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BNITM
Bernhard Nocht Institute for Tropical Medicine

From Infection to Transmission: Determinants of Arbovirus Vector Competence in Mosquitoes

Dissertation

Submitted by

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for the attainment of the degree *Doctor rerum naturalium*

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– Dein Burli

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The declarations of personal contribution are presented on the following pages. The full-length versions of these papers are provided in the supplementary material.

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Affidavit

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Patrick Höller

Abstract

Arthropod-borne viruses (arboviruses), specifically those transmitted by mosquitoes, are an increasing public health threat. This trend is amplified by globalization and climate change, which enable their spread into new regions. However, the transmission dynamics and vector competence of many arboviruses remain poorly understood, with more than 90% of potential virus-mosquito combinations remaining untested. This lack of knowledge limits accurate risk assessment and preparedness. Understanding how key determinants shape the transmission of arboviruses by mosquitoes is therefore of increasing importance. In this dissertation, the transmission dynamics of arboviruses endemic to (West Nile virus, Tahyna virus) and exotic to Europe (Snowshoe hare virus, Oropouche virus, Barmah Forest virus) were assessed, including a comparison of the two major West Nile virus lineages. Across the 29 virus-mosquito combinations tested, infection was observed in 97% of combinations, whereas transmission occurred in only 41%, demonstrating that infection does not necessarily lead to transmission. Vector competence varied substantially across mosquito taxa, with *Aedes albopictus* exhibiting the broadest transmission profile, and differed between viruses and viral lineages. All three exotic viruses were able to be transmitted by mosquitoes from Europe, highlighting the potential threat of those viruses. Effects of temperature on vector competence were heterogeneous and dependent on the specific virus-mosquito combinations. The minimum infectious dose was lower for West Nile virus lineage 2, compared with lineage 1. Overall, this dissertation demonstrates that vector competence is highly context-dependent and shaped by interactions between mosquito species, virus, temperature, and viral dose. These findings enable a more realistic assessment of arbovirus risk and highlight the need for context-specific evaluations of vector competence, as results cannot be generalized even across closely related mosquitoes or viruses.

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1 Introduction

1.1 Arboviruses

Arthropod-borne viruses (arboviruses) represent an increasing threat to global health. More than half of the world's population is currently estimated to be at risk of infection with at least one arbovirus (Labeaud et al., 2011; Lim et al., 2025). Due to the increase in urbanization and globalization, and the ongoing climate change, including rising temperatures and more frequent extreme weather events, arboviruses are expected to expand into new ecological and geographic regions, increasing their global health burden (Sant'Anna et al., 2025).

The role of mosquitoes in transmitting viral diseases was first hypothesized in the late 19th century. Notably, the Cuban physician Carlos J. Finlay proposed in 1881 that the mosquito *Aedes aegypti* acts as the vector of yellow fever (Finlay, 1881). This hypothesis was confirmed two decades later by the Walter Reed Commission, which established mosquito-borne transmission of the yellow fever virus and marked the beginning of our efforts to study arboviruses (Reed et al., 1900, 1901).

Since then, substantial research efforts have focused on understanding medically important arboviruses. Nevertheless, many arboviruses remain poorly understood and are often considered to be neglected. Globally, more than 500 arboviruses have been described, of which 150 are associated with human disease (Madewell, 2020). In Europe, a subset of these arboviruses are endemic and known to cause disease in humans, including the mosquito-borne viruses West Nile virus (WNV), Usutu virus, Sindbis virus, and Tahyna virus (TAHV) (Suvanto et al., 2022; Angeloni et al., 2023; Mravcová et al., 2023; European Centre for Disease Prevention and Control, 2025e). In addition to these endemic viruses, there are sporadic outbreaks caused by tropical viruses like dengue virus and chikungunya virus, and travel-associated cases of exotic viruses, such as Oropouche virus (OROV), highlighting the potential for the emergence of new arboviral threats in Europe (European Centre for Disease Prevention and Control, 2024b, 2025b, 2025c).

Transmission of arboviruses typically occurs through blood-feeding arthropods, such as mosquitoes, ticks, and biting midges. Mosquito-borne viruses are generally transmitted when a

female mosquito feeds on a viremic vertebrate host, becomes infected, and later transmits the virus to another vertebrate host during a subsequent blood meal. For many arboviruses, the virus naturally circulates between mosquitoes and vertebrate amplifying hosts, in what is known as a sylvatic or enzootic cycle (Figure 1). Humans may become infected in a spillover event when bitten by a mosquito, but are often considered incidental or dead-end hosts, as viremia in humans does not reach levels high enough to infect new mosquitoes. This is the case for viruses such as WNV, TAHV, and snowshoe hare virus (SSHV). Alternatively, arboviruses can be directly transmitted in a mosquito-human-mosquito cycle without the need for a non-human vertebrate host as an intermediate. This so-called urban transmission cycle is characteristic of viruses such as dengue virus and chikungunya virus (Figure 1). Some arboviruses can circulate in both sylvatic/enzootic and urban transmission cycles, depending on the ecological conditions (Kuno and Chang, 2005; Foster and Walker, 2019). More rarely, some arboviruses can also be transmitted between humans via vertical transmission or through the use of substances of human origin (Pealer et al., 2003; Braga et al., 2025).

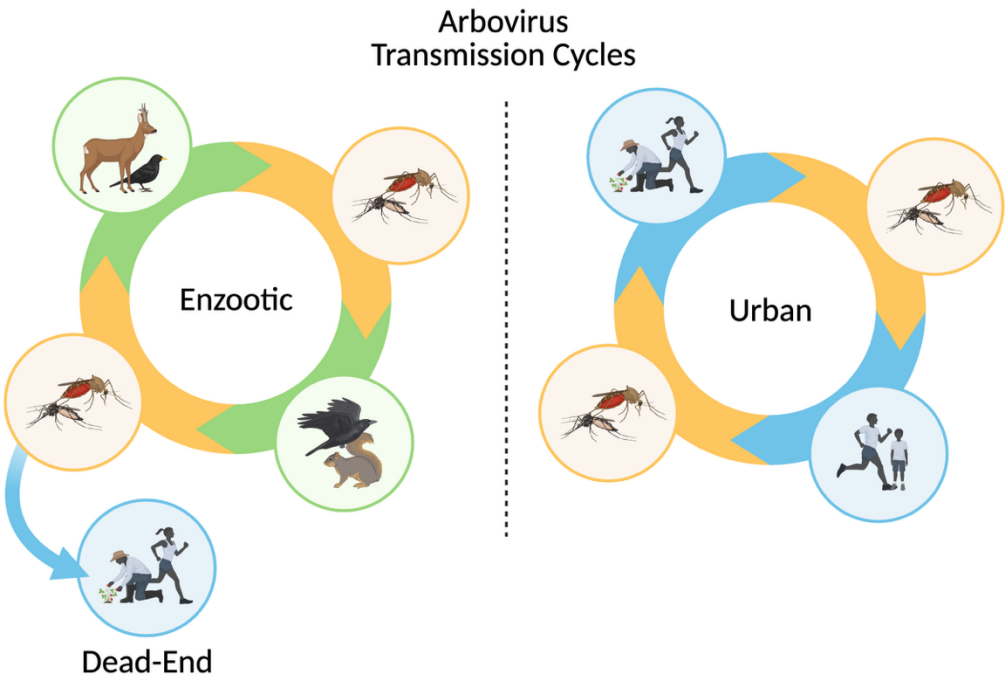

Figure 1 | Arbovirus transmission cycles. A schematic overview of the enzootic and urban arbovirus transmission cycles. On the left, in the enzootic cycle, arboviruses are transmitted between mosquitoes and vertebrate hosts. During a spillover event, the arbovirus can be transmitted to, for example, humans, which act as dead-end hosts. On the right, in the urban cycle, arboviruses cycle between mosquitoes and human hosts. Species shown are for illustrative purposes only and do not represent a specific arbovirus cycle. Figure created with BioRender.

Arboviruses are a diverse polyphyletic group of viruses, spanning multiple families and genera. Among these, members of the genera *Orthobunyavirus*, *Alphavirus*, and *Orthoflavivirus* include some of the most important mosquito-borne viruses and are repeatedly associated with human diseases and outbreaks (Foster and Walker, 2019). Understanding the transmission dynamics of these viruses is essential to assessing current and future arboviral risks.

1.1.1 Orthobunyaviruses

The genus *Orthobunyavirus*, within the family *Peribunyaviridae*, is the largest genus of arboviruses, with at least 80 species (Li et al., 2025). While several orthobunyaviruses are well characterized, many remain poorly studied despite their proven threat to human and animal health. Examples include TAHV (*Orthobunyavirus tahynaense*) and SSHV (*Orthobunyavirus khatangaense*), which have repeatedly caused human disease but remain neglected in research (Mravcová et al., 2023; Walker and Yuill, 2023). Both TAHV and SSHV belong to the California serogroup, which comprises 18 viruses with a broad geographic range that have been isolated from a wide range of mosquitoes of different genera (Evans and Peterson, 2019). In contrast to these neglected viruses, OROV (*Orthobunyavirus oropoucheense*) has recently attracted considerable attention due to a large outbreak in South and Central America, with travel-related cases worldwide (World Health Organization, 2024). Together, these viruses illustrate the neglected and emerging nature of this diverse arbovirus genus.

Like all orthobunyaviruses, these three viruses are enveloped, and their genomes consist of a tri-segmented, single-stranded, negative-sense RNA. The three segments are categorized as small, medium, and large, and encode all structural and non-structural viral proteins (Li et al., 2025). This genome segmentation can lead to genome reassortment when two related orthobunyaviruses infect the same cell simultaneously. During the viral replication, segments from the different parental viruses can be packaged together, resulting in a reassortant with a novel genomic combination. This process can lead to the emergence of a new virus, with increased or decreased virulence (Briese et al., 2013; Heitmann et al., 2021). The most prominent example is Ngari virus, which was revealed to be a reassortant between the large and small segments of Bunyamwera virus and the medium segment of Batai virus (Briese et al., 2013). While

both parental viruses are typically associated with mild illness, Ngari virus has caused outbreaks of hemorrhagic fever, including fatal infections (Briese et al., 2006). Therefore, the co-circulation of closely related othobunyaviruses may pose a unique risk that exceeds that of the individual viruses.

Several orthobunyaviruses were first identified in the mid to late 20th century, including the three covered in this thesis. OROV was isolated in 1955 in Trinidad from a febrile forest worker (Anderson et al., 1961). The first large documented outbreak occurred in 1961 in northern Brazil with 11,000 estimated cases (Pinheiro et al., 1981; Tilston-Lunel, 2024). Since then, outbreaks have become more frequent and geographically widespread in South America (Tilston-Lunel, 2024). Before the emergence of chikungunya virus and Zika virus in the 2010s, OROV was the second most common arbovirus in South America (World Health Organization, 2024). In 2024 to 2025, the largest OROV outbreak occurred, with more than 29,000 confirmed cases across 15 countries and territories in the Americas (Pan American Health Association and World Health Organization, 2025a, 2025b). This outbreak spread to previously non-endemic areas in the Americas and resulted in several travel-associated cases in Europe (European Centre for Disease Prevention and Control, 2024b; Tilston-Lunel, 2024; Pan American Health Association and World Health Organization, 2025a). The outbreak was likely fueled by climatic factors, specifically the climate phenomenon El Niño and the associated increase in temperatures (Fischer et al., 2025). TAHV and SSHV were discovered shortly after OROV. TAHV was first isolated in 1958 in former Czechoslovakia from *Ae. vexans* and *Ae. caspius,* presenting the first mosquito-borne virus identified in Europe (Bárdoš and Danielová, 1959). While TAHV is predominantly found in Europe, it has also been detected in parts of Asia and Africa. However, some of these reports are based on serological evidence and may reflect cross-reactivity with closely related viruses (Evans and Peterson, 2019). SSHV was isolated in 1959 in the northern United States of America from its namesake, the snowshoe hare (*Lepus americanus*) (Burgdorfer et al., 1961). It was later also detected in Canada and Russia (Evans and Peterson, 2019). Despite their early discovery, both SSHV and TAHV have received comparatively little attention in research.

Many orthobunyaviruses are maintained in an enzootic transmission cycle, as described above, involving arthropods and vertebrate hosts. For TAHV, lagomorphs, specifically hares, and large

mammals, such as deer and wild boars, are considered the most likely amplifying hosts (Bárdoš, 1969, 1969; Camp et al., 2021; Mravcová et al., 2023). For SSHV, the snowshoe hare and small rodents, such as squirrels, are regarded as the primary amplifying hosts (Walker and Yuill, 2023). While both TAHV and SSHV have been isolated from various field-caught mosquitoes, only a few laboratory vector competence studies have been conducted, leaving gaps in the understanding of their transmission potentials (Mravcová et al., 2023; Walker and Yuill, 2023). For TAHV, *Ae. vexans* from Europe and *Ae. albopictus* from China have been shown to be vector competent in laboratory studies (Bárdoš, 1969; Rödl et al., 1979; Cai et al., 2023). Experimental studies demonstrated that SSHV can be transmitted by *Ae. provocans, Ae. abserratus-punctori,* and *Ae. triseriatus* at temperatures between 19 °C and 23 °C (Heard et al., 1991; Hewlett et al., 1992). In addition, *Ae. aegypti* and *Culiseta inornata* showed vector competence at temperatures as low as 13 °C following intrathoracic injection (McLean et al., 1975). Considering the near-Arctic distribution of SSHV, the ability of the virus to be transmitted at low temperatures likely facilitates its persistence in northern ecosystems.

In contrast, OROV's primary vector is not a mosquito, but a biting midge (*Culicoides paraensis*), which can transmit the virus in a human-vector-human cycle without an intermediate host (Tilston-Lunel, 2024). While several vertebrate species, including sloths, are considered host animals, their epidemiological role is not well understood (Guagliardo et al., 2024; Tilston-Lunel, 2024). Studies investigating the vector competence of mosquitoes reported no to low transmission of OROV by *Culex* and *Aedes* mosquitoes prior to the 2024 outbreak (Gallichotte et al., 2025). The role mosquitoes may play in the transmission of OROV is likely low but incompletely understood (Ayres et al., 2025). It has been hypothesized that OROV might have shifted from a transmission cycle predominantly involving biting midges to one potentially involving mosquitoes, which could have contributed to the recent outbreaks (de Mendonça et al., 2021; U.S. Centers for Disease Control and Prevention, 2025). At the time the experiments for this thesis were conducted, no data were available on the vector competence of European mosquitoes. This left it unclear whether local mosquitoes, in combination with infected travelers introducing the virus, could enable autochthonous transmission and outbreaks in Europe.

