figure 7) (Gothe et al., 2026), indicating that both cell lines mount an RNAi response upon arbovirus infection.

Table 12. sRNA sequencing reads.

Replicate	Cell line	Virus	Clean reads	Total virus reads	% Viral clean reads	% Viral 21-nt	% Viral 24–29-nt
A	CPE/LULS50	WNV	14557753	985	0.01	42.34	6.40
	CPL/LULS56	WNV	12706582	2234	0.02	51.88	3.45
	CPE/LULS50	SINV	16284954	316272	1.94	60.85	2.98
	CPL/LULS56	SINV	15168432	177180	1.17	60.70	2.96
	CPE/LULS50	BUNV (seg. L)	17106309	607417	0.76	27.83	19.26
		BUNV (seg. M)		117184	2.62	16.42	26.65
		BUNV (seg. S)		447131	3.55	23.39	19.36
	CPL/LULS56	BUNV (seg. L)	14558210	518494	0.80	25.87	20.18
		BUNV (seg. M)		985	3.07	16.32	25.65
		BUNV (seg. S)		2234	3.56	21.50	20.40
B	CPE/LULS50	WNV	28867020	794	$2.75\text{^}10^{-3}$	18.77	4.41
	CPL/LULS56	WNV	16288959	949	0.01	25.08	5.27
	CPE/LULS50	SINV	16498489	3353	0.02	53.56	3.43
	CPL/LULS56	SINV	13052134	12382	0.09	55.95	2.92
	CPE/LULS50	BUNV (seg. L)	15916137	124485	0.782	20.47	23.13
		BUNV (seg. M)		310881	1.953	14.65	24.32
		BUNV (seg. S)		432849	2.720	20.28	18.00
	CPL/LULS56	BUNV (seg. L)	15750990	79255	0.503	26.84	14.32
		BUNV (seg. M)		209130	1.328	19.29	17.85
		BUNV (seg. S)		370297	2.351	30.39	13.11

Across both cell lines, BUNV generated the highest proportion of virus-specific reads, followed by SINV, while WNV consistently produced the lowest levels of viral sRNAs (Table 12). For

BUNV, virus-derived reads mapped to all three genome segments, with the M and S segments contributing the majority of viral reads in both cell lines.

Analysis of sRNA length distributions revealed that 21-nt virus-derived siRNAs constituted the dominant population for all viruses tested (Figure 7), consistent with Dicer-2-mediated processing of the siRNA pathway. The proportion of 21-nt vsiRNAs was highest for BUNV, intermediate for SINV, and lowest for WNV in both cell lines (Table 12). For BUNV infections, 21-nt vsiRNAs resulted predominantly from the L and S segments, with lower representation from the M segment.

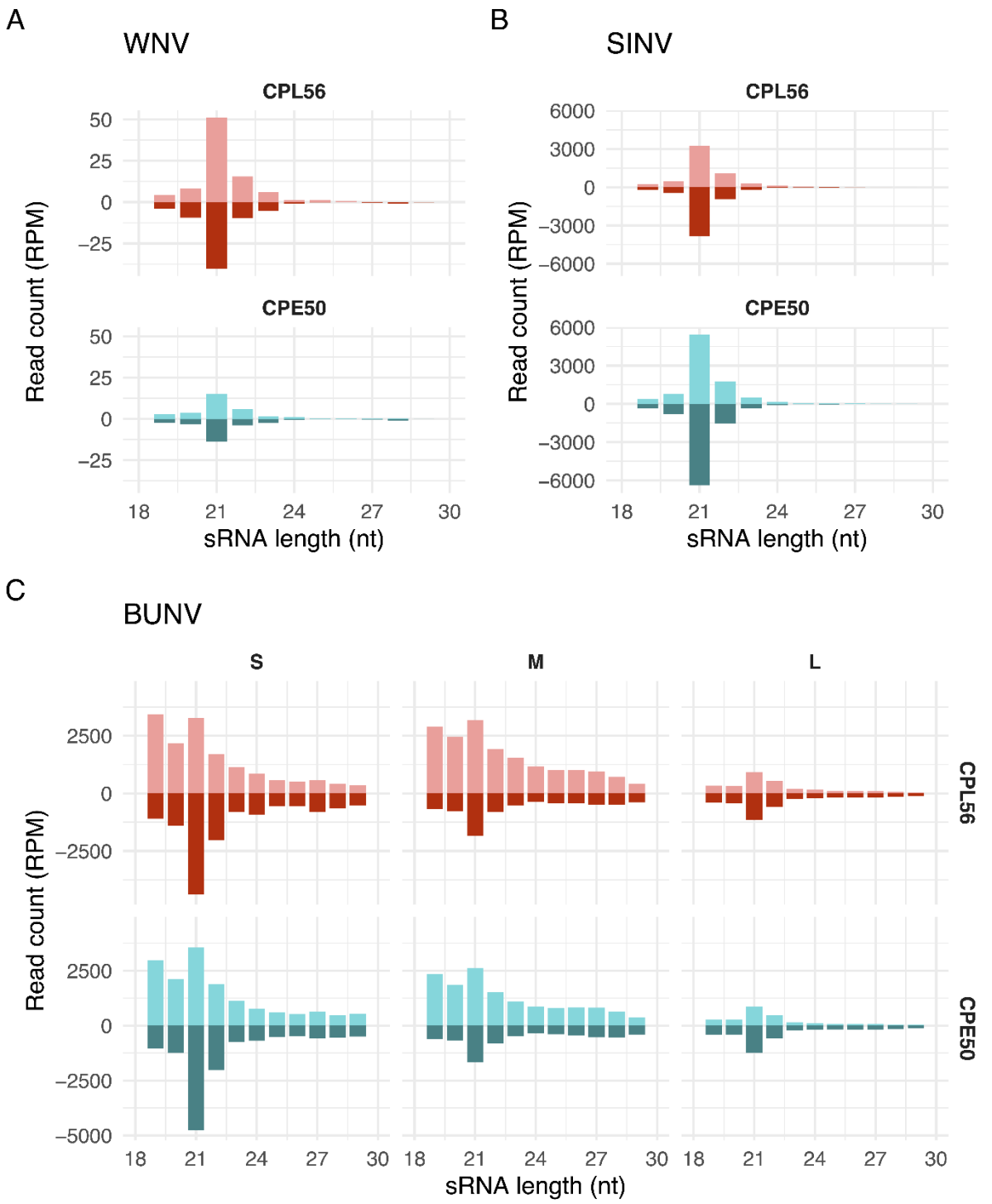

Figure 7. Replicate A small RNA length distribution in virus-infected CPE/LULS50 (CPE50) and CPL/LULS56 (CPL56) cells. (A) Positive strand WNV- and (B) positive strand SINV-specific small RNAs: positive numbers indicate mapping to the virus genome, and negative numbers indicate mapping to the antigenome. (C) Segmented negative strand BUNV-specific small RNAs: positive numbers indicate mapping to the BUNV antigenome, and negative numbers indicate mapping to the BUNV genome. RPM: Reads per million total clean reads. Two independent biological experiments were carried out, replicate A is shown in the current figure. For replicate B see (Gothe et al., 2026).

The mapping of 21-nt long vsiRNA to the genome of BUNV (Figure 8A), WNV (Figure 8B) and SINV (Figure 8C) revelled hot and cold spot patterns, with both genome and antigenome coverage. The antigenome from the M and S segment of BUNV presented higher concentration compared to the genome. As off WNV, a prominent peak of antisense vsiRNAs was detected in the 3′ untranslated region of the antigenome, coinciding with the known subgenomic flavivirus RNA (sfRNA) locus (Figure 8B) (Göertz et al., 2016).