The clinical manifestations of orthobunyavirus infections vary greatly. OROV infections are generally self-limiting in immunocompetent patients and are characterized by fever, headache, myalgia, arthralgia, diarrhea, vomiting, and extreme weakness. In severe cases, infections may progress to neurological disease. Recent evidence also suggests that OROV can be transmitted vertically in humans, which can lead to miscarriage and microcephaly (Braga et al., 2025; Pan American Health Association and World Health Organization, 2025a). In contrast, SSHV and TAHV infections are often asymptomatic or characterized by mild flu-like symptoms. However, in severe cases, infections can invade the central nervous system, causing meningitis or encephalitis (Bárdoš, 1969; Evans et al., 2019; Zhu et al., 2025). TAHV disease is also known as “Valtice fever”, and was first described in the 1960s (Bárdoš, 1969, 1974). Symptomatic cases of both TAHV and SSHV are most frequently reported in children, and cases of TAHV disease are most often observed in late summer and early autumn in Europe (Bárdoš, 1969; Mravcová et al., 2023; Walker and Yuill, 2023). Although only a few cases of SSHV disease in humans have been reported, primarily in Canada from the 1970s and 1980s, but also a more recent case in 2016, it is likely that the true burden of this virus is underestimated (Artsob et al., 1981, 1982; Embil et al., 1982, 1985; Meier-Stephenson et al., 2007; Lau et al., 2017). Serological studies further support this assumption that both SSHV and TAHV infections occur more frequently than reported. Studies investigating SSHV seroprevalence found a positivity rate of 42% and 6.8% in humans in Alaska (United States of America) in the 1980s and 1990s (Zarnke et al., 1983; Walters et al., 1999). Similarly, studies conducted on TAHV in France, Italy, and former Czechoslovakia found low to high seropositivity rates in the 1960s, with the highest reported rate being 90% (Bárdoš, 1969, 1974; Heinz and Asmera, 1972; Hubalek et al., 2014). More recent studies showed that 0.3% of blood donors in the Alps (Italy, Austria) and approximately 10% of patients with neuroinvasive disease of unknown etiology in Croatia, tested positive for TAHV antibodies (Sonnleitner et al., 2014; Stevanovic et al., 2022; Vilibić-Ćavlek et al., 2022). Together, the outbreak of OROV and the uncertain burden of SSHV and TAHV highlight the public health relevance of orthobunyaviruses.

1.1.2 Barmah Forest virus

Barmah Forest virus (BFV, *Alphavirus barmah*) is a member of the family *Togaviridae*, genus *Alphavirus*. Other members of this genus include chikungunya virus and Sindbis virus. Alphaviruses are enveloped, and their genome is organized as a single-stranded, positive-sense RNA (Gould et al., 2010). BFV was first isolated from *Cx. annulirostris* in the Barmah Forest, Australia, in 1974 (Marshall et al., 1982). The first symptomatic human cases were documented in 1984, and the virus was first isolated from a patient shortly after (Boughton et al., 1988; Phillips et al., 1990). It has been estimated that approximately 0.23% of the population in Queensland, Australia, got infected with the virus annually (Phillips et al., 1990).

BFV is the second most common cause of arboviral disease in Australia, after Ross River virus, and infections are notifiable (Parliament of Australia, 2018). More than 20,000 confirmed or probable BFV cases have been reported over the last two decades in Australia, with a peak of over 4,000 cases in 2013 (Australian Centre for Disease Control, 2026). The virus was previously considered endemic only to Australia, however, one case has been reported in a patient from Papua New Guinea with no travel history. This BFV strain showed substantial divergence from Australian strains, with a potential introduction dating back to the early 1900s. These findings support the hypothesis that BFV has long been present outside of Australia (Caly et al., 2019). As with other arboviruses, BFV cases are positively associated with increased precipitation, likely because rainfall favors mosquito reproduction. A study investigating seroconversion after heavy precipitation in Australia showed evidence of recent infections with BFV in up to 1.7% of blood donors (Faddy et al., 2015).

BFV is maintained in an enzootic transmission cycle between vertebrate hosts and mosquitoes as vectors and reservoirs. Marsupials, particularly kangaroos, are believed to be important reservoirs and amplifying hosts for the virus. However, antibodies against BFV have been detected in several other wildlife and livestock species, demonstrating that vertebrates other than marsupials are also involved in the transmission cycle (Taylor-Robinson, 2024). Under epidemic conditions, humans are hypothesized to also serve as amplifying hosts, potentially enabling a temporary urban transmission cycle (Russell and Dwyer, 2000; Australian Centre for Disease Control, 2025).

Several mosquito species have been implicated in the transmission of BFV, both through virus isolation from field-caught mosquitoes, and through laboratory vector competence studies. The virus has been isolated from several mosquito genera, including *Aedes*, *Culex, Coquillettidia*, and *Anopheles* (Marshall et al., 1982; Lindsay et al., 1995). Importantly, the isolation of a virus from a mosquito does not necessarily prove that the mosquito is infected, as the virus may originate from a recent blood meal. Laboratory studies have shown that *Ae. vigilax* and *Cx. annulirostris* exhibit a medium to high vector competence for BFV (Boyd and Kay, 1999, 2000; Ryan and Kay, 1999). The mosquito species most often associated with BFV are *Ae. camptorhynchus* and *Ae. vigilax* in coastal regions, and *Cx. annulirostris* in inland areas. All three species are primarily found in Australasia (Russell, 1995; Doggett et al., 1999; Russell and Dwyer, 2000; Taylor-Robinson, 2024).

The clinical presentation of BFV infections resembles that of other arthritogenic alphaviruses. Symptomatic BFV infections include arthralgia, arthritis, and myalgia, accompanied by fever and rash, with joint pain sometimes lasting several months (Flaxman et al., 1998; Australian Centre for Disease Control, 2025). These arthritogenic symptoms are similar to those of other Old-World alphaviruses, such as chikungunya virus and Sindbis virus, and resemble those of Ross River virus, which is also endemic in Australia (Gould et al., 2010). The broad host and vector range of BFV, together with increasing travel and trade, highlights its potential impact on public health in Australia and beyond.

1.1.3 West Nile virus

WNV (*Orthoflavivirus nilense*) is a member of the family *Flaviviridae* and the genus *Orthoflavivirus*. Like all members of this family, WNV is enveloped and has a single-stranded, positive-sense RNA genome (International Committee on Taxonomy of Viruses, 2026). WNV was first isolated from a febrile patient in the West Nile district, Uganda, in 1937 (Smithburn et al., 1940; Sejvar, 2003). Shortly after, the arboviral nature of WNV was first investigated, and it was found that the virus could be transmitted by mosquitoes, specifically *Ae. albopictus* (Philip and Smadel, 1943). The first outbreaks were documented in the 1950s in Israel and Egypt, which

further improved the understanding of the epidemiology and clinical characterization of the virus (Murgue et al., 2001; Sejvar, 2003).

Currently, nine distinct WNV lineages have been described, of which lineages 1 (L1) and 2 (L2) are most frequently associated with human disease. WNV-L1 was first detected in Europe in the 1960s and caused multiple outbreaks in countries around the Mediterranean basin. WNV-L2 was detected for the first time in 2004 in Europe, where it subsequently spread through Southern and Central Europe and eventually became the dominant lineage (Camp and Nowotny, 2020; Lu et al., 2024). In 2018, the largest European WNV epidemic to date occurred, with 1,548 reported human cases in 11 countries. In recent years, WNV transmission in Europe has again remained above average (Figure 2). While WNV-L2 is the dominant WNV lineage, WNV-L1 continues to circulate and cause outbreaks in Europe and is distributed more broadly around the globe (Mencattelli et al., 2021; Lu et al., 2024; Silverj et al., 2024). In Germany, WNV was first detected in 2018 in birds and horses, and in 2019 in humans. Since then, up to a few dozen human cases have been reported annually, mostly in eastern regions (Friedrich-Loeffler-Institut, 2025; Robert Koch Institut, 2025). The true number of infections is likely much higher due to underreporting and asymptomatic cases.

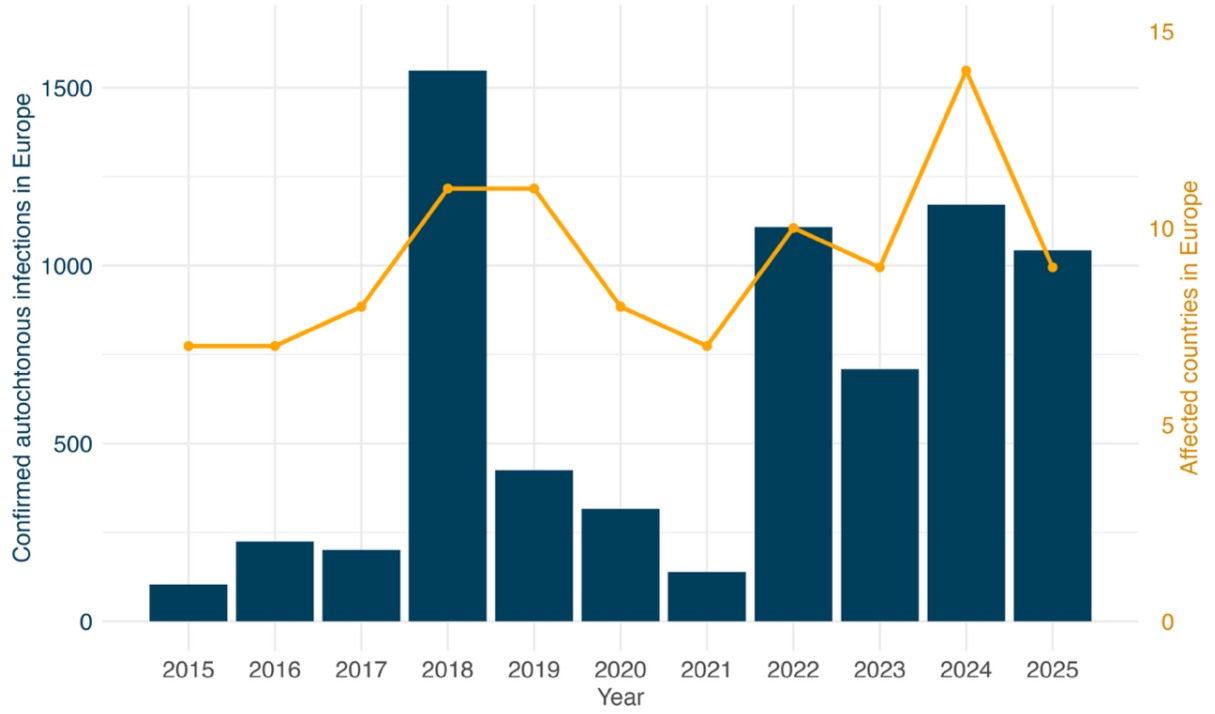

Figure 2 | Development of WNV in Europe over time. Temporal development of confirmed autochthonous human WNV infections in the European Union and European Economic Area (blue bars, left y-axis), and the number of countries that reported the infections (yellow line, right y-axis). Data from (European Centre for Disease Prevention and Control, 2017, 2019a, 2019b, 2019c, 2021a, 2021b, 2022, 2023a, 2024a, 2024c, 2025e)

WNV is maintained in an enzootic transmission cycle between mosquitoes as vectors and reservoirs, and birds as amplifying hosts. Vertebrates, such as humans and horses, are considered incidental dead-end hosts, as their viremia is not sufficiently high to cause onward infection in mosquitoes (Foster and Walker, 2019). The primary vectors are mosquitoes of the genus *Culex*, with the most important being *Cx. pipiens* s.s. in Europe, however, *Cx. torrentium* is increasingly recognized as an important vector (Hesson et al., 2014; Foster and Walker, 2019; Jansen et al., 2019, 2023). Bird species can develop highly varying levels of viremia, ranging from less than 10^4 to more than 10^8 plaque-forming units (PFU)/mL. Such differences of several orders of magnitude influence the probability that mosquitoes become infected when feeding on an avian host (VanDalen et al., 2013; Pérez-Ramírez et al., 2014). In contrast, viremia in humans is typically low, with reported levels below 80 PFU/mL, and is therefore insufficient to enable onward transmission (Pealer et al., 2003). Importantly, these data are derived from asymptomatic blood donors and likely do not reflect peak viremia levels during acute infections. In comparison, experimental infections in horses have shown peak viremias of approximately 460 PFU/mL

(Bunning et al., 2002). Nevertheless, a recent report of a patient with a co-infection of WNV lineages 1 and 3 detected an unusually high viral load of $10^{5.5}$ PFU equivalents/mL, raising the question of whether human-mosquito-human transmission could occur under exceptional circumstances (Davis et al., 2024). These differences highlight the importance of understanding which levels of viremia are sufficient to infect different mosquito species, also referred to as the minimal infectious dose required for transmission (MID).

Laboratory studies have extensively investigated the vector competence of various mosquito species from Europe for WNV, with most studies focusing on WNV-L1. In general, *Cx. pipiens* s.s. has been shown to transmit WNV-L1 at low to high transmission rates (Fortuna et al., 2015; Jansen et al., 2019, 2023; Martinet et al., 2023). *Culex torrentium* showed higher transmission efficiencies for WNV-L1, up to 33%, in studies comparing it to *Cx. pipiens* s.s., highlighting that this often neglected species might play an important role in WNV transmission (Jansen et al., 2019, 2023). While *Ae. albopictus* is not regarded as a major WNV vector, experimental studies demonstrated moderate transmission rates for WNV-L1 (Fortuna et al., 2015; Martinet et al., 2023). In general, fewer studies have investigated WNV-L2. Nevertheless, *Cx. pipiens* s.s. has been demonstrated to be vector competent for this lineage, with reported transmission efficiencies ranging from low to high (Brustolin et al., 2016; Vogels et al., 2017a, 2017b; Holicki et al., 2020b). *Aedes albopictus* was likewise shown to be vector competent for WNV-L2, albeit at a low transmission efficiency (Brustolin et al., 2016; Holicki et al., 2020b). Higher incubation temperatures and longer incubation periods are often associated with increased transmission for both lineages. However, not all studies report consistent results, leaving doubts on the generalizability of these associations (Fortuna et al., 2015; Brustolin et al., 2016; Vogels et al., 2017a, 2017b; Jansen et al., 2019, 2023; Holicki et al., 2020b; Martinet et al., 2023).

In general, comparing different vector competence studies remains challenging due to the large degree of experimental variation between studies and laboratories. Factors that frequently vary include the blood source used for infection, incubation temperature and period, whether the temperature is fluctuating or constant, and the methods used to calculate different rates. While a minimum data standard has been proposed, experimental studies remain largely unstandardized (Wu et al., 2022).

Human infection with WNV can lead to a wide spectrum of clinical manifestations, ranging from asymptomatic to severe neurological disease and death. Approximately 20% of infected humans develop symptomatic disease, referred to as West Nile fever, which is characterized by fever, myalgia, fatigue, and headache. In about 1% of infections, the virus causes West Nile neuroinvasive disease, which can manifest as meningitis, encephalitis, and acute flaccid paralysis, with case fatality rates of up to 17%. In addition to humans, horses are also frequently affected by WNV, with approximately 10% of infections resulting in neurological symptoms (European Centre for Disease Prevention and Control, 2023b). Vaccines for horses have been available since the early 2000s, whereas no vaccine for humans has progressed beyond phase II clinical trials, and no specific antiviral therapy is currently available, leaving clinical management limited to supportive care (Ronca et al., 2021).

The widespread distribution of competent mosquito vectors across Europe, the proven ability of WNV to cause large outbreaks, and the consistently high number of human cases highlight the continued public health importance of WNV in Europe, and emphasize the need to better understand its transmission dynamics.

1.2 Interactions of Mosquitoes and Arboviruses

1.2.1 Infection and Transmission Barriers in Mosquitoes

Arboviruses are not simply passively carried by mosquitoes before being transmitted to a new host. Instead, successful transmission requires that the virus infects, replicates, and disseminates within the mosquito. This process involves a series of complex interactions between the virus and the mosquito (Hardy et al., 1983). Typically, a mosquito becomes infected when it takes an infectious blood meal from a viremic host. The ingested blood meal reaches the midgut, where the virus establishes an infection in the epithelial cells. After replication, viral particles exit the midgut cells and enter the hemolymph to disseminate throughout the mosquito's body. Following replication in secondary tissues, the virus infects the salivary glands, where it replicates further. During a subsequent blood meal, the virus can then be released into the saliva and transmitted to a new vertebrate host (Kramer and Ciota, 2015). Although this infection sequence may appear straightforward, in reality, arboviruses must overcome several physiological and anatomical

barriers within the mosquito before onward transmission is possible. Four major barriers are commonly described: the midgut infection barrier, the midgut escape barrier, the salivary gland infection barrier, and the salivary gland escape barrier (Figure 3). These barriers include dose-independent mechanisms, which prevent the virus from passing through the barrier regardless of the ingested dose, and dose-dependent mechanisms, for which the likelihood of passage increases with viral dose (Hardy et al., 1983; Franz et al., 2015). Together, these barriers and processes are among the key factors influencing the vector competence of a mosquito for a given arbovirus.

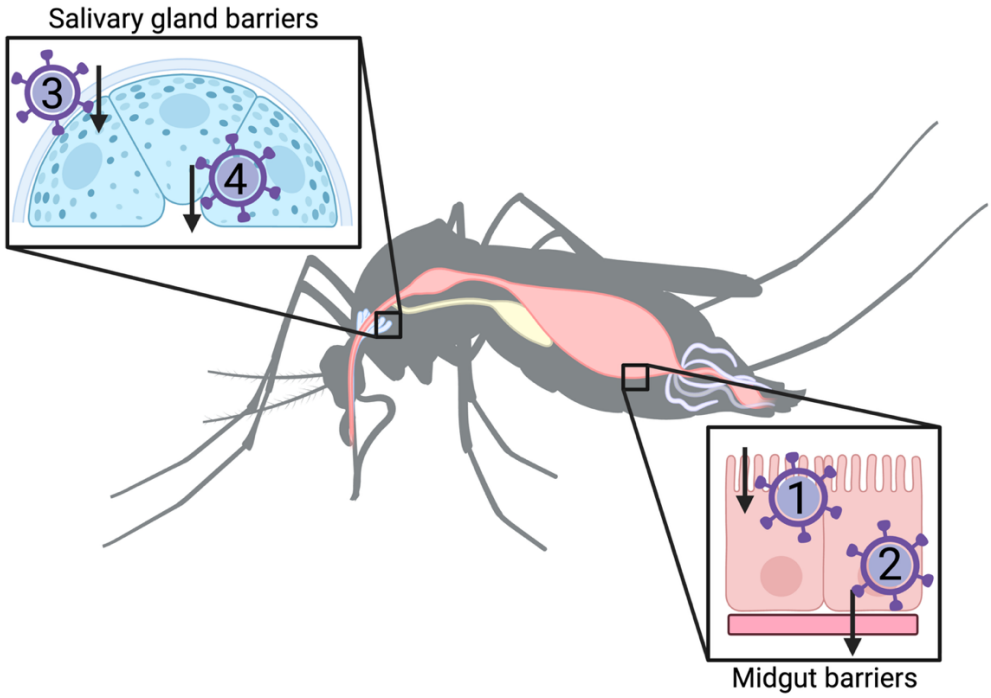

Figure 3 | Infection and transmission barriers within the mosquito. The following barriers must be crossed by an arbovirus to be successfully transmitted: (1) Midgut infection barrier: The virus must enter midgut epithelial cells and establish an infection. (2) Midgut escape barrier: The virus must exit epithelial cells and cross the basal lamina. (3) Salivary gland infection barrier: The virus must cross the basal lamina, infect salivary gland cells, and replicate within them. (4) Salivary gland escape barrier: The virus must be released into the apical cavity of the salivary glands, from where it is expelled with saliva. Figure created with BioRender.

The first barrier is the mosquito's midgut (mesenteron). The midgut consists of a single layer of epithelial cells that is surrounded on the basal side by an extracellular matrix, known as basal lamina (Kramer and Ciota, 2015). To establish an infection, the virus must first overcome the midgut infection barrier by entering the cells and replicating within them. It is generally assumed that arboviruses enter the epithelial cells via receptor-mediated endocytosis, and therefore, the

absence of a suitable receptor presents a dose-independent barrier that can prevent infection (Franz et al., 2015). The virus must then successfully replicate within the midgut epithelial cells, which represents the second layer of the midgut infection barrier. At this stage, the mosquito's immune response may suppress viral replication and prevent infection (Franz et al., 2015).

If a virus has successfully established an infection in the midgut, the next step is the dissemination from the midgut epithelial cells into the hemolymph. This process is limited by the midgut escape barrier, which is mainly formed by the basal lamina. This extracellular matrix has an exclusion size smaller than most arboviruses, making passive diffusion of virions unlikely (Franz et al., 2015). Several hypotheses have been proposed to explain how viruses overcome this barrier. One proposed mechanism involves structural changes of the basal lamina following a blood meal. Dilation of the basal lamina during blood feeding may create temporary pores large enough for virions to pass through (Carpenter and Clem, 2023; Ferdous et al., 2024). Alternative routes include escape through the foregut, a region where the basal lamina may be more porous, or infection of the tracheal system, a tubular network for gas exchange that penetrates and spans the basal lamina. Viruses likely overcome the midgut escape barrier through a combination of these mechanisms, which may vary depending on the specific virus-mosquito pairing (Hardy et al., 1983; Carpenter and Clem, 2023). Following the successful escape from the midgut, arboviruses disseminate through the hemolymph and can infect secondary tissues and subsequently enter the next stages of infection (Franz et al., 2015).

The final steps required for successful transmission are the infection of the salivary glands and the subsequent release of viral particles into the saliva. The salivary gland infection barrier primarily consists of the basal lamina surrounding the glands. It has been suggested that the trachea system and nerve cells might aid the virus in overcoming this basal lamina. Additionally, compatibility between viral and cellular surface molecules is required for successful infection (Franz et al., 2015). Following this infection, the virus must be released into the apical cavity of the glands, from where it can be expelled during blood feeding. Factors preventing this release are referred to as the salivary gland escape barrier. Although viral proteins appear to influence this process, the underlying molecular mechanisms remain poorly understood (Stauft et al., 2022). Finally, infectious viral particles are transmitted to a new vertebrate host via the mosquito's

saliva, which contains immunomodulatory molecules that can enhance the arbovirus infectivity (Schneider and Higgs, 2008).

Overall, these barriers include both dose-dependent and dose-independent components. Therefore, specific infection and transmission thresholds likely exist for each virus-mosquito combination, meaning that different minimum viral doses may be required for a mosquito to become infected and to subsequently transmit the virus (Hardy et al., 1983; Franz et al., 2015; Carpenter and Clem, 2023).

1.2.2 Vectorial Capacity and Vector Competence

The relationship between arboviruses and their mosquito vectors is shaped by a complex interplay of intrinsic and extrinsic factors. In general, extrinsic factors, such as environmental conditions, determine whether a mosquito comes into contact with the vertebrate host, and therefore with the arbovirus. These factors include the frequency of mosquito-host contact, the lifespan of a mosquito, and the density of mosquitoes relative to the host population. In contrast, intrinsic factors are largely traits that influence vector competence, such as the inherent ability of a mosquito to become infected after ingesting an infectious blood meal and subsequently transmit the virus. To describe this multifaceted relationship between vectors, hosts, and viruses, the concepts of vector competence and vectorial capacity are commonly used (Hardy et al., 1983; Kramer and Ciota, 2015).

Vector competence describes the inherent ability of a mosquito to become infected with and subsequently transmit an arbovirus (Kramer and Ciota, 2015). It is typically evaluated in laboratory studies using one of several approaches. The most common method is forced salivation, in which the individual mosquito's saliva is collected by inserting the proboscis into a capillary tube (Figure 4). The presence of the virus is then determined by detecting infectious viral particles or viral RNA (Heitmann et al., 2018; Krambrich et al., 2024). Alternatively, peripheral tissue (e.g., legs or salivary glands) is screened for the presence of the virus, assuming that a disseminated infection corresponds to transmissibility (Gloria-Soria et al., 2022). An ethically and logistically challenging method, most commonly used in the earlier studies, is to allow mosquitoes

to feed directly on laboratory animals, typically mice, and later assess the animals for infection (Rödl et al., 1979; Cai et al., 2023).

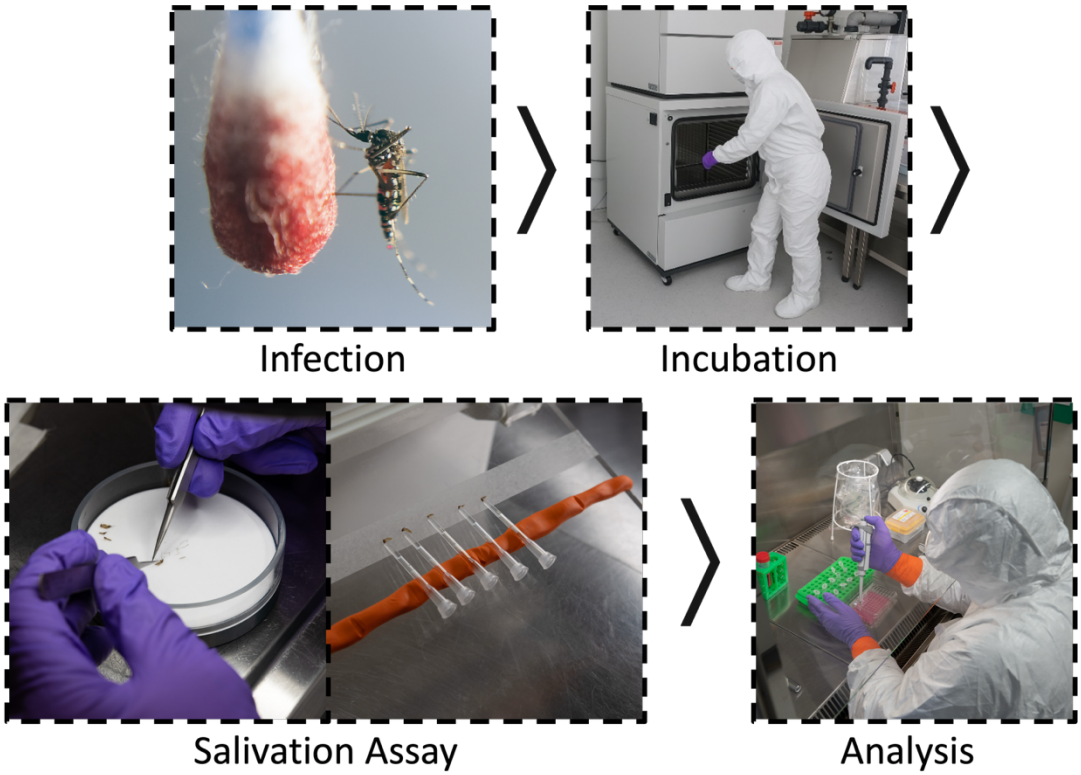

Figure 4 | Exemplary workflow of an experimental vector competence study using the forced salivation approach. Infection: Mosquitoes are exposed to a blood meal containing the virus of interest. Incubation: Blood-fed females are incubated for a defined period under controlled temperature conditions. Salivation assay: Typically, mosquitoes are immobilized, and their proboscis is inserted into a capillary tube to collect saliva. Analysis: Mosquito bodies and saliva are analyzed for the presence of the virus. These steps enable the determination of infection rates and transmission efficiencies. Importantly, variations in this workflow exist between studies, which can influence the observed outcomes. This figure depicts the work in the biosafety level 3 insectary at the Bernhard Nocht Institute for Tropical Medicine. Images © BNITM.

Vectorial capacity describes the potential of a mosquito to transmit a pathogen as the number of potentially infectious bites per time, given the introduction of an infectious mosquito into a susceptible population. Vectorial capacity (VC) can be expressed by the following formula: $VC = ma^2bp^n/-\ln p$, which is described by (m) the vector density, (a) the vector biting rate, (p) the probability of vector survival, (n) the pathogen extrinsic incubation period, and (b) the vector competence (Figure 5) (Macdonald, 1961; Garrett-Jones, 1964; Kramer and Ciota, 2015). Importantly, this equation illustrates that even mosquito populations with a relatively low vector competence can sustain an outbreak if other parameters compensate for it. One such example

has been recorded when a population of *Ae. aegypti* with a transmission efficiency of less than 2% was associated with a large yellow fever epidemic in West Africa, likely facilitated by high vector density and biting rates (Miller et al., 1989). Although three parameters, (m) the vector density, (a) the vector biting rate, and (p) the probability of vector survival, appear to solely describe a mosquito population, they can also be influenced by an arboviral infection. Studies have shown that infection with an arbovirus can alter mosquito behavior, such as host seeking and blood feeding activity, and may also change mosquito survival (Lambrechts and Scott, 2009; Maire et al., 2024). Therefore, these parameters cannot always be considered independently of the virus involved.