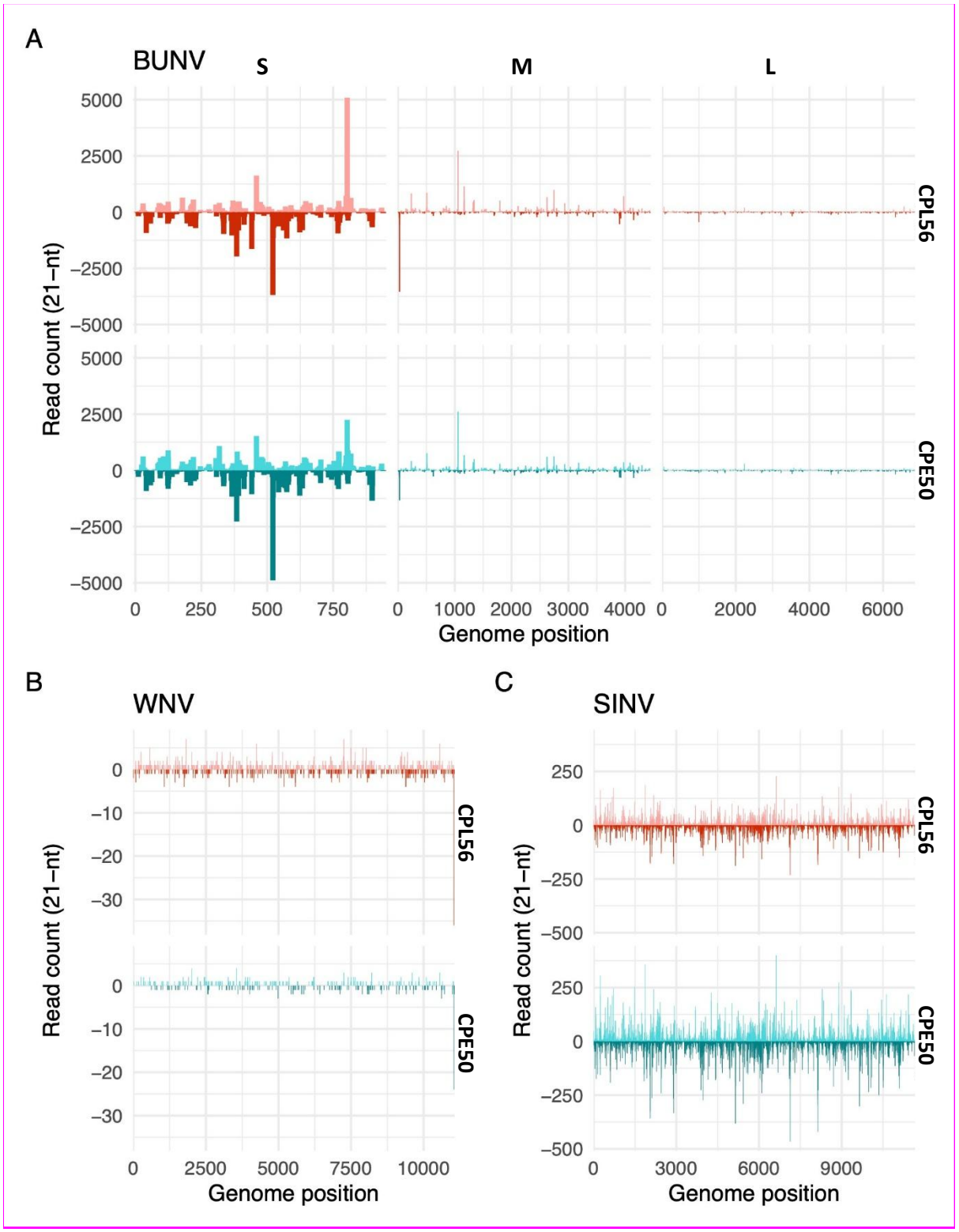

Figure 8. Distribution of viral specific 21 nt siRNAs across the viral genome and antigenome in infected *Cx. pipiens ss* CPE/LULS50 and CPL/LULS56 cells. BUNV (A), WNV (B) and SINV (C)-specific siRNAs mapped along the genome and antigenome. For the positive strand WNV and SINV, positive numbers indicate mapping to the virus genome, and negative numbers indicate mapping to the antigenome. For the negative strand BUNV -specific siRNA mapping to the three segments (L, M and S), positive numbers indicate mapping to the virus antigenome and negative numbers indicate mapping to the BUNV genome. Two

independent biological experiments were carried out for each experimental condition; plots from replicate 1 (shown in the main manuscript) is presented here.

In addition to 21-nt long vsiRNAs, BUNV infection resulted in substantial production of 24-29-nt piRNA-sized small RNAs, whereas SINV and WNV generated only minor proportions (Table 12; Figure 7). The mapping of 28-nt long sRNAs revelled the strongest genome coverage for BUNV (Figure 9A). For both cell lines a hot and cold spot patterns from the genome and antigenome coverage of BUNV could be observed, while for SINV this was very low, and inexistent for WNV. Additionally, for BUNV, these longer small RNAs mapped tendentially more to the M and S segments in both cell lines (Figure 8A).

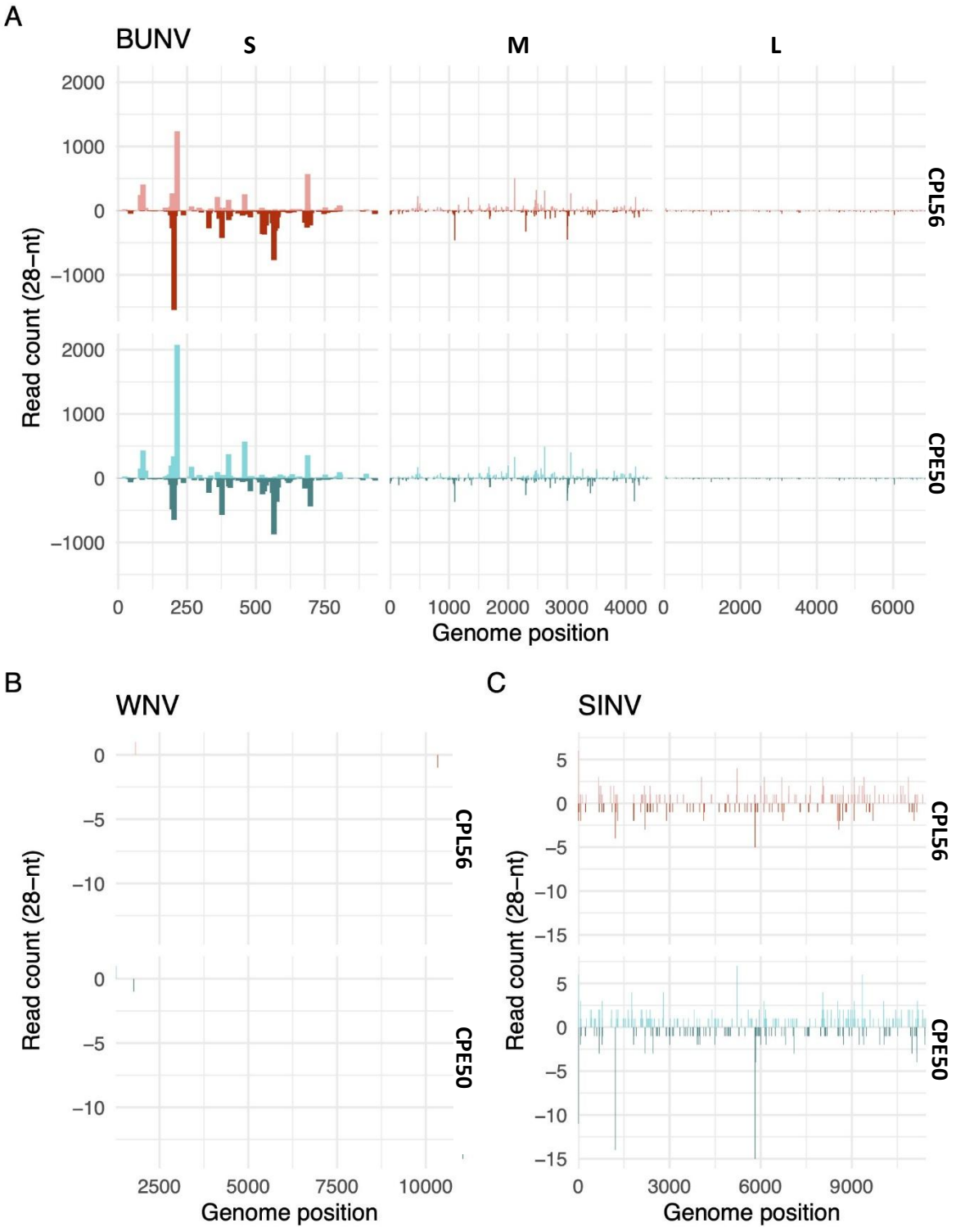

Figure 9. Distribution of viral specific 28 nt piRNA-sized RNAs across the viral genome and antigenome in infected *Cx. pipiens ss* CPE/LULS50 and CPL/LULS56 cells. BUNV (A), WNV (B) and SINV (C) -specific 28 nt RNAs mapped along the genome and antigenome. For the positive strand WNV and SINV, positive numbers indicate mapping to the virus genome, and negative numbers indicate mapping to the antigenome. For the negative strand BUNV -specific siRNA mapped to all three segments (L, M and S), positive numbers indicate mapping to the virus antigenome and negative numbers indicate mapping to the BUNV genome. Two

independent biological experiments were carried out for each experimental condition; plots from replicate 1 (shown in the main manuscript) are presented here.

Importantly, even though some differences on the sRNA profiles were observed between the two technical replicates, overall vsRNA could be observed for all viruses tested on both cell lines.

3.2.2.2. Ping-pong amplification activity for BUNV, SINV, and WNV on CPE/LULS50 and CPL/LULS56

Along with the typical 24-30 nts long end product of the piRNA pathway, mature piRNA, when processed by the PIWI family proteins, result in a ping-pong- amplification cycle where the produced virus derived piRNA (vpiRNAs) hold a recognition bias for either uridine at position one or adenine at position ten in the antisense and sense sequences, respectively (U1 and A10), and a complementary region of 10 nucleotides. These specific ping-pong cycle dependent vpiRNAs amplification characteristics can be detected by looking for vpiRNAs nucleotide frequency and read overlaps, and interestingly, during infection in mosquitoes, each arbovirus family exhibits diverse characteristics of the virus derived piRNA response.

To understand if the ping-pong cycle is active in CPE/LULS50 and CPL/LULS56 cell lines, the vpiRNA reads resulting from BUNV, SINV and WNV infection were analysed for nucleotide frequency at position U1 and A10, and overlap picks with 10 nucleotides were characterized.