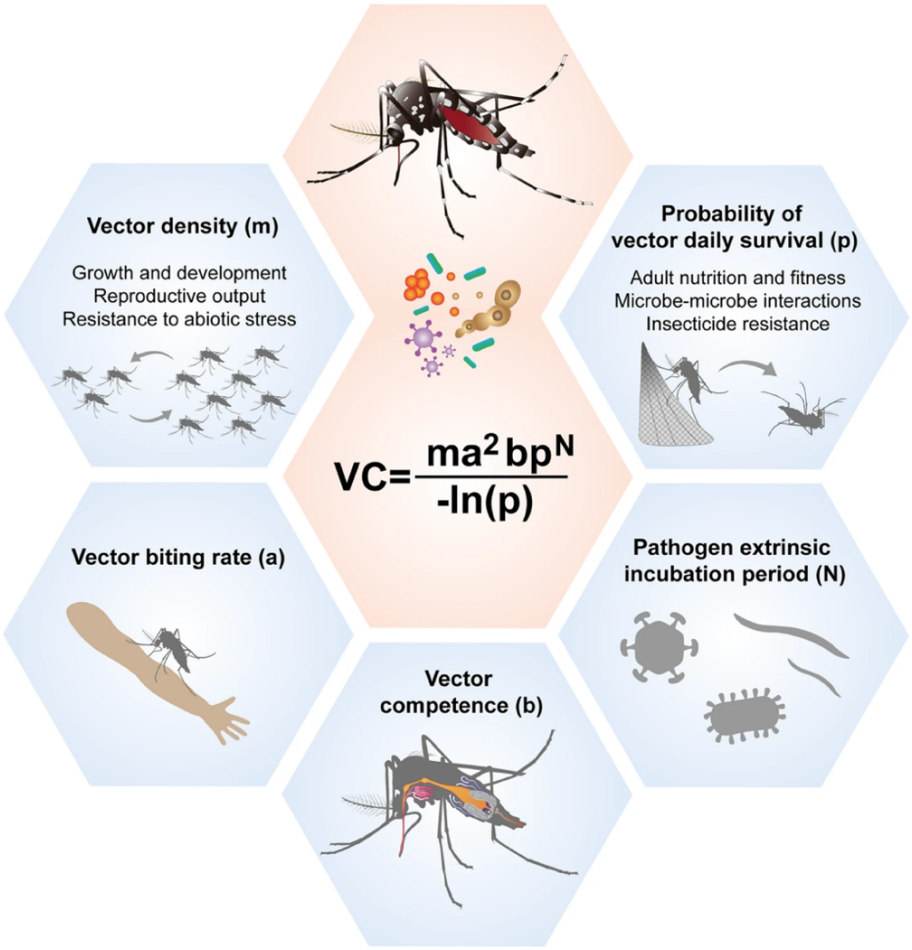

Figure 5 | Determinants and equation of vectorial capacity. Schematic representation of the key parameters influencing the vectorial capacity (VC). Figure from (Cansado-Utrilla et al., 2021)

Environmental conditions strongly influence vector competence and other parameters of the vectorial capacity equation. Among these environmental factors, temperature is the most influential. As mosquitoes are ectotherm organisms, temperature affects multiple aspects of their biology, including developmental time, survival, and biting frequency (Ciota et al., 2014; Foster and Walker, 2019). Increased temperatures generally lead to faster larval and pupal development, often leading to more generations per season and smaller adults. However, once an optimum is surpassed, higher temperatures lead to increased mortality (Ciota et al., 2014; Foster and Walker, 2019; Doeurk et al., 2025). The temperature during larval development has also been shown to influence the vector competence of adult mosquitoes, although the direction and magnitude of this influence are not yet fully understood (Westbrook et al., 2010; Muturi et al., 2012).

Additionally, temperature strongly influences viral replication and therefore affects factors such as the vector competence and the extrinsic incubation period. Higher temperatures are often associated with a shorter extrinsic incubation period, defined as the time between a mosquito ingesting a virus and becoming infectious (Hardy et al., 1983; Foster and Walker, 2019). A recent review reported that for several important arboviruses, including WNV, the extrinsic incubation period generally decreases with increasing temperature, although the strength of this effect varies between viruses and mosquito species. For other viruses, such as Ross River virus and Usutu virus, no consistent temperature-dependent trend has been observed (Hartner et al., 2025). Overall, the extrinsic incubation period depends strongly on the specific virus-mosquito combination. Similarly, for many arboviruses, increasing temperature is often associated with higher transmission (Foster and Walker, 2019). This association has been demonstrated for several virus-mosquito pairings, including multiple WNV-mosquito combinations (Vogels et al., 2017b; Jansen et al., 2023). However, this relationship is not consistent across all systems. For example, one study showed that increased temperature led to higher WNV transmission in *Cx. pipiens* biotype *pipiens*, but not in the closely related biotype *molestus* (Vogels et al., 2016). Similarly, a recent systematic review on the influence of temperature on the transmission of dengue virus, chikungunya virus, and Zika virus found that, although the majority of studies reported a positive association between temperature and transmission, several studies found no, or even negative correlations (Delrieu et al., 2023). Importantly, laboratory studies are most often

conducted under constant temperature, whereas in natural environments, temperatures fluctuate. These fluctuations have been shown to influence vector competence and other mosquito traits, with effects that vary depending on the virus-mosquito combination (Lambrechts et al., 2011; Carrington et al., 2013a, 2013b; McGregor et al., 2021).

Overall, considering both the mosquito and virus biology, transmission does not seem to linearly increase with temperature, but rather it often follows a unimodal relationship, peaking at a thermal optimum and declining at both lower and higher temperatures. For example, the thermal optimum is estimated to be around 24 °C for WNV transmission by various *Culex* species, and higher, between 26 °C and 29 °C, for dengue virus, chikungunya virus, and Zika virus transmission by different *Aedes* species in nature (Mordecai et al., 2019). However, these relationships are primarily based on a limited number of well-studied viruses and mosquito taxa, leaving neglected arboviruses and less-studied mosquito taxa underrepresented.

1.3 Mosquitoes – Key Vectors of Arboviruses

Mosquitoes, the family *Culicidae*, are the most important arbovirus vectors worldwide. They are divided into two subfamilies, *Anophelinae* and *Culicinae*, which together encompass approximately 3,500 recognized species. However, only a fraction of these are relevant for the transmission of arboviruses, primarily, those of the genera *Culex* and *Aedes,* which are the most widespread and epidemiologically important arbovirus vectors worldwide (Foster and Walker, 2019; Aardema et al., 2022).

1.3.1 *Culex* as Vectors

In Europe, *Cx*. *pipiens* s.s. and *Cx. torrentium* are some of the most common mosquito species (Hesson et al., 2014; Aardema et al., 2022). The term *Cx. pipiens* can be ambiguous, as it might either refer to the single species or to the *Cx. pipiens* species complex. This complex consists of four closely related species and one subspecies: *Cx. pipiens pallens* (subspecies), *Cx. quinquefasciatus*, *Cx. australicus*, *Cx. globocoxitus*, and *Cx. pipiens* sensu stricto (s.s., indicating the species) (Aardema et al., 2022). The species *Cx. pipiens* s.s. comprises two biotypes, *pipiens* and *molestus*, which are not reliably morphologically distinguishable. However, they differ

markedly in their traits and ecology. For example, *Cx. pipiens* biotype *molesuts* is autogenous, meaning females can lay eggs without a preceding blood meal. Despite their differences, the biotypes are often not differentiated in studies (Foster and Walker, 2019; Jansen et al., 2019, 2022).

A similar species, *Cx. torrentium*, further complicates the correct identification. Females of *Cx. torrentium* cannot be distinguished morphologically from those of *Cx. pipiens* s.s., and are therefore often collectively labeled as *Cx. pipiens* in research, despite their substantial ecological and vector competence differences (Wehmeyer et al., 2024). Both species are widely distributed in Europe and often share habitats. However, *Cx. pipiens* s.s. dominates south of the Alps, while *Cx. torrentium* is more abundant in the north, with a transition zone in central Europe where both occur at similar levels (Hesson et al., 2014). They also differ in their ecological traits, with larvae of *Cx. torrentium* being found more often in artificial bodies of water, and *Cx. torrentium* appearing earlier in the growing season than *Cx. pipiens* s.s. (Hesson et al., 2014).

Importantly, these three taxa, *Cx. pipiens* biotypes *pipiens* and *molestus*, as well as *Cx. torrentium*, differ in their vector competence, highlighting the need for their accurate distinction (Jansen et al., 2019, 2022). All three taxa show an opportunistic feeding behavior, taking blood meals from both mammals and birds (Wehmeyer et al., 2024). This broad host range makes them important bridge vectors for arboviruses.

1.3.2 *Aedes* as Vectors

In Europe, both native and invasive *Aedes* species are present. While some native species are implicated in arbovirus transmission, such *Ae. vexans* for TAHV, most research focuses on the potential threat posed by invasive *Aedes* species (Lühken et al., 2023; Mravcová et al., 2023). To date, four invasive *Aedes* species have become established in Europe: *Ae. albopictus*, *Ae. aegypti*, *Ae. japonicus,* and *Ae. koreicus*. Of these, *Ae. albopictus* is most widely distributed, currently established in 16 countries within Europe (European Centre for Disease Prevention and Control, 2025d). This species originates from tropical and subtropical rural regions in Asia, with the first recorded introduction into Europe being in 1979 in Albania (Adhami and Reiter, 1998; Kraemer et al., 2019). With ongoing climate change, *Ae. albopictus* is predicted to expand further, both

geographically and in the duration of its seasonal activity in Europe (Kraemer et al., 2019). This ongoing spread of *Ae. albopictus* is of great public health concern, as the species is a competent vector of multiple arboviruses, including chikungunya virus and dengue virus (Walker and Yuill, 2023). *Aedes albopictus* exhibits an opportunistic feeding behavior with a preference towards humans, making it an efficient arbovirus vector. This preference, however, might partly reflect a sampling bias, as many studies are conducted in urbanized areas, where mosquitoes are in high contact with humans (Garamszegi, 2024; Cebrián-Camisón et al., 2020). Furthermore, long-established populations show a broader host range than recently introduced ones, suggesting a higher potential as a bridge vector for arboviruses (Garamszegi, 2024).

Together, its wide distribution and vector competence highlight the public threat posed by this species, and the importance of further investigating the relationship of *Ae. albopictus* and arboviruses.

2 Objectives

For many arboviruses, vector competence, transmission dynamics, and risks remain poorly researched. Understanding how key determinants shape transmission and whether exotic arboviruses can be transmitted outside their endemic settings is increasingly important.

In this thesis, I aimed to assess the transmission potential of selected exotic arboviruses (SSHV, OROV, BFV) by determining the vector competence of mosquitoes from Europe (Papers I, II, V). SSHV and BFV were selected to represent distinct ecological backgrounds, whereas OROV was investigated in response to the large outbreak in the Americas. In addition, I aimed to assess the vector competence of mosquitoes from Europe for the endemic TAHV, which remains largely unstudied despite the virus's long-established presence in Europe (Paper III).

Vector competence itself is influenced by several factors, one of the most important being the viral titer of an infectious blood meal, as mosquitoes encounter highly variable levels of host viremia in nature. To investigate how the viral titer affects transmission, I aimed to determine the minimum infectious dose required for WNV transmission across different mosquito species, with a particular focus on distinguishing between the two major WNV lineages, given their relevance in Europe (Paper IV).

Taken together, this thesis aimed to provide a comprehensive assessment of how key parameters, such as mosquito species, temperature, virus, and infectious dose, influence vector competence. In doing so, it aims to deliver essential insights to improve risk assessments, surveillance, and mitigation strategies in Europe.

3 Discussion

Arboviruses continue to emerge and expand their geographic range, driven by factors such as climate change, urbanization, globalization, and the associated spread of invasive mosquito species. Despite this public health threat, the vector competence of many arboviruses remains poorly understood. In fact, more than 90% of potential virus-mosquito combinations remain untested (Chen et al., 2023). Vector competence is influenced by a complex interplay between arboviruses, mosquitoes, and the environment. In this thesis, I investigated the transmission dynamics of various mosquito species across a range of arboviruses with different backgrounds, and assessed the factors determining vector competence. This included the three orthobunyaviruses OROV, SSHV, and TAHV, the alphavirus BFV, and the orthoflavivirus WNV.

3.1 Infection Does Not Equal Transmission

While infection of a mosquito is a prerequisite for transmission, it is not equivalent to vector competence (Franz et al., 2015). Nevertheless, the detection of viruses in wild-caught mosquitoes is sometimes interpreted as evidence for vector competence. Such interpretations should be treated with caution, as they overlook the biological barriers within the mosquito that must be overcome for successful transmission.

Across all five investigated viruses, infection was observed in nearly all mosquito species, whereas transmission was detected by less than half of the virus-mosquito combinations. Specifically, out of 29 combinations tested, 28 (97%) resulted in infection, whereas just 12 (41%) combinations led to transmission. Notably, even OROV, a virus traditionally associated with biting midges rather than mosquitoes, was able to infect several mosquito species, but was only transmitted by one (*Ae. albopictus*) (Paper II). Similarly, SSHV, TAHV, and BFV showed broad infection across all mosquito species tested. However, this broad susceptibility did not translate into equally broad transmission. While SSHV was transmitted by species of both *Aedes* and *Culex*, transmission of TAHV and BFV was restricted to *Aedes* mosquitoes (Papers I, III, V).

Importantly, several virus-mosquito combinations showed low transmission efficiencies of 2-4%. Although these values might, at first glance, seem insignificant, even such limited transmission

efficiencies are of importance. For example, an *Ae. aegypti* population, which caused a major yellow fever virus outbreak in Africa, exhibited a transmission efficiency of just 2% (Miller et al., 1989). Therefore, low transmission efficiencies do not negate epidemiological relevance, particularly when other factors, such as vector density and biting rate, are high.

These findings highlight an important pattern. While infections occur frequently, transmission remains restricted, suggesting that the midgut infection barrier is often overcome, and subsequent barriers, such as the midgut escape barrier and the salivary gland infection and escape barriers, likely present critical bottlenecks (Franz et al., 2015). Importantly, the detection of transmission via forced salivation, the method used in this thesis, might underestimate the transmission of some virus-mosquito combinations (Gloria-Soria et al., 2022). Therefore, the transmission efficiencies reported here may represent conservative estimates. To determine the key limiting barriers, future studies could separately analyze the mosquito body, extremities, salivary glands, and saliva.

A key factor that may determine whether the final barriers of transmission are overcome, and therefore a source for the discrepancy between infection and transmission, is the viral load within the mosquito (Franz et al., 2015). For WNV, the odds of transmission doubled with every tenfold increase in body titer. In addition, the viral RNA concentrations in mosquito bodies formed two distinct clusters: a low- and a high-titer group. Nearly all mosquitoes that transmitted WNV belonged to the high-titer group (Figure 1 and Table 2 in Paper IV). A similar pattern was observed for OROV and TAHV, where transmitting mosquitoes showed some of the highest body titers, however, due to the limited sample size, no statistical conclusions could be drawn (Paper II, Figure 1 in Paper III). While a wide range of body titers was also detected for BFV, transmission was not consistently associated with high body titers (Figure 1 in Paper V).

A recent study reported a comparable correlation between body titers and transmission of WNV by *Cx. theileri*, supporting that this relationship extends across mosquito species (Burgas-Pau et al., 2025). Differences in mosquito body titers spanning several orders of magnitude have been described previously (Hodoameda et al., 2022). Studies implicated the midgut barriers to be largely responsible for these variations, as bypassing them via intrathoracic injection led to lower

titer variation. These bottlenecks likely restrict dissemination to just a few founding virions (founder effect), which in turn determine viral replication and ultimately the vector competence (Forrester et al., 2014; Grubaugh et al., 2016; Hodoameda et al., 2022). However, this may only explain the titer variations in part, as additional host-specific factors have also been found to contribute to this variation (Hodoameda et al., 2024a, 2024b). Importantly, these studies did not assess whether the individual mosquitoes are vector competent, thereby limiting their comparability to the findings of Paper IV. Notably, the distinct clustering, rather than a continuous distribution, of viral body titers observed in Paper IV represents a previously undescribed pattern. This suggests that only a subset of infected mosquitoes may reach viral loads sufficient for transmission, and provides a hypothetical explanation for the large discrepancy between infection and transmission. Importantly, many studies only report mean body titers or do not provide viral load data, possibly obscuring such patterns. Further research is therefore needed to assess whether this pattern is consistent across virus-mosquito combinations, and to determine the underlying mechanisms. A recent study investigating WNV in field-caught mosquitoes also discovered a wide range of Cq-values, a proxy for viral load, in mosquito bodies (Alahakoon et al., 2025). This suggests that the observed variation in body titers is not a laboratory artefact, but reflects biological heterogeneity in natural settings.

These trends suggest that transmission may be driven by the viral titer within the mosquito, with a minimum threshold required to cross key barriers. Further research on the relationship between transmission and the body titer, as well as the underlying mechanisms, is therefore of particular interest. If high body titers can be reliably linked to increased chances of transmission, it could become possible to estimate the likelihood of transmission based on the mosquito's body titer. Such an approach would be highly useful for the analysis of field-caught mosquitoes, as current methods do not differentiate between mere infection and actual vector competence.

Together, these findings demonstrate that infection is a poor proxy for transmission, that vector competence is largely determined by barriers downstream of initial infection, and that viral replication dynamics within the mosquito are decisive for successful transmission.

3.2 Mosquito Taxa Shape Vector Competence

Mosquito taxa are highly diverse in their ecological niches, geographic ranges, and biological traits. Vector competence must therefore be assessed at the individual taxa (species and biotype) level, as generalizing across taxa might obscure important differences. These details are essential for accurately evaluating the risks posed by arboviruses.

Among the most widespread mosquito species in Europe is *Cx. pipiens* s.s.. There are two distinct biotypes of this species, biotypes *pipiens* and *molestus* (Aardema et al., 2022). In Paper I, *Cx. pipiens* biotype *pipiens* showed higher infection rates for SSHV, and transmission at a higher temperature compared with *Cx. pipiens* biotype *molestus*, however, transmission rates were equal between the two biotypes. Similarly, other studies have reported differences between the biotypes, but no consistent pattern has emerged, suggesting that these variances are virus- and context-dependent (Vogels et al., 2016; Heitmann et al., 2017; Jansen et al., 2019; Holicki et al., 2020b; Pilgrim et al., 2024). Furthermore, such variations can even extend to differences between distinct mosquito populations (Holicki et al., 2020a, 2020b).

A key strength across this thesis is the inclusion of *Cx. torrentium* across all vector competence experiments, a species that is often overlooked in studies or pooled with *Cx. pipiens* s.s.. *Culex torrentium* demonstrated low to medium susceptibility to infection across all tested viruses, and was vector competent for SSHV (Paper I) and both WNV lineages (Paper IV). To date, *Cx. torrentium* has only been considered in a limited number of studies, which confirmed vector competence for Batai virus, Sindbis virus, Usutu virus, and WNV (Heitmann et al., 2017, 2024; Jansen et al., 2019, 2022, 2023; Holicki et al., 2020a; Pilgrim et al., 2024). Compared with the results of Paper IV, previous studies reported lower infection rates but similar transmission efficiencies for WNV-L1 in *Cx. torrentium* (Jansen et al., 2019, 2023). Notably, Paper IV provides the first assessment of vector competence of *Cx. torrentium* for WNV-L2, the dominating WNV lineage in Europe. The distinction of the different *Culex* species becomes particularly important in light of these findings, as *Cx. torrentium* exhibited up to three times higher transmission efficiencies for WNV-L2, compared with *Cx. pipiens* biotype *pipiens* (Table 1 in Paper IV). This highlights the potential of *Cx. torrentium* as a potent vector for WNV, particularly given its

widespread distribution in Northern Europe, a region where WNV is currently largely absent but is projected to expand (Hesson et al., 2014; Farooq et al., 2023). These results underscore the relevance of *Cx. torrentium* as a vector species that should be considered in future vector competence and risk assessment studies for WNV in Europe.

Another mosquito species of high importance is *Ae. albopictus*, also known as the Asian tiger mosquito. In this thesis, *Ae. albopictus* emerged as the mosquito species with the broadest vector competence, as it was susceptible to infection by all tested viruses at medium to high rates, and was the only mosquito species capable of transmitting all five viruses (Papers I-V). Beyond the viruses investigated in this thesis, *Ae. albopictus* is an efficient vector for other major human pathogenic viruses, such as dengue virus and chikungunya virus (Foster and Walker, 2019). *Aedes albopictus* is already the most widespread invasive mosquito in Europe, and is projected to continue expanding its geographic range northwards, likely spreading the viruses it transmits (Kraemer et al., 2019; Farooq et al., 2025). This threat is exemplified by the rapid global expansion of chikungunya virus over the last few decades, where *Ae. albopictus* contributed to the widespread transmission following an adaptation of the virus to this mosquito species (Tsetsarkin et al., 2007). The risk of its broad vector competence is further increased by *Ae. albopictus'* opportunistic feeding behavior, which includes non-human mammals, humans, and also birds (Cebrián-Camisón et al., 2020; Garamszegi, 2024). This makes the species an excellent bridge vector for viruses such as TAHV, BFV, and SSHV, which are amplified in mammals, and potentially relevant for WNV, which amplifies in avian hosts. Importantly, the current geographic distribution of several of the tested viruses does not fully overlap with that of *Ae. albopictus*, which suggests that their absence is unlikely to be caused by the absence of a suitable vector, but rather by ecological factors or limited introduction events (Evans and Peterson, 2019; Kraemer et al., 2019; Australian Centre for Disease Control, 2025; European Centre for Disease Prevention and Control, 2025a). However, the demonstrated broad vector competence of *Ae. albopictus* highlights the threat posed by exotic arboviruses, as they might encounter a competent vector upon introduction, thereby increasing the risk of autochthonous transmission.

Together, the findings of this thesis demonstrate that vector competence cannot be generalized across taxa, and that overlooking relevant mosquito species and biotypes can lead to

misinterpretation of transmission risk. Two species emerged as particularly concerning: *Cx. torrentium* due to its high vector potential for WNV, and *Ae. albopictus*, due to its broad transmission profile.

3.3 Variation of Vector Competence Among Related Viruses

Unsurprisingly, the virus itself is an important determinant of vector competence. However, the extent to which closely related viruses share their infection and transmission profiles across mosquitoes remains incompletely understood for many viruses. To address this, viruses at multiple taxonomic levels were compared, including viruses of the same genus, the same serogroup, and different lineages within the same virus species. Specifically, the vector competence of three viruses within the genus *Orthobunyavirus* (TAHV, SSHV, OROV), with SSHV and TAHV belonging to the same serogroup, was assessed. Additionally, differences between the two major lineages of WNV were examined.

SSHV and TAHV are both members of the same serogroup, the California serogroup. Most members of this group have been isolated from *Aedes* mosquitoes, but several species have only been isolated a limited number of times, in some cases, only once (Evans and Peterson, 2019). Therefore, conclusions about the primary vectors often remain inconclusive. To address this limitation, the vector competence of two neglected California serogroup viruses, TAHV and SSHV, was assessed.

Both SSHV and TAHV exhibited a broad infection profile, as they infected all tested *Aedes* and *Culex* species, with the highest infection rates observed in *Ae. albopictus* for both viruses. In contrast, transmission was more restricted. While TAHV was only transmitted by two *Aedes* species (*Ae. albopictus* and *Ae. rusticus*), SSHV showed an exceptionally broad transmission profile, with three *Culex* mosquitoes (*Cx. pipiens* biotype pipiens, *Cx. pipiens* biotype *molestus*, *Cx. torrentium*) and *Ae. albopictus* transmitting the virus with similar efficiencies (Table 2 in Paper I, Table 2 in Paper III). This pattern indicates that vector competence is not conserved within the California serogroup, but is highly virus-specific. These findings are consistent with previous experimental vector competence studies. TAHV transmission has been shown for *Ae. vexans* and *Ae. albopictus*, whereas *Cx. pipiens pallens* was able to become infected but did not transmit the

virus (Rödl et al., 1979; Cai et al., 2023). For SSHV, transmission has been demonstrated in multiple *Aedes* and *Culiseta* species, while *Culex* mosquitoes have not been investigated in laboratory experiments before (McLean et al., 1975; Heard et al., 1991; Hewlett et al., 1992). Interestingly, the observation in Paper I that SSHV is transmitted by *Aedes* and *Culex* with similar efficiencies contrasts with patterns described for other California serogroup members, which often show a clear difference between mosquito genera. La Crosse virus, for example, showed transmission efficiencies in *Aedes* species to be up to four times higher than in *Culex* mosquitoes (Harris et al., 2015). Similarly, California encephalitis virus and Jamestown Canyon virus are primarily linked to transmission via *Aedes* mosquitoes, however, *Culex* species have rarely been assessed for vector competence (Kramer et al., 1992, 1993). These findings challenge the previously described classification of SSHV as a "non-*Culex*" virus (Snyman et al., 2023). Accordingly, *Culex* mosquitoes should be considered in future studies and risk assessments for SSHV, as they might play a role in its transmission.

Such a classification into *Culex*- and *Aedes*-associated viruses has also been established for mosquito-borne viruses of the genus *Orthoflavivirus*, where phylogenetic relationships correlate with epidemiology and disease associations. *Culex*-associated orthoflaviviruses, such as WNV, were linked to avian hosts and neuroinvasive disease, whereas *Aedes*-associated orthoflaviviruses, such as dengue virus, were linked to primate hosts and hemorrhagic disease. However, these classifications have exceptions, for example, some viruses within the *Aedes* clade are actually transmitted by *Culex* mosquitoes while still causing hemorrhagic disease (Gaunt et al., 2001). This highlights that, while such classifications can be useful for understanding broad patterns, they must be applied with caution to avoid overgeneralizing.

Like TAHV and SSHV, OROV belongs to the genus *Orthobunyavirus*, however, unlike these viruses, OROV is not part of the California serogroup. In light of the large 2024 OROV outbreak, a risk assessment of potential mosquito-borne transmission was performed (Paper II). The circulating outbreak strain was not yet available at the time. However, as a timely evaluation of the transmission potential was prioritized, a prototype viral strain (TR 9760) was used. This raises the question of how well the vector competence determined for the prototype reflects the currently circulating virus. In Paper II, OROV was able to infect *Culex* (*Cx. pipiens* biotype *pipiens*,

Cx. torrentium) and *Aedes* (*Ae. aegypti*, *Ae. albopictus*) mosquitoes at low levels, with transmission being only observed in *Ae. albopictus*. These findings are largely consistent with previous studies investigating pre-2024 viral isolates, as they also reported no to low infection across mosquito species (Gallichotte et al., 2025; Payne et al., 2025; Rosales-Rosas et al., 2026). However, transmission of the prototype virus has previously been reported only for *Cx. quinquefasciatus*, while *Ae. albopictus* repeatedly tested negative for transmission (Gallichotte et al., 2025; Payne et al., 2025; Rosales-Rosas et al., 2026). This discrepancy might be explained by differences in *Ae. albopictus* populations or experimental conditions. Mosquito population-specific variations have been shown to influence vector competence for other arboviruses, such as chikungunya virus and Zika virus, and might also affect OROV transmission (Gloria-Soria et al., 2021). The low infection and transmission rates are likely due to high midgut barriers, as a previous study showed the infection rates and transmission efficiencies to be higher after bypassing the midgut barriers by intrathoracic injection (de Mendonça et al., 2021). Recent studies evaluated the vector competence of mosquitoes for the 2024 outbreak strain. Similarly to Paper II, no to low infection across mosquito species was found, with transmission only being reported for *Cx. pipiens* s.s. at a low efficiency (Payne et al., 2025; Pilgrim et al., 2025; Rosales-Rosas et al., 2026). Taken together, these comparisons indicate that the currently circulating OROV strain has not evolved substantial adaptation to mosquitoes as vectors. Therefore, despite the use of the prototype strain, the results of Paper II likely provide a reasonable approximation of the transmission risk of OROV.

Together, the findings for TAHV, SSHV, and OROV reduce key knowledge gaps for these neglected arboviruses and demonstrate that vector competence is highly diverse across the genus *Orthobunyavirus*, and even within the California serogroup. This emphasizes that such traits cannot be generalized at the genus or serogroup level and highlights the need to investigate additional orthobunyaviruses to better understand their transmission dynamics.

While genetic diversity is limited or insufficiently characterized for some arboviruses, WNV is known to comprise nine distinct lineages. Among these, lineages 1 and 2 are most widely distributed and epidemiologically relevant (Mencattelli et al., 2023). In Paper IV, differences in

vector competence between strains of the two major lineages were assessed in three key mosquito species from Europe. The WNV strain TOS-09 of lineage 1 and the WNV strain 1382 of lineage 2 were used and are hereafter referred to as WNV-L1 and WNV-L2, respectively. Clear differences between WNV-L1 and WNV-L2 were observed. Although infection was observed for both lineages across all mosquitoes, the infection rates were consistently higher for WNV-L2. This pattern also translated into higher transmission, with WNV-L2 showing up to four-fold higher transmission efficiencies across mosquito species. The difference between WNV-L1 and -L2 was particularly pronounced in *Ae. albopictus*, where a moderate transmission efficiency was observed for WNV-L2, whereas WNV-L1 resulted in no transmission (Table 1 in Paper IV). Furthermore, differences in the dose-response relationships were observed, as discussed in detail below (section 3.5, p. 34).

These results align with several previous studies reporting higher infection rates among *Culex* and *Aedes* mosquitoes for lineage 2 compared with lineage 1, although infection rates in *Ae. albopictus* were similar for both lineages in those studies (Brustolin et al., 2016; Körsten et al., 2022). However, findings across studies are not consistent. For example, a higher infection rate for lineage 1 compared with lineage 2 was observed in *Cx. theileri*, without significant difference in transmission efficiencies (Burgas-Pau et al., 2025). Similarly, a study comparing *Cx. pipiens* s.s. from Europe and from North America found higher infection rates for lineage 1 than for lineage 2. Interestingly, the transmission efficiency was dependent on the origin of the mosquitoes, with mosquitoes from North America transmitting lineage 1 more efficiently, while the European population showed similar transmission efficiencies for both lineages. This pattern reflects the natural geographic distribution of the two lineages (Fros et al., 2015).

The variability across studies suggests that lineage-specific differences in vector competence are influenced by the origin of the mosquito population and experimental conditions. It remains important to assess to what extent these differences can be associated with lineage-specific rather than strain-specific variation. To further investigate these patterns, future studies should include a broader range of viral strains and mosquito populations. This is especially important for lineage 1, which exhibits a greater genetic diversity compared with lineage 2 (Mencattelli et al., 2023). Nevertheless, the results of Paper IV consistently indicate a higher vector competence of

WNV-L2 in mosquitoes from Europe, which potentially contributes to the spread and current dominance of this lineage in Europe.