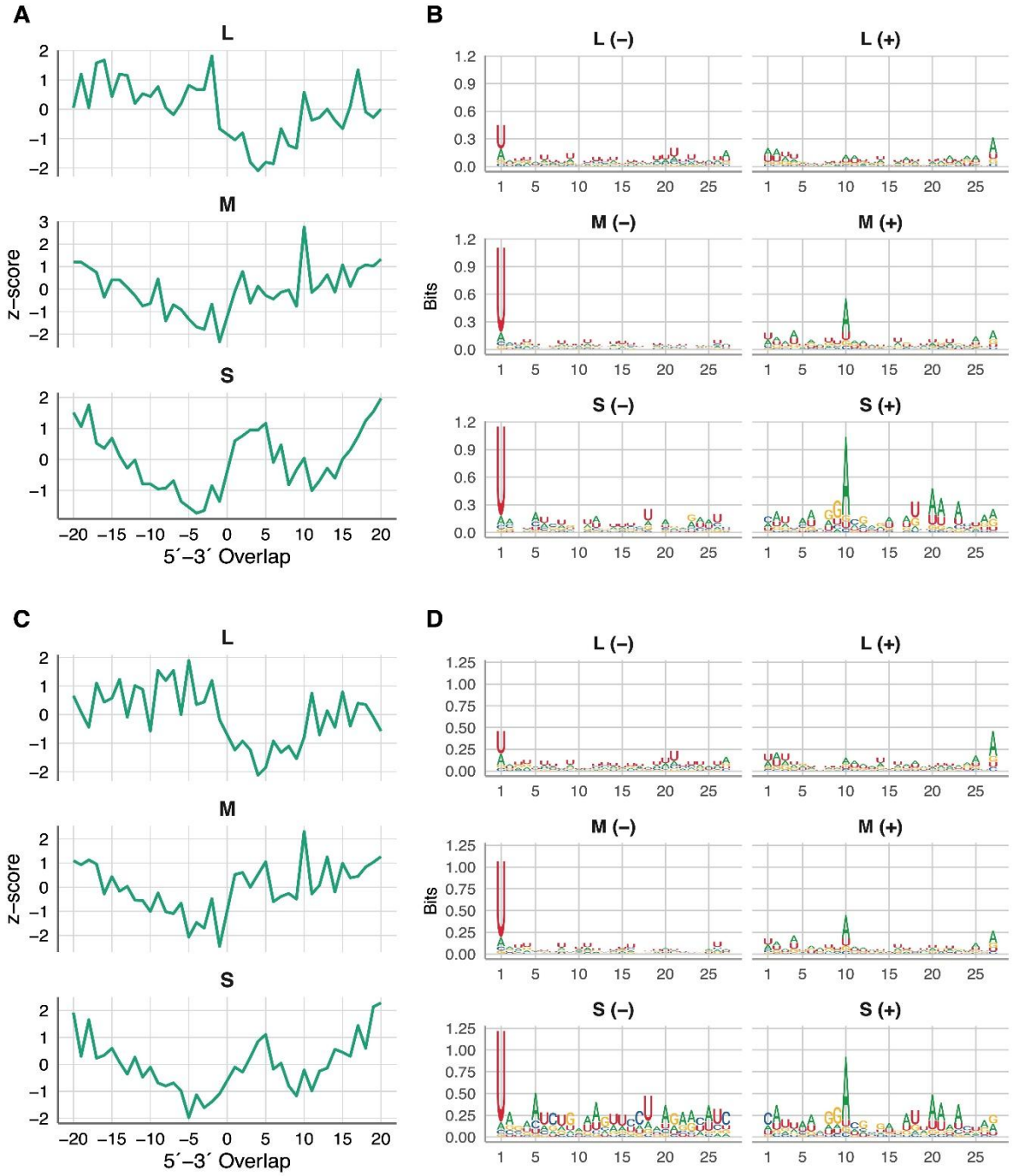

Figure 10. Replicate A ping-pong signature of piRNA-sized (24-29 nt) small RNAs produced during BUNV infection in CPE/LULS50 and CPL/LULS56 cells. Overlap z-score indicating the probability of overlap between the genome and antigenome of BUNV L, M or S segment-specific piRNA-sized small RNAs in infected CPE/LULS50 (A) and CPL/LULS56 (C) cells; relative nucleotide frequency and conservation per position of BUNV L, M or S segment-specific piRNA-sized small RNAs in infected CPE/LULS50 (B) and CPL/LULS56 (D) cells. Two independent biological experiments were carried out, and the results of replicate A are shown here. For replicate B see (Gothe et al., 2026).

While piRNA-sized sRNAs (24-29 nt) were produced in both cell lines during infection with all tested viruses (Table 12), the characteristic ping-pong amplification signature of piRNA amplification was only present in BUNV-infected cells. The reads mapping to BUNV M and S

genome segments displayed hallmarks of canonical piRNAs, including the ping-pong signature (10-nt overlap), and nucleotide biases (U1 and A10) in both cell lines (Figure 10B, Figure 10D).

3.2.3. *Cx. pipiens ss* cell line virome analysis

The development of high-throughput small RNA sequencing in combination with bioinformatic analyses permits the identification of active replicating (persistent) viral infections in insect cell lines and mosquitoes (Q. Wu et al., 2010). Research has shown that siRNAs produced in response to infection map to at least 70% of viral genome and antigenome and therefore can be used to create viral contigs and detect virus infections (Aguiar et al., 2015; Q. Wu et al., 2010, 2012). This method can even be used to detect viral infections that do not result in any cytopathic effects or morphological changes (Franzke et al., 2018). Therefore to investigate potential ongoing insect-specific viral infections in the established cell lines, virus discovery analysis was performed as described previously (Franzke et al., 2018) using the small RNA sequencing datasets.

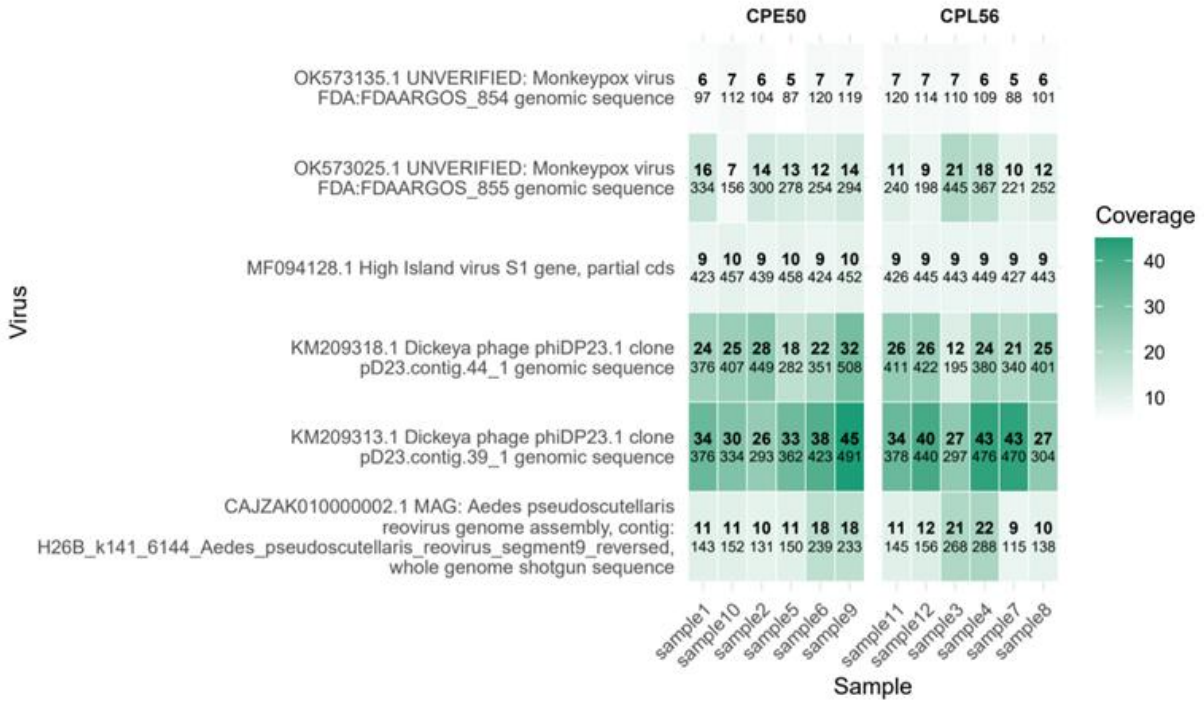

Figure 11. Heat Map analysis shows virus diversity across CPE/LULS50 and CPL/LULS56 cell lines. Virus genome coverage by siRNAs (21nt) counts across CPE/LULS50 and CPL/LULS56 cell line samples. Only viral sequences detected in ≥ 3 samples are shown. Genome coverage (up to 45%) is represented by shading intensity, and siRNA read counts are indicated numerically within each cell.

The 21nt siRNAs were mapped to a database with known viruses and the percentage coverage of these siRNAs to the tested viral genomes was determined. Several viral sequences were identified to which siRNAs mapped with a coverage of 9-40%, however none were detected

with a coverage of 70% or more (Figure 11); suggesting the absence of active ISV replication in both cell lines.

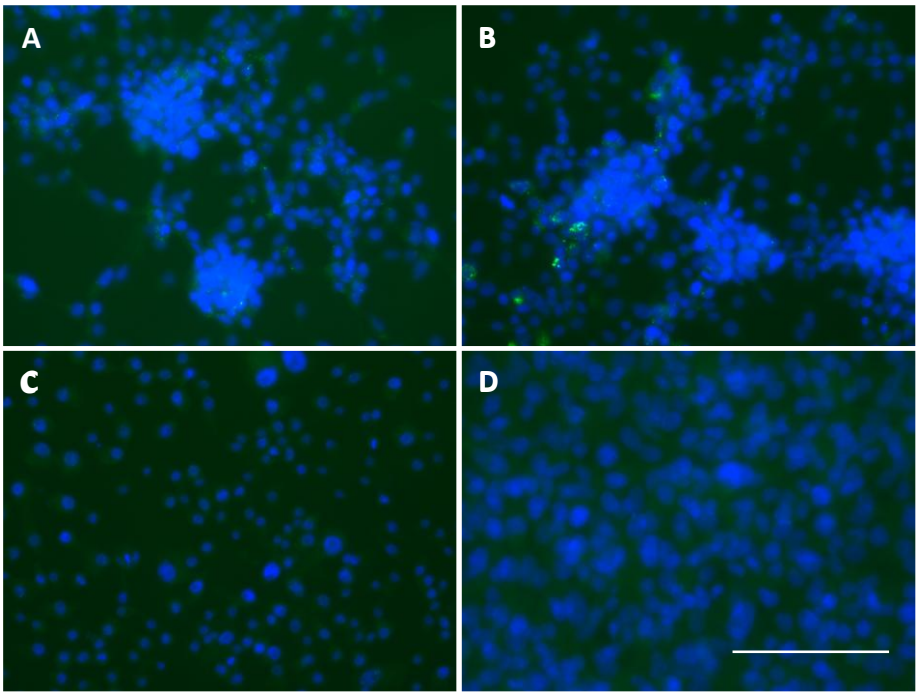

Figure 12. 3G1 immunofluorescence assay for dsRNA detection in different cell lines. Immunofluorescence assay performed on *Ae. aegypti*-derived Aag2 persistently infected with Cell fusing agent virus (A), Aag2 infected with Eilat virus MOI 1 and fixed at 24 hours post infection as positive control for the detection of viral dsRNA by mAb 3G1 (B), CPE/LULS50 (C), BHK-21 (D). Each panel shows a representative image for its respective experimental condition. Cells were fixed by paraformaldehyde and incubated with dsRNA-specific mAb 3G1, followed by goat anti-mouse Alexafluor 488 (green). DNA are labelled with DAPI stain (blue). Plates were imaged at 40x magnification. Scale bar denotes 100 μm.

The lack of active ISV replication was further supported by immunofluorescence assays, in which no dsRNA staining was detected in the tested CPE/LULS50 cells, in contrast to the positive controls (Aag2 with persistent CFAV infection and Aag2 actively infected with EILV) (Figure 12).

3.3. *In vivo* ISV-WNV co-infections

To investigate the potential influence of ISV infections on the replication and transmission dynamics of arboviruses in mosquitoes, we focused on WNV, the principal arbovirus transmitted in Europe. Initially, wild-caught *Cx. torrentium* specimens were selected, as this species is recognized as one of the primary WNV vectors in Europe (Jansen et al., 2019, 2023; Leggewie et al., 2016; Vanslembrouck et al., 2024). At first, *in vivo* experiments were designed to assess mosquito permissiveness to the selected set of ISVs. To this end oral and intrathoracic injections were performed to evaluate which ISV administration route would result if higher infection rates.

3.3.1. Characterization of wild caught *Cx. torrentium* permissiveness to selected ISV replication

For ISV oral administration, an ISV-PBS meal containing $1x10^7$ $TCID_{50}$/mL of either EILV, or ASALV, was offered to female *Cx. torrentium*. Engorged individuals were selected and sampled at 4, 7, 8 and 14 dpi for EILV, and at 3 dpi for ASALV. Presence of viral RNA was determined by real-time PCR of the mosquito body homogenate.

EILV and ASALV oral administration resulted in poor infection rates (Table 13). After oral exposure to the different ISVs, only two mosquitoes were found to be positive for EILV at 4 dpi, and all mosquitoes tested negative for ASALV specific viral RNA (Table 13).

Table 13. ISV infection rate on wild caught *Cx. torrentium* mosquitoes.

Virus administration rout	ISV	Infection rate (dpi)					
		0	**3**	**4**	**7**	**8**	**14**
Oral	EILV	–	–	13% *n*=15	0% *n*=15	0% *n*=10	0% *n*=15
	ASALV	–	0% *n*=13	–	–	–	–
Injection	EILV	100% *n*=5	100% *n*=15	–	100% *n*=12	–	100% *n*=15
	ASALV	100% *n*=4	100% *n*=15	–	100% *n*=13	–	100% *n*=15
	NIEV	50% *n*=8	–	–	50% *n*=10	–	40% *n*=5

Note: dpi - days post infection; – - no data point; *n* - total number of individuals tested on that time point.

Since previous studies show that *Culex* spp. get better ISV infection rates through intrathoracic injection, EILV and ASALV were further tested via this route (Jagtap et al., 2023; Nasar, Gorchakov, et al., 2014; Schulze, 2023). Additionally NIEV infectibility was only tested via intrathoracic injection since previous work demonstrated poor virus infectivity through oral administration (Schulze, 2023). To this end, 10^3 PFU of the respective ISV was administrated via intrathoracic injection on *Cx. torrentium* adult female mosquitoes. Mosquitoes were sacrificed at -0, -3, -7, and -14 dpi for EILV and ASALV, and -0, -7, and -14 dpi for NIEV. Virus RNA was quantified by real-time PCR of the mosquito body homogenate (Figure 13).

Infection rates of 100% were observed for both EILV and ASALV on all time points tested (Table 13). Both viruses presented approximately 10^3-10^4 viral copy number at 0 dpi which represent the approximate amount of virus injected to the mosquitoes (10^3 PFU). Virus copy numbers greatly increased from 0 dpi to 3 dpi for both viruses suggesting virus replication in the mosquito, yet EILV copy numbers decreased slightly more on 7 and 14 dpi when compared with ASALV copy numbers (Figure 13).

NIEV presented low ratio of positive mosquitoes throughout the tested time points (Table13), resulting in the discard of this virus as a potential candidate for the ISV-WNV co-infection experiments, and was no longer considered for the current work.