Taking all these findings together, knowledge of the vector competence of a single virus cannot be generalized even to closely related viruses. Vector competence is not only highly virus-specific, as demonstrated for TAHV and SSHV, but substantial variations can also occur between viral strains, as shown by the differences between WNV-L1 and -L2. For OROV, relatively little variance in vector competence was found when comparing the findings of Paper II with those of other studies. This might reflect the nature of OROV as a non-mosquito-associated virus, making the limited variation less surprising. In conclusion, vector competence data must always be interpreted in the context of differences between viruses. This is especially important for modelling and risk assessments, which must consider the circulating lineages or even strains, rather than relying on generalized data.

3.4 Heterogeneous Effects of Temperature on Vector Competence

Temperature is perhaps the most important abiotic factor influencing arbovirus transmission, as it affects every component of the vectorial capacity, modulating virus replication and mosquito physiology, behavior, and ecology (Bellone and Failloux, 2020). It is often assumed that higher temperatures generally increase transmission efficiency (Foster and Walker, 2019). However, this thesis demonstrates that this assumption simplifies the variance of temperature effects across virus-mosquito combinations.

For SSHV, infection rates increased with temperature in four out of five mosquito taxa, but transmission varied between the taxa, occurring either at both tested temperatures or only at one (Table 2 in Paper I). Similarly, OROV infection rates in *Ae. albopictus* increased with temperature at 21 days post-infection, but not at 14 days, whereas transmission was observed across temperatures and time points (Table 1 in Paper II). In contrast, the infection rates for BFV in *Ae. aegypti* and *Ae. albopictus* remained relatively stable across temperatures. However, when looking at the transmission, different patterns emerged, BFV in *Ae. albopictus* showed a clear increase in efficiency with temperature, whereas no effect was seen in *Ae. aegypti* (Table 2 in Paper V). For TAHV, a clear positive relation with temperature was observed in *Ae. albopictus* as

both infection and transmission increased with temperature (Table 2 in Paper III). Importantly, SSHV transmission was observed at 18 °C, which is consistent with previous reports of transmission at temperatures as low as 13 °C (McLean et al., 1975). This likely reflects SSHV's adaptation to cooler climates, as it is naturally distributed in colder regions of the Northern Hemisphere. Furthermore, the ability of mosquitoes from Europe to transmit SSHV under moderate temperatures makes the travel-associated introduction into Europe a potential scenario.

Differences between mosquito taxa, as shown for SSHV and BFV, highlight that temperature effects are not uniform, demonstrating that temperature is not a universal driver of infection or transmission across all virus-mosquito combinations. This heterogeneity is consistent with previous studies showing variable temperature effects across virus-mosquito combinations (Bellone and Failloux, 2020; Hartner et al., 2025). Temperature likely influences the barriers of infection and transmission differently. As shown by these findings, the midgut infection and escape barriers appear to be more easily overcome at higher temperatures for most virus-mosquito combinations. However, the underlying mechanisms shaping these responses remain unclear. Temperature can affect infection and transmission at multiple levels, including by affecting viral replication and the mosquito immune system, both of which are known to be critically temperature-dependent, as well as other aspects of mosquito biology (Bellone and Failloux, 2020; Wimalasiri-Yapa et al., 2021; Bisht and te Velthuis, 2022). Overall, the observed temperature responses are likely shaped by a combination of these factors.

Besides vector competence, temperature also influences the interaction between arbovirus and mosquito by shaping the extrinsic incubation period. However, similar to vector competence, the effects are highly heterogeneous, with some virus-mosquito combinations showing a strong temperature response, while others exhibit little variation between temperatures (Bellone and Failloux, 2020; Hartner et al., 2025).

Importantly, all mosquito infection experiments of this thesis were conducted using fluctuating temperature profiles, meaning temperatures systematically fluctuated around a set mean, following a day-night cycle. This is in contrast to most other vector competence studies, which

are performed under constant temperatures. Such fluctuations provide a more realistic simulation of the environmental conditions mosquitoes experience in nature (Lambrechts et al., 2011). Several studies have shown that both the mean temperature and the degree of fluctuation can influence infection, dissemination, and body titers of mosquitoes. However, the magnitude and direction of these effects differ between virus-mosquito combinations (Lambrechts et al., 2011; Carrington et al., 2013b, 2013a; Jian et al., 2023). One study on WNV in *Culex* mosquitoes found no effect of fluctuating temperatures on transmission, although body titers were influenced (McGregor et al., 2021). Using biologically appropriate temperature conditions is therefore important to determine relevant vector competence.

Overall, the findings of this thesis demonstrate that the influence of temperature on vector competence is highly complex, heterogeneous, and context-dependent. As observed across the investigated virus-mosquito combinations, temperature does not drive vector competence in a specific direction, but rather affects competing processes within the mosquito. As a result, transmission outcomes depend on whether factors promoting or hindering transmission outweigh. These patterns highlight the need to better understand temperature-dependent dynamics, which are becoming increasingly important in the context of the ongoing climate change and its potential to change arboviral transmission dynamics.

3.5 Effects of Viral Dose on Vector Competence

While viral replication and the associated barriers within the mosquito have been established as fundamental determinants of vector competence, replication dynamics within the vertebrate host are equally important for transmission. In particular, the viremia encountered by blood-feeding mosquitoes plays a key role, as it can vary by several orders of magnitude (Pérez-Ramírez et al., 2014). This variation does not only depend on the peak viremia in the host, but also on the timing of the blood meal relative to the course of infection.

To assess the influence of the viral dose, represented by the blood meal titer, on infection and transmission, as well as to determine the MIDs, WNV was selected as a model system, due to the well-documented variation in viremia in its natural avian hosts (Pérez-Ramírez et al., 2014).

The results of Paper IV clearly demonstrate the dose-dependency of vector competence. The odds of infection more than doubled for each ten-fold increase of blood meal titer for both WNV lineages, with a stronger effect on WNV-L2. Transmission showed a similar dose-dependent trend, with the odds of transmission for WNV-L2 doubling for each ten-fold increase of blood meal titer. For WNV-L1, transmission was only observed at the highest blood meal titer, however, a detailed statistical analysis was not possible for this lineage due to the limited number of positive saliva samples.

The positive correlation between increasing infection rates and blood meal titers is aligned with existing literature that demonstrated similar dose-dependent effects across a range of lineage 1 isolates and mosquito species, although the strength of this effect varies between studies (Turell et al., 2001; Goddard et al., 2002; Reisen et al., 2006; Richards et al., 2007; Vanlandingham et al., 2008; Anderson et al., 2010; Brustolin et al., 2016; Giayetto et al., 2021). However, not all of these studies included an assessment of the vector competence, i.e., the detection of infectious virus in saliva. Nevertheless, a general trend of an increasing transmission with higher blood meal titers has been observed (Turell et al., 2001; Goddard et al., 2002; Reisen et al., 2006; Giayetto et al., 2021). Besides WNV, this association has also been reported for other arboviruses (Althouse and Hanley, 2015; Beranek et al., 2024). Furthermore, it has also been shown that higher blood meal titers can lead to shorter extrinsic incubation periods, which again increases the overall vectorial capacity (Mahmood et al., 2006; Oviedo et al., 2011).

The observed dose-dependent increase in infection and transmission provides insight into the dynamics of the different barriers within the mosquito. Across both viral lineages and all tested mosquito species, higher blood meal titers generally led to higher infection rates (Table 1 and Figure 1 in Paper IV). This indicates that the midgut infection barrier is highly dose-dependent, as previously proposed (Hardy et al., 1983; Franz et al., 2015). The more limited increase of transmission efficiencies suggests that barriers following the initial infection, i.e., the midgut escape, salivary gland infection, and salivary gland escape barriers, represent the primary restrictions on successful transmission.

This pattern is particularly evident when considering the interaction between *Ae. albopictus* and WNV-L1. While it exhibited high infection rates at low blood meal titers, transmission remained

absent even at the highest dose (Table 1 in Paper IV). This suggests a relatively weak midgut infection barrier, but substantially stronger downstream barriers preventing transmission. I hypothesize that the transmission barriers in this virus-mosquito combination might be dose-independent, potentially reflecting restricted dissemination from the midgut, and limited replication within or escape from the salivary glands. However, previous studies have demonstrated successful transmission of lineage 1, indicating that this lineage is not generally unable to be transmitted by *Ae. albopictus*, but that there might be viral strain or mosquito population-specific differences (Fortuna et al., 2015; Brustolin et al., 2016; Martinet et al., 2023). The discrepancy might also be explained by differences in experimental conditions.

In addition to observing changing infection rates and transmission efficiencies, depending on the blood meal titer, the MID was evaluated by determining the lowest blood meal titer at which transmission occurred. Interestingly, this MID differed strongly between the two lineages, with WNV-L2 consistently requiring lower doses. Our results showed that the MID was 10^7 $TCID_{50}$/mL for WNV-L1 and 10^5 $TCID_{50}$/mL for WNV-L2 in both *Culex* species tested (*Cx. pipiens* biotype *pipiens*, *Cx. torrentium*) (Table 1 in Paper IV). While no direct comparisons are available for these species, one study investigating *Cx. quinquefasciatus* found a MID of 10^6 PFU/mL for lineage 1 (Giayetto et al., 2021). In *Ae. albopictus,* the MID was 10^6 $TCID_{50}$/mL for WNV-L2, while no tested concentration was sufficient to cause transmission of WNV-L1 (Table 1 in Paper IV). While previous findings on lineage 2 are comparable to these results (Brustolin et al., 2016), findings on lineage 1 are contradictory, as a dose of $10^{6.7}$ PFU/mL was reported as the MID in *Ae. albopictus* (Martinet et al., 2023). This discrepancy could be explained by the higher incubation temperature used by Martinet et al., which is known to increase transmission potential for certain mosquito-WNV combinations (Vogels et al., 2017b).

As already established, viremia levels in avian hosts vary substantially, and the barriers for transmission within a mosquito are largely dose-dependent (Pérez-Ramírez et al., 2014; Franz et al., 2015). As a result, the probability that a mosquito becomes infected and subsequently transmits the virus is determined by the viremia of the host at the time of feeding, which must be equal to or greater than the respective MID. Specifically, contrary to the lifelong infection of mosquitoes, the natural infection of WNV in birds is acute and follows a typical unimodal viremia

curve, with a rise, one short peak, and subsequent decline (Lim et al., 2014, 2015; Pérez-Ramírez et al., 2014). The duration of viremia in birds is typically below one week, with peak viremia levels varying widely between species and ranging from < 10^4 PFU/mL to > 10^6 PFU/mL. No substantial difference in either the duration or peak viremia has been observed between the WNV lineages in avian hosts (VanDalen et al., 2013; Lim et al., 2014, 2015; Pérez-Ramírez et al., 2014). Therefore, the lower MIDs observed for WNV-L2 suggest two advantages over WNV-L1: (i) mosquitoes can acquire WNV-L2 from a wider variety of avian hosts, as they are not restricted to species and individuals developing the highest peak viremia, and (ii) the temporal window during which the virus can be acquired is broader, as the viremia remains above the MID for a longer period of time, including both the rising and declining phases of viremia. Together with the higher transmission efficiencies of WNV-L2 (discussed in section 3.3, p. 28), these results may provide a mechanistic explanation for the spread and current dominance of lineage 2 in Europe.

Building on this, it is well described that there is often a trade-off between the duration and magnitude of viremia, also described as “tortoise vs. hare” viral replication strategies. It was suggested that arboviruses following the “tortoise” strategy, characterized by lower magnitude but longer duration of viremia, may be more successful in spreading through vector and host populations (Althouse and Hanley, 2015). In the context of Paper IV, this further suggests that the 100-fold lower MID of WNV-L2 could be a critical advantage, indicating that even moderate viremia levels, which may be too low for WNV-L1, are likely sufficient to maintain WNV-L2 circulation.

Overall, the viral dose has been demonstrated to be a key determinant of vector competence, shaping both infection and transmission. Knowledge of host viremia is essential for translating experimental vector competence data into real-world transmission dynamics. Without integration of realistic host viremia, the assessment of transmission risks might be biased, leading to either over- or underestimation of the true risk. Importantly, reliable host viremia data remain limited for many arboviruses, especially neglected viruses. However, MIDs determined under laboratory conditions with infection via an artificial blood meal are likely inflated, as the infectious dose required for infection and transmission is lower when mosquitoes feed on a living host compared with artificial feeding (Kramer et al., 1981; Lord et al., 2006; Althouse and Hanley,

2015). Therefore, the thresholds determined in Paper IV provide comparative insights into viral lineage- and mosquito species-specific differences in transmission dynamics and should be interpreted relative to each other, rather than absolute. Overall, viral dose has been demonstrated to be a key determinant of vector competence, shaping both infection and transmission.

3.6 Relevance and Implementation

Ultimately, this thesis provides data that improve the understanding of vector competence dynamics in mosquitoes, particularly the importance of variability across virus-mosquito combinations. Beyond advancing our knowledge on a scientific basis, these findings can be directly applied to transmission modelling, risk assessment, and mapping, with the potential to influence future control strategies.

This thesis presents insights into the transmission potential of neglected and emerging arboviruses. Given the limited surveillance of these and other arboviruses, a majority of infections likely remain undetected. This is particularly concerning, considering that more than half of all central nervous system infections worldwide lack an identified causative pathogen, a disease spectrum that many arboviruses can cause (Erdem et al., 2017). Reports such as those from Croatia, where 10% of patients with neuroinvasive disease of unknown etiology tested positive for TAHV antibodies, emphasize the need to consider neglected arboviruses as a relevant public health threat (Vilibić-Ćavlek et al., 2022). For TAHV, Paper III provides the first assessment of vector competence in mosquitoes from Europe since 1979, addressing important knowledge gaps for this widely circulating virus. In contrast, SSHV and BFV are not endemic in Europe, yet the results of Papers I and V demonstrate that transmission in Europe is possible from a vector perspective. For both viruses, these results represent the first evidence of vector competence outside their native distributions. Together, these findings suggest that neglected arboviruses may pose a greater public health risk than currently recognized and underscore the need to better understand the transmission dynamics of neglected arboviruses.

The risk of OROV transmission by mosquitoes from Europe was assessed as a rapid response to the large 2024/2025 outbreak in South and Central America. At the time of experimentation, no

data on the vector competence of mosquitoes from Europe were available. While the results of Paper II identified large areas of Southern Europe as theoretically suitable for OROV transmission, the overall likelihood of autochthonous transmission was considered low, indicating a limited public health risk in Europe.

Finally, Paper IV provides important new insights into the differences between the two human pathogenic WNV lineages, L1 and L2. In particular, the understanding of WNV-L2 was deepened, which remains comparatively understudied, despite being the dominant lineage in Europe. Differences in the dose-response relationship and the vector competence between the lineages were identified, with WNV-L2 consistently showing more efficient transmission at lower minimum infectious doses, likely contributing to its dominance in Europe. Another key finding was the high vector competence of *Cx. torrentium* for WNV-L2, highlighting the elevated vector potential of this widely distributed species.