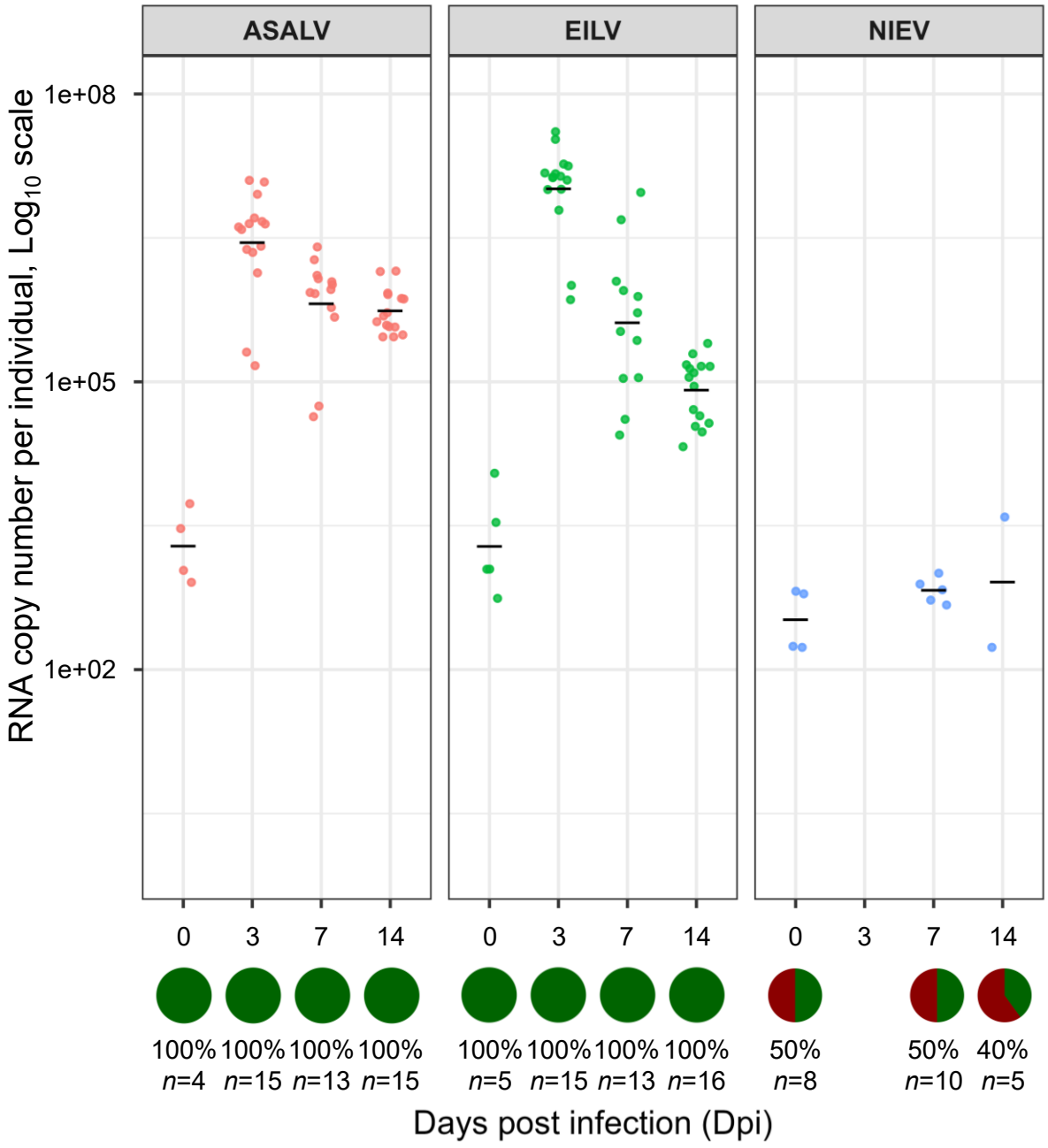

Figure 13. Intrathoracic infection of ISV in *Cx. torrentium* mosquitoes. One to four days old female *Cx. torrentium* were injected with 10^3 PFU of ASALV, EILV, or NIEV, and mosquitoes bodies were sampled at 0-, 3-, 7-, and 14-days post infection (Dpi). Total RNA was extracted from individual mosquitoes, and virus copy numbers were determined by virus-specific real-time PCR. Pie charts indicate the infection rate of the ISV tested (infected samples over the total number tested for each time point). n- equal to total mosquitoes sampled for the corresponding time point.

3.3.2. ASALV co-infection with WNV experiment *in vivo* wild caught *Cx. torrentium*

To determine the effect of ASALV on WNV infection in the *Cx. torrentium*, WNV viral copy numbers were compared between two experimental groups, the ASALV infected group and the mock group. Adult female *Cx. torrentium* were either injected with 10^3 PFU of ASALV

(ASALV + WNV group), or injected with PBS (PBS + WNV group). Three days after injection all mosquitoes were offered a blood meal containing $1x10^7$ $TCID_{50}$/mL of WNV, and engorged mosquitoes were kept at 27°C +/-5°C, with 70% RH.

Due to challenges associated with seasonal mosquito availability and limited field collection yields, the number of *Cx. torrentium* individuals available for this experiment rapidly became scarce and the few mosquitoes that were available were harvested only at 21 dpi since it has been showed that WNV infection rates in *Cx. torrentium* are at the highest at 21 dpi (Jansen et al., 2023). WNV viral RNA was quantified by real-time PCR of the mosquito body homogenate. Additionally, mosquito's saliva was collected and tested for CPE induction on HuH7 cells.

No WNV RNA could be detected in mosquitoes either of the ASALV infected, or the mock (PBS) group. Additionally, no positive CPE was observed in the forced salivation assay (Table 14).

Table 14. ASALV and WNV co-infection assay with wild caught *Cx*. *torrentium* mosquitoes.

Group	WNV RNA at 21 dpi	Salivation assay (CPE)
PBS + WNV	0% *n*= 3	0% *n*=3
ASALV + WNV	0% *n*=9	0% *n*=9

Note: dpi - days post infection; CPE - cytopathic effect; *n* - total number of individuals tested on that time point.

To overcome the *Cx. torrentium* mosquitoes' scarcity limitation, an additional mosquito species, *Cx. p*. biotype *pipiens*, was incorporated into the study. Together with *Cx. torrentium*, *Cx. p*. biotype *pipiens* is considered a key WNV vector in Northern Europe (Jansen et al., 2019, 2023; Leggewie et al., 2016; Vanslembrouck et al., 2024).

3.3.3. Characterization of *Cx. p.* biotype *pipiens* permissiveness to ASALV replication

Given the low infection rates observed for orally ISV infected *Cx. torrentium* mosquitoes, *Cx. p.* biotype *pipiens* laboratory colony mosquitoes were only tested for ASALV susceptibility, directly via intrathoracic injection.

Table 15. ASALV infection rate on *Cx. p.* biotype *pipiens* mosquitoes.

Virus	Injection rate (dpi)		
	3	**7**	**14**
ASALV	100% *n*=15	100% *n*=30	100% *n*=9

Note: dpi - days post infection; CPE - cytopathic effect; *n* - total number of individuals tested on that time point.

For all injected mosquitoes, ASALV viral RNA could be detected throughout the time points tested (Table 15; Figure 14). Even though 0 dpi was not tested, ASALV presented similar copy numbers at 3 and 7 dpi comparatively with what was observed for *Cx. torrentium*. Although ASALV copy numbers seem to decrease on 14 dpi this could be due to lower mosquitoes sampled for this time point. Overall ASALV replication seems to be supported in *Cx. p.* biotype *pipiens* mosquitoes (Figure 14).

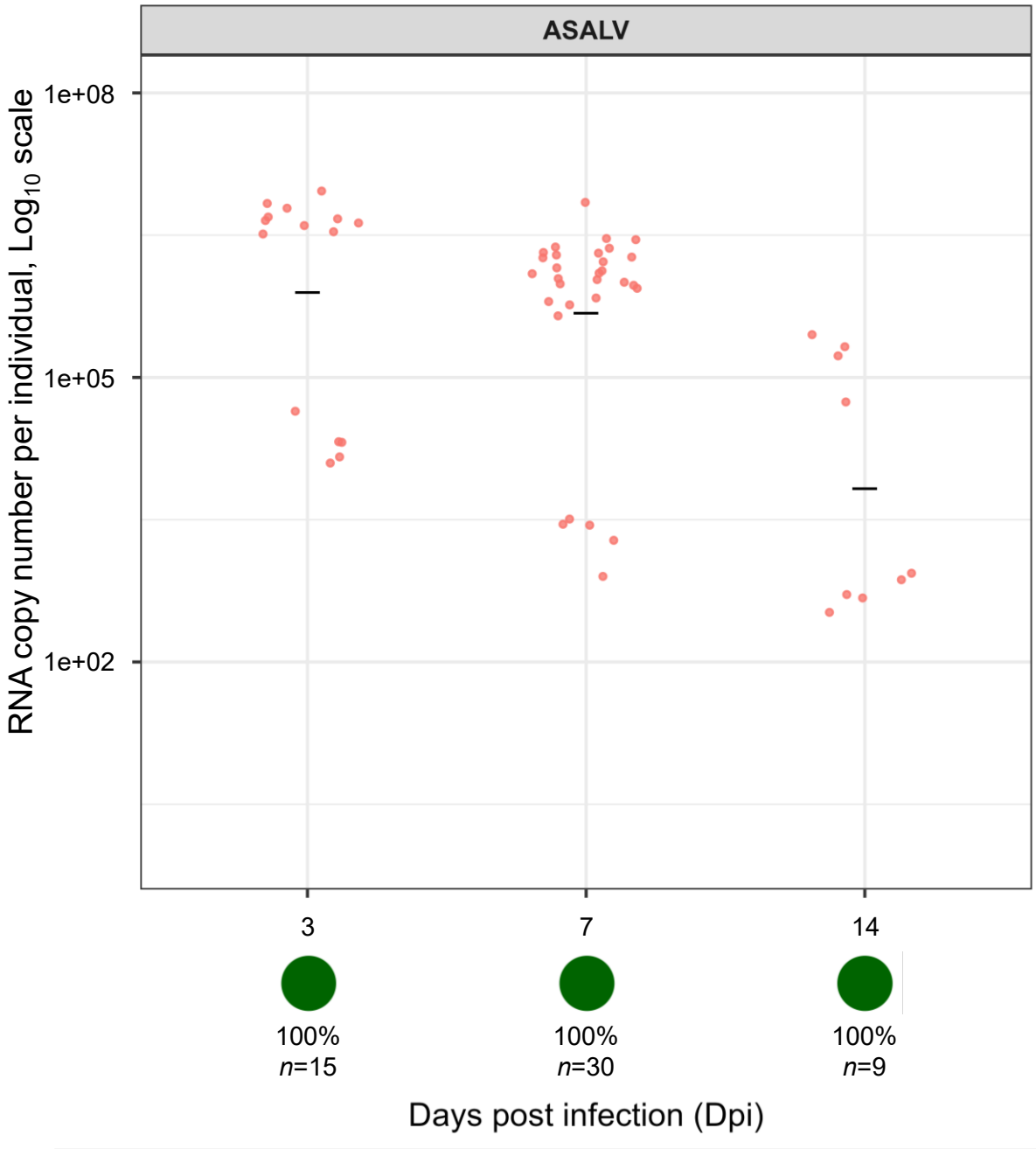

Figure 14. ASALV copy numbers in intrathoracic infection of *Cx. p.* biotype *pipiens* mosquitoes. One to four days old female mosquitoes were injected with 10^3 PFU of ASALV, and mosquitoes bodies were sampled at 3-, 7-, and 14-days post infection. Total RNA was extracted of individual mosquitoes and virus copies numbers were determined by virus-specific real-time PCR. Pie charts indicate infection rates of the ISV tested (infected samples over the total number tested for each time point). *n*- equal to total mosquitoes sampled for the corresponding time point.

3.3.4. ASALV-WNV co-infection in *Cx. p.* biotype *pipiens* mosquitoes

To evaluate the potential interference dynamics of ASALV on WNV in *Cx. p.* biotype *pipiens* mosquitoes the same experimental layout as described previously was performed, shortly two experimental groups where compared; the ASALV + WNV infected group and the PBS + WNV group, for WNV viral copy numbers at 7, 14, and 21 dpi. Additionally, the saliva was collected at 21 dpi and added to HuH7 cells to evaluate CPE induction.

Table 16. WNV infection rates for ASALV-WNV co-infection on *Cx. p.* biotype *pipiens* mosquitoes

Group	Infection rate (dpi)			Salivation assay (CPE)
	7	14	21	
PBS + WNV	17% *n*=30	25% *n*=31	20% *n*=30	17% *n*=30
ASALV + WNV	17% *n*=30	17% *n*=30	27% *n*=30	23% *n*=30

Note: dpi - days post infection; CPE - cytopathic effect; *n* - total number of individuals tested on that time point.

Throughout the tested time points, WNV-positive mosquitoes where detected for both the PBS group and ASALV group (Table 16.). WNV copy numbers were similar between both groups, as well as the number of CPE causing saliva (Figure 15). Interestingly, a positive saliva was observed for the PBS group, where the WNV copy numbers were below the detection limit.

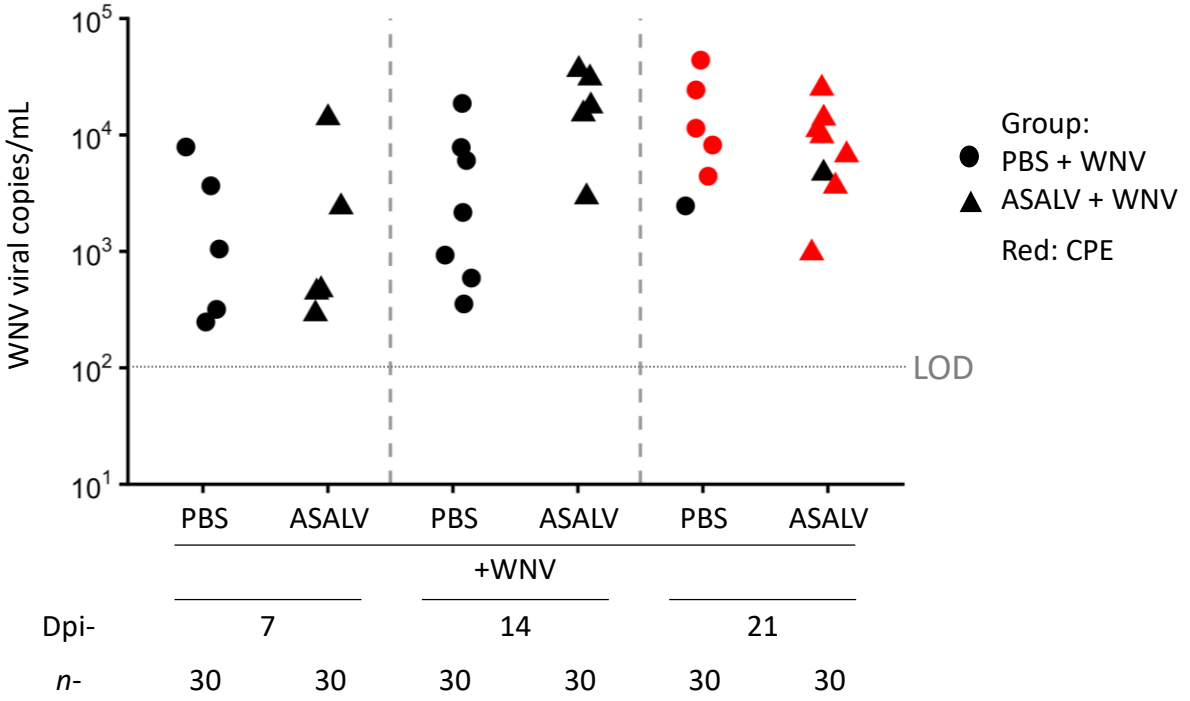

Figure 15. ASALV and WNV co-infection assay on *Cx. p.* biotype *pipiens* mosquitoes. Female mosquitoes (4-14 old females) were either intrathoracically injected with 10^3 PFU of ASALV (ASALV + WNV group), or PBS (PBS + WNV group), followed by an artificial blood meal containing WNV at $1x10^7$ TCID50/mL. At 7-, 14-, and 21-day post-infection (Dpi), total RNA of individual mosquito body homogenate was extracted. WNV copy numbers were determined by real-time PCR, and normalised to GAPDH (housekeeping gene) through delta-delta CT approach. LOD: limit of detection. At dpi 21, the saliva of the mosquitoes was sampled for CPE on HUH7 cells (red). *n*- total individuals per group, per time point. Data is a combination of three biological replicates.

4. Discussion

Arboviruses such as WNV and USUV represent an increasing public health concern in Europe, where mosquitoes of the *Cx. pipiens* complex and *Cx. torrentium* play a central role in virus transmission. Despite their epidemiological importance, the molecular determinants of arbovirus infection and vector competence in *Culex* mosquitoes remain comparatively understudied, partly due to the limited availability of well-characterized experimental systems. At the same time, ISVs are increasingly recognized as important components of the mosquito virome that may influence arbovirus replication and transmission. The work presented in this thesis aimed to address these knowledge gaps by investigating ISV-arbovirus interactions and characterizing newly established *Cx. pipiens ss*-derived cell lines as experimental models for virus-vector research. Using a combination of *in vitro* infection experiments, sRNA profiling, virome analysis, and *in vivo* mosquito infections, this study evaluated viral replication

dynamics, virus specific RNAi responses, and potential interference between ISVs and medically relevant arboviruses. Overall, the results demonstrate that the newly established *Cx. pipiens ss* cell lines are broadly permissive to arboviruses and ISVs and possess functional virus-derived RNAi responses. Additionally, ISV infections did not consistently alter arbovirus replication in either cell culture or mosquito systems. Together, these findings highlight the complexity and context dependency of ISV-arbovirus interactions and provide new experimental tools to investigate virus-vector dynamics in *Culex* systems.

4.1. Characterization of *Cx. pipiens ss*-derived cell lines

Mosquito cell culture systems provide highly tractable platforms to study viral replication dynamics, host antiviral pathways, and virus-vector interactions under controlled conditions (Rückert, 2026). However, studies using *Culex* mosquitoes have historically relied on only two widely available cell lines: the *Cx. quinquefasciatus*-derived Hsu line (Hsu et al., 1970) and the *Cx. tarsalis*-derived CT line (J. & G.H., 1976). Both lines exhibit limited susceptibility to several arboviruses and are commonly persistently infected with several ISVs, which can confound experimental interpretation (Rückert et al., 2019; Weger-Lucarelli et al., 2018).

Given these limitations, the development of European *Cx. pipiens ss*-derived lines (Bell-Sakyi et al., 2021), provide an unprecedented opportunity to investigate virus-vector interactions within a temperate European context. *Cx. pipiens ss* mosquitoes are widespread in Europe and increasingly implicated in the transmission of medically important viruses such as WNV and USUV. Understanding their interactions with arboviruses and ISVs is therefore critical for predicting vector competence dynamics and the development of potential vector biocontrol strategies. Collectively, the work presented here establishes a foundation for integrating *Cx. pipiens* cell culture systems into the broader framework of arbovirology and vector competence research. In this study, both lines (CPE/LUL50 and CPL/LUL56) were infected with a panel of arboviruses and ISVs representing the families *Flaviviridae*, *Togaviridae*, and *Peribunyaviridae* (including WNV, USUV, SINV, BUNV, NIEV, EILV, and HERBV). Viral replication kinetics were measured, small RNA responses were profiled following infection with representative viruses, and virome analyses were performed to test for persistent ISVs. These approaches allowed direct assessment of viral permissiveness, intrinsic antiviral immunity, and baseline virome status prior to evaluating ISV-arbovirus interactions.

4.1.1. Viral replication kinetics

Both cell lines supported replication of a broad spectrum of positive-strand and negative-strand arboviruses, including SINV, BUNV, WNV, USUV, EILV, NIEV, and HERBV. This broad susceptibility is notable, particularly in comparison to other *Culex*-derived cell lines, such as HSU, which have shown more restricted viral permissiveness (Schulze, 2023; Weger-Lucarelli et al., 2018). Overall, replication kinetics were broadly similar between the two lines; however, subtle differences were observed.

While WNV replicated robustly in both lines, other flaviviruses such as USUV and NIEV reached lower titer, suggesting different virus-specific replication fitness. The WNV strain used in this study belongs to lineage 2, which has previously been reported to replicate more efficiently in mosquito systems, and may therefore partially explain the higher replication levels observed here (Höller et al., 2026; Schulze, 2023). A similar replication kinetic profile was previously reported for WNV, USUV, and NIEV on CT cells (Schulze, 2023). For WNV and USUV, these results contribute to a consistent pattern previously observed in multiple cellular systems (mammalian, avian and mosquito cells = CT and C6/36 cells), and for *Cx. p.* biotype *pipiens* mosquitoes (H. Wang et al., 2020).