Taken together, this thesis demonstrates that arbovirus vector competence is highly complex and is shaped by specific virus-mosquito combinations. During a time of rapid globalization, urbanization, and climate change, it has become increasingly important to consider both endemic and exotic arboviruses as expanding public health threats. By moving beyond simplified assumptions and providing detailed data, this thesis advances the nuanced understanding of the determinants of vector competence for both endemic and exotic arboviruses. The implementation of these findings can strengthen evidence-based risk management and guide vector control strategies.

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Documentation of gAI Resources

In accordance with the affidavit (p. x), gAI resources were used exclusively for proofreading, wording suggestions, and language refinement (grammar, punctuation). The following resources were used: Grammarly, 2026. ChatGPT, 2026.

Supplementary Material

The following pages contain the full-length versions of the five papers on which this dissertation is based.

Paper I

viruses

MDPI

Article

Mosquitoes from Europe Are Able to Transmit Snowshoe Hare Virus

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Abstract: Snowshoe hare virus (SSHV) is a zoonotic arthropod-borne virus (arbovirus) circulating in colder areas of the Northern Hemisphere. SSHV is maintained in an enzootic cycle between small mammals and mosquitoes, assumably of the genera *Aedes* and *Culiseta*. Symptoms of SSHV human infection can range from asymptomatic to severe neuroinvasive disease. Studies on SSHV transmission are limited, and there is no information available on whether mosquitoes of the genus *Culex* are able to transmit SSHV. Therefore, we investigated six mosquito species via salivation assay for their vector competence. We demonstrated that SSHV can be transmitted by the abundant European *Culex* species *Cx. pipiens* biotype pipiens, *Cx. pipiens* biotype molestus, and *Cx. torrentium* with low transmission efficiency between 3.33% and 6.67%. Additionally, the invasive species *Ae. albopictus* can also transmit SSHV with a low transmission efficiency of 3.33%. Our results suggest that local transmission of SSHV after introduction to Europe seems to be possible from a vector perspective.

Keywords: snowshoe hare virus; vector competence; *Aedes*; *Culex*

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1. Introduction

Arthropod-borne viruses (arboviruses) have been an increasingly emerging global health threat over the last decades, as the recent epidemics of dengue and chikungunya have shown. The risk of arbovirus transmission increases due to factors such as climate change, environmental changes, and increased traveling and trading, which contributes to the spread of both, invasive mosquito species and the arboviruses themselves [1,2].

Snowshoe hare virus (SSHV) belongs to the *Orthobunyavirus* genus in the *Peribunyaviridae* family within the *Bunyavirales* order, which forms the largest genus of arboviruses worldwide [3]. *Orthobunyavirus* virions are enveloped and have a single-stranded negative-sense tripartite genome. All members of the *Orthobunyavirus* genus are transmitted by arthropods, especially mosquitoes [3]. Based on their serological and genetical relationship, the genus of *Orthobunyavirus* is subdivided into different groups/complexes. The California serogroup (CSG) with SSHV has currently 18 members. The prototype virus of the CSG is the California Encephalitis virus (CEV), isolated from different mosquito pools in the 1940s in the San Joaquin Valley, California (US) [4,5]. In 1952, the first human case of encephalitis caused by the California Encephalitis virus was described [6]. The first isolation of SSHV took place in 1959 in Bitterroot Valley, Montana (US) from the blood of a *Lepus americanus*, the snowshoe hare [7]. This was the first time a CSG member was isolated from a vertebrate and not from mosquitoes.

Orthobunyaviruses are found across various regions, ranging from tropic to arctic areas on all continents, with the exception of Antarctica [8]. SSHV is distributed in colder regions in the Northern Hemisphere, i.e., Canada, USA, and Russia [8,9]. The clinical course of SSHV infection in humans can range from asymptomatic to mild illnesses to severe neuroinvasive diseases. In fact, in the US, mainly three members of the CSG are causing neuroinvasive diseases: La Crosse Virus (LACV), Jamestown Canyon Virus (JCV), and SSHV [10]. While LACV and SSHV affect mainly children, JCV affects primarily adults, and the reason for this is still unknown [11]. There are only a few reports of SSHV infection in humans from the 1970s and 1980s from various Canadian provinces, one pediatric neuroinvasive case from Novia Scotia in 2006, and one case of meningoencephalitis from Manitoba in 2016 [12–17]. Serological studies conducted in Alaska (US), an endemic area of SSHV, revealed antibody positivity rates of 42% in the 1980s and 6.8% in the 1990s, indicating a significant number of undetected human cases [18,19].

SSHV circulation is sustained through an enzootic cycle involving mosquitoes and mammals. It is assumed that the primary and amplifying hosts are small mammals. For instance, serological studies conducted in Alaska and Wyoming (US), as well as in Newfoundland (Canada), have indicated high seroprevalences in snowshoe hares [18,20,21]. As SSHV-positive mosquitoes have been detected northwards of the distribution area of snowshoe hares, other mammalian species must be involved in the transmission cycle [22]. In Montana (US), ground squirrels have tested positive for SSHV antibodies, while voles, chipmunks, rats, and other small mammals in this area were negative [23]. Hares, rabbits, lemmings, and red-backed voles have been found SSHV-antibody-positive in Alaska (US) [24]. Additionally, larger wild animals such as bison, dall sheep, bovines, sheep, deer, and moose have shown positive serological results [18,20,25,26]. Laboratory studies on small mammals revealed snowshoe hares, squirrels, rats, and voles susceptible to SSHV, but marmots and the white-footed mouse do not develop viremia [23]. However, laboratory studies have provided no evidence of SSHV infection in larger mammals, and the experimental infection of deer, elk calves, and dogs failed [23,27]. Similar to several arboviruses, e.g., West Nile virus, among larger mammals, horses appear uniquely susceptible to SSHV, potentially developing encephalitis [28–30]. Serological studies in Newfoundland (Canada) have shown a low seroprevalence in horses, suggesting SSHV infection of horses is predominantly asymptomatic; only a small number of horses develop encephalitis, but spontaneous recovery is possible [20,29].

Field studies identified several mosquito species carrying SSHV, but this does not necessarily confirm them as competent vectors. Various *Aedes* species, including *Ae. fitchii*, *Ae. canadensis*, *Ae. communis*, *Ae. cinereus*, *Ae. hexodontus* complex, *Ae. punctor* complex, or *Ae. vexans* have tested positive for SSHV [23,31–36]. Additionally, SSHV-positive *Culiseta* species were also collected, e.g., *Cs. impatiens* and *Cs. inornata* [23,34]. Notably, there are no reports of SSHV in *Anopheles* or *Culex* mosquitoes, even though these have been analyzed in studies [34,37]. This resulted in the suggestion that SSHV is a "non-*Culex*" virus, and the current distribution of SSHV aligns with the high abundance of *Aedes* and *Culiseta* mosquitoes [38]. However, further research is needed to confirm this hypothesis.

SSHV is found in regions with long harsh winters and short summers. LeDuc et al. proposed that the virus winter maintenance could occur through transovarial transmission, persistence in infected vertebrates, or overwintering in mosquitoes [39]. In support of the transovarial transmission theory, SSHV-positive *Aedes* larvae have been collected in the field, indicating this as a method for SSHV overwintering [36,40]. Another plausible mechanism is the overwintering of infected adult mosquitoes. *Culiseta* mosquitoes are susceptible to SSHV by intrathoracic injection and able to transmit SSHV, even after incubation at temperatures as low as 0 °C or 13 °C [41,42]. SSHV maintenance in vertebrates is also possible, as SSHV could be detected in the mosquito-free season in several small mammals, like hares and voles [24]. However, there are limited laboratory studies on the vector competence of different mosquito species for SSHV. Transmission of SSHV to suckling mice was shown for *Ae. Provocans*, *Ae. Abserratus-punctori*, and *Ae. triseratus* at 19 °C/23 °C [43,44].

Replication of SSHV was also detected in intrathoracic-injected *Ae. Canadensis*, as well as *Cs. iornata* mosquitoes incubated at temperatures from 13 °C to 24 °C [45]. *Aedes aegypti* and *Cs. iornata* showed a vector competence even at low temperatures: 13 °C/24 °C for *Ae. aegypti* and 13 °C for *Cs. iornata* [45]. Further studies with *Cs. iornata* incubated at temperatures around the freezing point showed positive specimens even after 194 days [45].

Three members of the CSG are distributed in Europe: Tahyna virus (TAHV) on the whole continent, as well as Inkoo virus (INKV) and Chatanga virus (CHATV), which both only occur in the northern areas of Europe. While it is assumed that transmission of TAHV takes place by a broad range of mosquito species (*Aedes*, *Culex*, and *Culiseta*), the transmission of INKV and CHATV probably only occurs by *Aedes* mosquitoes, but this assumption is mainly based on virus detection in field-caught mosquitoes, which again does not necessarily confirm them as competent vectors [8]. Given the recent emergence of certain orthobunyaviruses with public and veterinary health relevance in new areas, such as the appearance of the Cache Valley virus in New York, it is crucial to possess basic knowledge about these viruses to prevent larger outbreaks/epidemics [46,47]. To address the substantial knowledge gap regarding the vector competence of mosquitoes for SSHV, we conducted this study, with a special focus on *Culex* species. This information will help to estimate the risk of SSHV transmission in currently nonendemic areas such as Europe.

2. Materials and Methods

Egg rafts of *Culex pipiens* s.s./*Culex torrentium* were collected in the field during the summer of 2023 in northern Germany (Lon: 53.467821/Lat: 9.831346). Larvae were reared at room temperature with a 12:12 light:dark photoperiod. Species identification as *Culex pipiens* biotype pipiens (*Cx. pipiens* pipiens) and *Cx. torrentium* was performed by extracting DNA of a pool of 5 L1/L2 larvae per egg raft (DNeasy blood & tissue kit, Qiagen, Hilden, Germany) and multiplex quantitative real-time PCR (qPCR) as described by Rudolf et al. (HotStarTaq master mix kit, Qiagen, Hilden, Germany) [48]. Pupae were placed in an insectary with a relative humidity of 70%, 26 °C, and a 12:12 light:dark photoperiod, including 30 min twilight. To exclude natural arbovirus infections, 10 randomly selected adult mosquitoes per species were tested by pan-Orthobunya-, pan-Flavivirus-, and pan-Alphavirus-PCR, confirming all specimens as negative [49–51]. Lab strains of *Culex pipiens* biotype molestus (*Cx. pipiens* molestus) (established since 2011 from egg rafts collected in Heidelberg, Germany), *Culex quinquefasciatus*, *Aedes aegypti* (both long-established colonies from Bayer, Leverkusen, Germany), and *Aedes albopictus* (established with eggs from the field in Heidelberg in 2016/2017) were reared in the insectary likewise.

Female mosquitoes with an age of 4–14 days were starved for 24 (*Aedes*) or 48 (*Culex*) hours. An artificial blood meal was performed at 24 °C for two hours, containing 50% human blood (expired blood preservation), 30% of an 8% fructose solution, 10% filtrated bovine serum (FBS), and 10% virus stock, final virus concentration was 2.2×10^6 FFU/mL. SSHV stock was propagated on BHK-21 cells (*Mesocricetus auratus*, CCVL L 0179, Friedrich-Loeffler-Institute, Riems, Germany), using the SSHV strain ATTC VR-711 [52]. A blood meal was offered either via cotton stick for all *Culex* mosquitoes, reaching a feeding rate (FR, number of engorged females per number of fed females) of 67.1% for *Cx. pipiens* pipiens, 55.7% for *Cx. pipiens* molestus, 50.0% for *Cx. torrentium* and 95.5% for *Cx. quinquefasciatus* or via two 50µL drops for *Aedes* mosquitoes, reaching an FR of 42.6% for *Ae. aegypti* and 81.0% for *Ae. albopictus* (Table 1). Only fully engorged females were used for the experiments. Mosquitoes were incubated for 14 days at 70% humidity and fluctuating temperature profiles of 18 °C or 24 °C with variations of +/−5 °C within 24 h. The highest temperature was reached in the middle of the light period, and the lowest temperature was reached in the middle of the dark period to mimic day and night fluctuation. Fructose was offered continuously via cotton pads and refreshed every 2–3 days. The survival rate (SR, number of alive mosquitoes 14 days post infection (dpi) per number of fed females) of all mosquito species was in a range of 68–100% (Table 1). All experiments were performed in 2 replicates.

Table 1. Number of mosquito specimens per experiment condition (total input), calculation of feeding rate (FR, number of engorged females per number of fed females), and survival rate (SR, number of alive mosquitoes 14 days post infection per number of fed females) for infection experiments with snowshoe hare virus 14 days post infection.

Species	Temperature (°C)	Total Input	FR (%)	SR (%)
Aedes aegypti	18° +/− 5 °C	96	60.4 (58/96)	69.0 (40/58)
	24° +/− 5 °C	122	42.6 (52/122)	80.8 (42/52)
Aedes albopictus	18° +/− 5 °C	60	83.3 (50/60)	68.0 (34/50)
	24° +/− 5 °C	84	81.0 (68/84)	76.5 (52/68)
Culex pipiens biotype pipiens	18° +/− 5 °C	83	53.0 (44/83)	77.3 (34/44)
	24° +/− 5 °C	97	55.7 (54/97)	87.0 (47/54)
Culex pipiens biotype molestus	18° +/− 5 °C	115	44.4 (51/115)	96.1 (49/51)
	24° +/− 5 °C	79	67.1 (53/79)	94.3 (50/53)
Culex torrentium	18° +/− 5 °C	56	96.4 (54/56)	87.0 (47/54)
	24° +/− 5 °C	56	94.6 (53/56)	94.3 (50/53)
Culex quinquefasciatus	18° +/− 5 °C	75	53.3 (40/75)	100.0 (40/40)
	24° +/− 5 °C	90	46.7 (42/90)	97.6 (41/42)

Vector competence was analyzed at 14 dpi via salivation assay as previously described [53]. Briefly, mosquitoes were anesthetized with CO_2 to remove legs and wings. The proboscis was put into a 10 µL tip containing 10 µL of phosphate-buffered saline (PBS) and incubated for 30 min. PBS-saliva solution was pipetted onto BHK cells in a 96-well plate to observe if the saliva contained infectious virus particles, which would induce a cytopathic effect during the next 7 days. If CPE was observed, RNA of the supernatant was extracted (QIAmp viral RNA mini kit, Qiagen, Hilden, Germany) and tested via qRT-PCR (QuantiTect Reverse Transcription kit, Qiagen, Hilden, Germany) using the Primers SHL80C (CAACAATTCTTAGCTAGGATTAA) and SHL146V (GATCGACATCTATATCTTTGGCA) located in the L-segment [54], with an addition of the VetMAX™ Xeno™ Internal Positive Control (Applied Biosystems, Thermo Fisher Scientific Corporation, Waltham, MA, USA). A series of 1.19×10^3, 1.19×10^4, 1.19×10^5 dilutions of a synthetic SSHV standard (5′-ATGCAACAATTCCTAGCTAGGATTAATGCTGCAAGAGATGCATGTGTTGCCAAAGA TATAGATGTCGATCCTA-‘3) was used as a positive control.