These observations raise the question of whether WNV possesses replication strategies that confer higher replication fitness in *Cx. pipiens ss* cells, whereas related flaviviruses may not exploit the same pathways. Or if the anti-viral pathways present in the cell lines act differently upon the different flavivirus infection. Indeed, the same mosquito antiviral pathway (JAK/STAT), was shown to differentially influence WNV and USUV replication in the past (Paradkar et al., 2012, 2015; Walsh et al., 2025). Furthermore, WNV has been shown to have a broad mosquito cell tropism, and transmitted horizontally and vertically within a *Cx. p.* biotype *pipiens* population (Anderson et al., 2008; Bell-Sakyi et al., 2021; Girard et al., 2004), which may explain its broad facility replicating here in both embryo- and larva-derived cell lines. Comparable tissue tropism and vertical transmission data are largely unavailable for USUV and NIEV (Schilling et al., 2025).

Among the alphaviruses, both SINV and EILV reached high virus titer, with subtle differences observed between the cell lines. The insect-specific alphavirus EILV replicated to higher titers in the *Cx. p.* biotype *molestus* (CPL/LULS56) cell line than in the mixed *Cx. p.* biotype *pipiens* and biotype *molestus* (CPE/LULS50) cells. However, there are no data available comparing the infectivity of EILV in mosquitoes of specific *Cx. pipiens* biotypes. SINV replication kinetics

showed the opposite trend, even though, it has been demonstrated that *Cx. p.* biotype *molestus* mosquitoes are more susceptible to SINV compared to biotype *pipiens* (Foy et al., 2004; Jansen et al., 2022). Additionally, the insect-specific alphavirus ASALV was unable to successfully replicate in the mixed *Cx. p.* biotype *pipiens* and biotype *molestus* (CPE/LULS50) cells, although later in the study it was possible to stablish ASALV infection on adult *Cx. p.* biotype *pipiens* mosquitoes, and another *Culex* spp. (*Cx. quiquefasciatus*) has been reported to be susceptible to ASALV infection (Jagtap et al., 2023). Perhaps the cell population/s present in the cell line may lack the necessary conditions for a successful replication of ASALV. Further investigation on the different cell populations present in the cell lines would be of interest to better understand virus replication patterns and dynamics.

Regarding the peribunyavirus group, a greater similarity was observed in the viral replication kinetic profiles for the arthropod-transmitted BUNV and insect-specific HERBV between the two cell lines, with no notable differences, representing the group of viruses with greater replication stability between the cell lines. Many viruses belonging to the *Peribunyaviridae* family have been described to undergo vertical transmission in a variety of arthropod vectors including mosquitoes (Bergren & Kading, 2018). However, no study has specifically looked into the vertical transmission potential of BUNV or HERBV. Here we show that in larva- and embryo-derived cell lines can support BUNV and HERBV replication.

Collectively, these findings raise the question if factors potentially influencing CPL/LULS56 and CPE/LULS50 cells susceptibility differences could include, amongst others, the cell line origin (embryo- or larva-derived), species, or intrinsic antiviral pathways, such as RNAi-mediated and JAK/STAT responses.

4.1.2 siRNA, piRNA, and ping-pong amplification profile for WNV/SINV/BUNV

Both *Cx. pipiens ss*-derived cell lines exhibited strong siRNA responses, producing abundant viral-specific 21-nt siRNAs across all tested viruses, indicating a functional RNAi activity and demonstrating their utility in RNAi research.

siRNA responses were largely similar between the two cell lines. During WNV infection, a hot spot of siRNAs was observed in the 3′ UTR of the antigenome. Suggesting targeted processing of the subgenomic flavivirus RNA (sfRNA) region (Bavia et al., 2016). The sfRNAs are responsible for viral pathogenicity, host adaptation, and emergence of new pathogenic strains, and are often targeted by the host antiviral responses (Slonchak & Khromykh, 2018). This observation is consistent with previous findings in *Cx. pipiens* mosquitoes (Göertz et al., 2019).

Notably, virus-specific small RNA abundance was lower in WNV-infected cells compared to SINV or BUNV infections. This could be due to the time point of sampling chosen which may have been closer for BUNV and SINV replication kinetics peak, versus an early time point chosen for WNV (96 hpi) when the replication kinetics reveal its peak at around 6 dpi. However, differences in the amount of virus-specific small RNAs produced during infection between virus families have previously been reported for other cell lines (Göertz et al., 2019). This may reflect intrinsic differences in how viruses interact with the RNAi machinery (Miesen et al., 2016; Rückert et al., 2019).

Analysis of piRNA-sized small RNAs revealed canonical vpiRNA production in BUNV-infected cells. These piRNAs originated from both M and S segments and displayed the ping-pong amplification signature, consistent with previous reports from *Aedes* and some *Culex* cell lines (Dietrich et al., 2017; Scherer, 2021; Varjak et al., 2018; Walsh et al., 2022). In contrast, WNV and SINV infections produced only low-abundance piRNA-sized reads without canonical ping-pong features, indicating limited engagement of the piRNA pathway, consistent with prior observations in *Cx. pipiens* mosquitoes and other *Culex* spp. cell lines (Miesen et al., 2016; Rückert et al., 2019). It has been observed that SINV infection strongly induces vpiRNA with ping-pong amplification in *Aedes* spp. cell lines (Blair, 2019; Girardi et al., 2017; Joosten et al., 2019, 2021; Kolliopoulou et al., 2019; Morazzani et al., 2012b; Varjak, Maringer, et al., 2017; Vodovar et al., 2012). The lack of SINV-induced piRNAs contributes to the notion that piRNA induction is not only virus dependent but that it can also be mosquito species dependent. And while SINV-induced vpiRNA it has been shown in different *Aedes* species, it seems that this is lacking in *Culex* spp. (Kolliopoulou et al., 2019).

4.1.3 Virome analysis of *Cx. pipiens ss* cell lines

The search for persistent ISVs in these lines showed no evidence of active viral infections, as siRNA mapping did not reveal significant matches to known viruses. This contrasts with the common characterization of mosquito cell lines, which usually are described to harbour at least one ISV (Chen et al., 2004; Franzke et al., 2018; Göertz et al., 2019; Jousset et al., 1993; Rückert et al., 2019; Weger-Lucarelli et al., 2018). The absence of ISVs in these lines may be linked to prior presence of the bacteria *Wolbachia* during cell establishment (Bell-Sakyi et al., 2021), later removed by antibiotic treatment. *Wolbachia* is described to suppress various RNA virus replication, including ISVs, which could explain the absence of ISVs in the tested cell lines (Bishop et al., 2020; McLean et al., 2019; Schnettler et al., 2016; Zhang et al., 2016). As our virus detection relied only on mapping viral siRNAs to deposited viral sequences, unknown or

weakly targeted viruses may have been overlooked. Moreover, the abundance of persistent viruses may vary over time, and our sampling may have coincided with a low replication phase for potential additional ISVs. Although this approach has been regularly successful in the past (Abel et al., 2023; Aguiar et al., 2015; Franzke et al., 2018; Q. Wu et al., 2010), additional longitudinal and in-depth metagenomic sequencing would be beneficial to confirm the results.

4.2. Insect-specific virus and arbovirus interactions

ISVs are a diverse group of RNA viruses that infect mosquitoes but are unable to replicate in vertebrate hosts. They are now recognized as key components of the mosquito virome, shaping host physiology and influencing vector competence for arboviruses (Blitvich & Firth, 2015; B. Bolling et al., 2015; B. G. Bolling et al., 2012; Goenaga et al., 2015, 2020; Hall-Mendelin et al., 2016; Hermanns et al., 2020; Hobson-Peters et al., 2013; Kenney et al., 2014; Kent et al., 2010; Kuwata et al., 2015; Nasar et al., 2012, 2015; Samina et al., 1986; Schultz et al., 2018; Talavera et al., 2018). Curiously, experimental evidence demonstrates that ISVs can suppress, enhance or have no effect on arboviral replication depending on viral strain, timing, and host background (B. G. Bolling et al., 2012; Joseph et al., 2024; Kenney et al., 2014), demonstrating the complexity of ISV-arbovirus-vector interactions highlighting the need on detailed research when it comes to consider ISVs as potential vector biocontrol strategy.

4.2.1. ISV-arbovirus co-infections in *Cx. tarsalis* derived CT cell line

The current study, reveals that *in vitro* co-infection experiments, where pre-existing or simultaneous EILV infection in CT (*Cx. tarsalis*) cells did not influence the replication of WNV-2. This is not in line with published reports; however, these reports show already conflicting results: On the one hand, superinfection exclusion of WNV with the same EILV virus [isolate EO329] as used in the current study, has been described in C6/36 (*Ae*. *albopictus*) cells and *Cx. tarsalis* mosquitoes (Joseph et al., 2024). On the other hand, another study showed *in vitro* increase of WNV infectious particles in the presence of a different EILV virus isolate (isolate MP458-NA-2018) on C7/10 (*Ae*. *albopictus*) cells (Guggemos et al., 2024). These differences of our results and the ones in the literature may be correlated to the different WNV isolates used in this study (WNV isolate ED-I-33/18-UM [lineage 2]), to those used in earlier studies reporting strong interference effects (WNV [lineage 1] strain WN02-1956, and NY99) (Joseph et al., 2024). Contributing to the hypotheses that EILV interference on WNV occurs in a strain-specific manner (Joseph et al., 2024). Furthermore, interference assays with the same EILV [isolate EO329] as used for the current study, showed reduced virus production and delayed

replication kinetics of different virus belonging to the *Togaviridae* virus family (SINV, VEEV, EEEV, CHIKV, WEEV) in previous studies in C7/10 (*Ae. albopictus*) cells (Nasar et al., 2015). Again, no such inhibition was observed in our studies on CT cells with a different *Togaviridae* virus: SFV. This could be due to differences in the experimental set ups (e.g. MOIs used and mosquito species); highlighting again experimental dependency of the reported EILV-arbovirus interactions. Similarly, no effects on arboviruses belonging to the *Flaviviridae* family, such as WNV and USUV were detected.

Similarly, no measurable interference of viral replication profiles for the tested arboviruses was observed for the insect-specific flavivirus: NIEV. As of NIEV, no interference studies have been reported yet, so no comparison data is available. ISV interference has been shown to occur more efficiently between phylogenetically related viruses (H. Wang et al., 2020). However, such correlations were not evident in our assays, as flaviviruses and alphaviruses tested were not affected by NIEV or EILV viruses, respectively. These findings suggest that ISV-arbovirus interactions are complex and may depend on several factors including specific virus strains, infection timing, and host cell context.

4.2.2. *Cx. torrentium* and *Cx. p.* biotype *pipiens* mosquito susceptibility to ISV infection

As *Cx. p.* biotype *pipiens* and *Cx. torrentium* are important North European vectors for WNV (Jansen et al., 2019, 2023; Leggewie et al., 2016; Vanslembrouck et al., 2024), *in vivo* mitigation of WNV transmission through pre-existing ISV infection was evaluated. To this end, first the mosquito susceptibility for ISVs was tested through two different infection routs (i) oral (for *Cx. torrentium*), and (ii) intrathoracic injection (for *Cx. torrentium*, and *Cx. p.* biotype *pipiens*). For *Cx. torrentium* oral infection with EILV and ASALV was tested, and only a few positive mosquitoes were found infected with either. However, intrathoracic injection with these viruses resulted in a 100% infection rate for all tested time points. The discrepancy between oral infection and intrathoracic infection outcome was also described elsewhere for EILV in *Cx. quinquefasciatus* (Nasar, Haddow, et al., 2014; Schulze, 2023), *Ae. albopictus, Ae. aegypti,* and *Ae. gambiae* (Nasar, Haddow, et al., 2014). Indicating a possible restriction by the midgut barrier for successful virus replication of these ISVs.

In contrast, very low infection rates resulted from intrathoracic infection of NIEV throughout the different time points tested. These results align with previous studies, where NIEV infection via intrathoracic injection or oral administration resulted in poor infection rates in *Cx. quinquefasciatus* (Schulze, 2023). Although NIEV was initially isolated from a pool of *Culex*

mosquitoes, our study and literature demonstrate that mosquitoes of the *Cx. torrentium* and *Cx. quinquefasciatus* species seem to poorly support its replication. However, we show that in *Culex* cell culture systems NIEV successfully replicates. Or the route of administration of the virus is not the appropriate, or there could be a possible need of the virus to establish a midgut infection to result in systemic infection. Also, some ISVs have been described as having a certain vector host range. With restriction going from mosquito genus (*Aedes* and *Culex*), down to species specific, as is the case with Parramatta River virus (McLean et al., 2015). Raising the question if NIEV could also hold such vector host range restrictions. Further infectivity assays with NIEV on different *Culex* spp. mosquitoes would be beneficial to better elucidate this virus biology.

As for *Cx. p.* biotype *pipiens* mosquito's susceptibility to ISV*,* only ASALV was tested via intrathoracic injection, and infection rates of 100% were obtained throughout all tested time points. Here, we describe for the first time that ASALV successfully replicates in adult *Cx. p.* biotype *pipiens* mosquitoes. Together with *Cx. declarator*, *Culex* (*Melanoconion*), *Cx. quinquefasciatus*, and *Ae. aegypti*, ASALV shows a broad spectrum of mosquitoes species as potential host (Hermanns et al., 2020; Jagtap et al., 2023; Ramos et al., 2024).

4.2.3. ASALV-WNV co-infection in *Cx. p.* biotype *pipiens*

In addition to ASALV, *Cx. p.* biotype *pipiens* adult mosquito's susceptibility to WNV-2 was inferred, and infection rates reached 20% at 14 dpi. The infection rates obtained in the current study are greatly lower than previously reported, where WNV-2 (Gr'10 isolate) infection rates at 14 dpi reach 46% and 77% on *Cx. pipiens* (Fros et al., 2015), and *Cx. p.* biotype *pipiens* (H. Wang et al., 2020), respectively. Viral infection rates can be influenced by a complex multivariable setting including differences in mosquitoes population, mosquito species, different geographic distribution, and virus family/lineage, amongst others (Blair, 2022; Sanchez-Vargas et al., 2004; Souza-Neto et al., 2019; Wei et al., 2020). The differences between the infection rates obtained in this study and the ones previously reported could therefore result from diverse strain fitness since it has been additionally demonstrated that different strains belonging to WNV-2 may result in different infection rates on *Cx. pipiens sl* (Romo et al., 2018). Furthermore, before exposure to a WNV meal, mosquitoes were injected with a PBS solution which could have possibly interfered with the mosquito's immune system and consequently altered its virus susceptibility.

The interference study of ASALV and WNV in *Cx. p.* biotype *pipiens* mosquitoes provided additional insights into the ecological relevance of ISV-arbovirus interactions. In these experiments, ASALV was used to assess potential modulation of WNV infection *in vivo*. Consistent with our cell culture findings, prior or concurrent ISV infection had no measurable effect on WNV infection or transmission in *Cx. p.* biotype *pipiens*. However, no interference assay was performed on cell culture for ASALV since the *Cx. p.* biotype *pipiens* and *molestus* cell line didn't support ASALV replication. Additional experiments on *Culex* cell systems would be beneficial for a more direct comparison.

These results indicate that although ISVs are often discussed as potential modulators of arbovirus dissemination and transmission, positive interference cannot be generalized across all virus-vector combinations. In fact, the impact of ISVs appears to be highly context-dependent, influenced by factors such as virus strain, infection titer, tissue tropism, infection route, and vector species. Specially for WNV a very inconsistent ISV-WNV interaction has been reported in the past where primary infections with Culex flavivirus (CxFV) showed a reduction on the secondary infections with WNV (B. G. Bolling et al., 2012; Kent et al., 2010; Kuwata et al., 2015). However, a positive ecological association between CxFV and WNV was described in mosquitoes in Chicago (Newman et al., 2011).

From an applied perspective, ISVs such as EILV and ASALV remain interesting candidates for ISV-arbovirus research, given their demonstrated ability in some contexts to reduce arbovirus replication. Engineered or naturally circulating ISVs could, in principle, establish persistent, non-pathogenic infections that lower vector competence for pathogens of public health relevance, such as WNV and USUV. However, realizing this potential will require a deeper understanding of ISV persistence, vertical transmission dynamics, and host specificity under realistic field conditions before any translational applications can be considered.

5. Conclusion

Arboviruses such as WNV and USUV continue to pose increasing public health threats in Europe, where *Culex* mosquitoes, particularly *Cx*. *pipiens ss* and *Cx*. *torrentium* are key vectors (Jansen et al., 2023). While numerous studies have examined determinants of arbovirus transmission in *Ae. aegypti*, comparable mechanistic insight into arbovirus-host interactions in *Culex* mosquitoes remains limited. This knowledge gap is largely attributed to the scarcity of suitable, well-characterized *Culex*-derived cell models and to an incomplete understanding of the ecological drivers that modulate vector competence, including the role of ISV.

In this study, this gap was partly addressed by characterizing European *Cx. pipiens ss*-derived cell lines regarding vector-virus interactions. Specifically, the work demonstrated that these cell lines are broadly permissive to both positive- and negative-strand arboviruses and insect-specific viruses, exhibit functional RNAi responses, do not harbour detectable persistent ISVs, and can therefore serve as robust tools for vector-virus studies. In parallel, *in vitro* and *in vivo* co-infection experiments showed that ISV infections did not (strongly) modulate replication or transmission of WNV, USUV or other tested arboviruses.

These findings, together with previous studies, emphasize the complex and context-dependent nature of ISV-arbovirus interactions, which are influenced by factors such as virus strain, infection timing, and host background. Therefore, comprehensive evaluation under diverse experimental and ecological conditions will be necessary before ISVs can be reliably considered as tools for vector control strategies.

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7. Publications

Some results of this project were submitted to peer-reviewed journal:

Gothe, S., Jagtap, S., Böhmer, P., Reuter, M., Frank, S., Sreenu, V. B., Bell-Sakyi, L., Merits, A., Altinli, M., & Schnettler, E. (2026). Characterization of Culex pipiens cell lines: Virus infection and RNAi response. *Parasites & Vectors*.

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Eidesstattliche Versicherung

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

Hamburg, den 17.03.2026

Sarah Gothe Esteves Viegas