The transmission rate (TR, number of SSHV-positive saliva per number of SSHV-positive bodies) and transmission efficiency (TE, number of SSHV-positive saliva per fed females) were calculated. To estimate the amount of infectious virus particles, saliva was titrated as described by Jansen et al. [55]. The RNA of mosquito bodies, excluding legs and wings, was extracted (MagMAX CORE nucleic acid purification kit, Applied Biosystems, Thermo Fisher Scientific Corporation, Waltham, MA, USA), and RT-qPCR was performed as mentioned above. Infection rate (IR, number of SSHV-positive bodies per fed females) and mean body titer of each specimen were calculated using a series dilution of the above-mentioned SSHV standard.

The RT-qPCR was validated in accordance with the “Minimum Information for Publication of Quantitative Real-Time PCR Experiments” guidelines as outlined by Bustin et al. [56]. A series of ten-fold dilutions from 1.19 to 1.19×10^9 copies/µL of the SSHV standard were analyzed in five replicates according to the above-mentioned RT-qPCR protocol. The limit of detection was determined to be 2.68×10^5 copies/mosquito body, and the standard deviation of C_qs at this concentration was 1.173. The linear dynamic range was established between 2.68×10^5 and a minimum of 2.68×10^{11} copies/mosquito body, meaning that the concentration is in a linear proportion to the PCR signal in this range and can therefore be considered reliable. From the calibration curves, the coefficient of determination was calculated to be 0.9968. The slope was −3.539, while the y-intercept was at 53.304. Finally, the PCR efficiency was 0.9184, i.e., 91.8% of the target molecules were amplified in each step.

3. Results

All investigated mosquito species were susceptible to SSHV and four of the six were capable of transmitting SSHV, i.e., *Ae. albopictus*, *Cx. pipiens* pipiens, *Cx. pipiens* molestus, and *Cx. Torrentium* (Table 2). No positive saliva was detected for *Ae. Aegypti* and *Cx. Quinquefasciatus*.

Table 2. Calculation of infection rate (IR, number of SSHV-positive bodies per fed females), mean body titer, transmission rate (TR, number of SSHV-positive saliva per number of SSHV-positive bodies), and transmission efficiency (TE, number of SSHV-positive saliva per fed females) for infection with snowshoe hare virus 14 days post infection; thirty specimens were investigated per condition (n.a. = not applicable for the mean if there were no positive specimens or for the confidence interval if there was only one positive body).

Species	Temperature (°C)	IR (%)	Mean Body Titre log10 Copies/Mosquito Specimen (95% Confidence Interval)	TR (%)	TE (%)
Aedes aegypti	18° +/− 5 °C	0.0 (0/30)	n.a.	0.0 (0/0)	0.0 (0/0)
	24° +/− 5 °C	10.0 (3/30)	3.6 (1.8–5.4)	0.0 (0/0)	0.0 (0/0)
Aedes albopictus	18° +/− 5 °C	50.0 (15/30)	7.0 (6.2–7.8)	6.7 (1/15)	3.3 (1/30)
	24° +/− 5 °C	96.7 (29/30)	7.0 (6.4–7.6)	3.5 (1/29)	3.3 (1/30)
Culex pipiens biotype pipiens	18° +/− 5 °C	23.3 (7/30)	4.7 (4.1–5.2)	0.0 (0/0)	0.0 (0/0)
	24° +/− 5 °C	26.7 (8/30)	5.4 (4.2–6.7)	12.5 (1/8)	3.3 (1/30)
Culex pipiens biotype molestus	18° +/− 5 °C	16.7 (5/30)	6.0 (3.9–8.2)	20.0 (1/5)	3.3 (1/30)
	24° +/− 5 °C	3.3 (1/30)	4.8 (n.a.)	0.0 (0/0)	0.0 (0/0)
Culex torrentium	18° +/− 5 °C	50.0 (15/30)	5.0 (4.7–5.4)	0.0 (0/0)	0.0 (0/0)
	24° +/− 5 °C	40.0 (12/30)	5.8 (5.0–6.6)	16.7 (2/12)	6.7 (2/30)
Culex quinquefasciatus	18° +/− 5 °C	3.3 (1/30)	6.1 (n.a.)	0.0 (0/0)	0.0 (0/0)
	24° +/− 5 °C	43.3 (13/30)	5.1 (4.8–5.5)	0.0 (0/0)	0.0 (0/0)

Aedes aegypti showed the lowest IR of all species, with an IR of 0.0% at 18 °C +/− 5 °C and 10.0% at 24 °C +/− 5 °C (Table 2). Both temperature profiles are presented as 18 °C and 24 °C, respectively, in the following results and discussion. Likewise, the mean body titer of 3.6 log10 copies per specimen was the lowest titer of all species, resulting in no transmission. In contrast, *Ae. albopictus* showed the overall highest IRs of 96.7% at 24 °C and 50.0% at 18 °C. Moreover, the body titers of 7.0 log10 copies per specimen at both temperature profiles are the highest values over all species. While the TR is higher at 18 °C with 6.7% in comparison with 3.5% at 24 °C, the TE with 3.3% is identical for both temperatures.

For *Cx. pipiens* pipiens, the IR was 23.3% at 18 °C and 26.7% at 24 °C, with mean body titers of 4.7 and 5.4 log10 copies per specimen, respectively. Transmission was only observed at 24 °C with a TR of 12.5% and a TE of 3.3%. *Culex pipiens* molestus showed lower IRs, with 16.7% at 18 °C and 3.3% at 24 °C. The titer was slightly lower at the higher temperature of 24 °C with 4.8 log10 copies per mosquito compared with 6.0 log10 copies per mosquito at 18 °C. Transmission only took place at 18 °C, with a TR of 20.0% and a TE of 3.3%. *Culex torrentium* had the highest IR of all investigated *Culex* species, with 50.0% at 18 °C and 40.0% at 24 °C. The body titer was higher at the higher temperature, with 5.8 log10 copies per specimen in comparison with 5.0 log10 copies per specimen at the lower temperature. Transmission was only observed at the higher temperature with a TR of 16.67% and a TE of 6.67%, which is the highest measured TE for all species. No transmission was observed for *Cx. quinquefasciatus*, but infection was detected at both temperatures with

an IR of 3.3% with a body titer of 6.1 log10 copies per specimen at 18 °C and an IR of 43.3%, with a body titer of 5.1 log10 copies per specimen at 24 °C.

In all detected saliva samples, a cytopathic effect was only present in the first well of the saliva dilution, which resulted in a concentration of <10 infectious virus particles per saliva sample.

4. Discussion

In this study, we found that *Culex pipiens* pipiens, *Cx. pipiens* molestus, and *Cx. torrentium* are capable of transmitting SSHV. To the best of our knowledge, this is the first demonstration of SSHV transmission by *Culex* mosquitoes. Moreover, this is the first demonstration of SSHV transmission by the highly invasive mosquito species *Ae. albopictus*. Although no positive saliva was detected in *Ae. aegypti* and *Cx. quinquefasciatus*, both species were susceptible to SSHV infection. The TE of SSHV by both *Aedes* and *Culex* genera was relatively low at around 3.3%, with a slightly higher rate of 6.7% observed for *Cx. torrentium*. This contrasts with other members of the CSG, which show a clear difference in transmission by the different mosquito genera. For example, transmission of the closely related LACV [57] has been demonstrated in *Culex*, specifically *Cx. restuans* and *Cx. pipiens*, with TE values also ranging under 10% [58]. However, vector competence studies with LACV and *Ae. triseriatus* showed significantly different results, yielding high TE values of 40% [58]. Another example of a CSG member is TAHV, for which recent studies with a newly discovered TAHV strain from China showed infection of *Ae. albopictus* and *Cx. pipiens pallens*, but SSHV-positive saliva was only detected in *Ae. albopictus* [59]. Other members of the CSG group, like CEV and JCV, are also known to be transmitted by several *Aedes* species, with transmission rates ranging from high to low levels [60,61]. The transmission of JCV by *Ae. albopictus* was recently demonstrated with TRs ranging from 13% to 30% (7 dpi, incubation temperature of 27 °C) [62]. The hypothesis that SSHV is a "non-*Culex*" virus and that the current distribution of SSHV corresponds with the high abundance of *Aedes* and *Culiseta* mosquitoes is probably not correct [38]. Risk assessment for SSHV in endemic areas may need to be reconsidered, as *Culex* mosquitoes as vectors need to be taken into account.

In this study, transmission was shown at 24 °C for three species (*Ae. albopictus*, *Cx. pipiens* pipiens, *Cx. torrentium*) and at 18 °C for two species (*Ae. albopictus*, *Cx. pipiens* molestus). Therefore, it seems that the transmission of SSHV is not limited by lower temperatures. This aligns with the observations of McLean et al., who detected SSHV transmission by *Ae. aegypti* and *Cs. Iornata* at incubation temperatures of 24 °C and 13 °C [45]. Abundant European *Culex* mosquito species, as well as the invasive *Ae. Albopictus*, are susceptible and able to transmit SSHV under the prevailing temperatures in Europe. Thus, travel-associated SSHV introduction via mosquitoes/humans or introduction by infected vertebrate hosts, migrating from Russia to Northern Europe, is a potential scenario. The theories of LeDuc et al. for winter maintenance of SSHV are also conceivable for Europe [39]. Transovarial transmission, a known mechanism for several CSG viruses in *Aedes* mosquitoes, appears feasible for SSHV in European *Aedes* species [63]. The investigated *Culex* mosquitoes from Germany, which overwinter as adults similar to *Culiseta* mosquitoes from Northern America, could also facilitate SSHV maintenance [64–66]. Whether SSHV can establish an enzootic cycle in Europe would also depend on the hosts. However, due to the broad host range of different species of small mammals [23], it can be assumed that European hares, squirrels, or other small mammals could serve as amplifying hosts. However, these experimental studies are missing.

Another factor that should be kept in mind is the risk of reassortment of viral genome segments, common with orthobunyaviruses [67]. If genetically and antigenic close orthobunyaviruses infect the same cell, an exchange of the three segments can take place, leading to a reassortment of segments and the emergence of novel viruses. The reassortment of orthobunyaviruses in the laboratory was first described for LACV in dually infected mosquitoes [68]. The phenotypes of reassortment viruses can vary significantly from their

parental strains, with either increased or decreased virulence. For example, the laboratory reassortment of LACV and JCV shows a loss of pathogenicity in mammals [69]. An example of the opposite effect is the natural reassortment of the Ngari virus (NRIV), containing the L- and S-segment of the Bunyamwera virus (BUNV) and the M-segment of the Batai virus (BATV), both belonging to the bunyamwera serogroup of the orthobunyaviruses [70]. NRIV shows a higher pathogenicity for mammals in comparison with the parental viruses, i.e., BUNV and BATV infections in humans are mainly asymptomatic or associated with febrile illness, while NRIV causes hemorrhagic fever [71]. Interestingly, laboratory studies with an NRIV-like virus showed an increased pathogenicity in mammalian cells in comparison with the parental viruses BUNV and BATV but a decreased growth in insect cells [72]. Therefore, the assessment of new reassortment viruses must always take the host as well as the vector into account in order to make a risk assessment. The observations of Beaty et al. on the reassortment viruses of LACV and SSHV revealed an important function for the M-segment: encoding for the glycoproteins and the nonstructural proteins NSm. Reassortment viruses containing the M-segment of LACV were efficiently transmitted by *Ae. triseratus*. In contrast, reassortment viruses containing the M-segment of SSHV were only inefficiently transmitted [73–75]. However, there are only a few states in the US where LACV and SSHV occur together and natural reassortment could take place, but so far, there have been no reports from the field. Two members of the CSG are distributed in Northern Europe, INKV and CHATV, whereby CHATV is phylogenetically most closely related to SSHV within the CSG, and the reassortment with SSHV in coinfected hosts/vectors is quite conceivable [8,57]. In fact, SSHV occurs in Russia, where INKV, CHATV, and TAHV co-circulate. A sequence analysis of several isolates in Russia belonging to the CSG showed potential SSHV reassortments [9]. With expanding mosquito populations and the presence of other CSG members in Northern Europe and Russia, the risk of novel SSHV reassortments increases.

5. Conclusions

SSHV can be transmitted by abundant, native *Culex* mosquitoes in Europe with low TE: *Cx. pipiens* biotype pipiens, *Cx. pipiens* biotype molestus, and *Cx. torrentium.* Additionally, the invasive species *Ae. albopictus* can also transmit SSHV with low TE. Both investigated tropical mosquito species, *Ae. aegypti* and *Cx. quinquefasciatus,* were not able to transmit SSHV. Considering these findings, the introduction of SSHV to Europe appears feasible from the vector as well as the climate perspective. However, further investigation of the potential vertebrate hosts and introduction pathways is needed.

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Paper II

The Journal of Infectious Diseases

BRIEF REPORT

Risk Assessment of Oropouche Virus Transmission by Mosquitoes in Europe

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Oropouche virus (OROV) emerged as a significant health threat in Central and South America in 2024. *Culicoides* (Diptera: Ceratopogonidae) is considered the primary vector, while the role of mosquitoes in the transmission cycle is still unclear. This study evaluates the vector competence of 5 mosquito species common in Europe for OROV (old strain). While *Culex torrentium*, *Culex pipiens* biotype *pipiens*, *Aedes aegypti*, and *Aedes japonicus* showed no transmission, *Aedes albopictus* demonstrated low transmission at higher temperatures. Temperature-based risk analysis for areas with established *A albopictus* populations suggests that regions around the Mediterranean are at potential risk for OROV transmission.

Keywords. Oropouche virus; vector competence; transmission risk.

The arbovirus Oropouche virus (OROV; *Orthobunyavirus oropoucheense*) has become a significant health concern in Central and South America [1, 2]. In February 2024, the Pan American Health Organization/World Health Organization issued an alert for an increasing number of human OROV infections. By the end of November 2024, >11 000 confirmed cases were reported, and for the first time, fatal outcomes and vertical transmission—likely leading to miscarriage and microcephaly—were described [1]. This prompted the Pan American Health Organization/World Health Organization to raise the alert level from medium to high, which emphasizes the urgency of preparedness, surveillance, and research to address critical knowledge gaps about OROV.

OROV belongs to the genus *Orthobunyavirus* (family Peribunyaviridae), the largest genus of known arboviruses. *Culicoides* (Diptera: Ceratopogonidae) is considered the primary vector of OROV, while the role of mosquitoes in the transmission cycle has not been clarified yet [2]. The few existing laboratory studies demonstrated low to no vector competence for *Culex quinquefasciatus*, *Culex pipiens*, *Anopheles quadrimaculatus*, *Aedes aegypti*, or *Aedes albopictus* [3–5]. However, there is a significant lack of knowledge, particularly regarding mosquitoes from Europe. At the same time, several travelers infected with OROV were identified in Europe importing OROV from Central and South America [1]. Therefore, we evaluated the vector competence for OROV for 5 mosquito species in Europe, including the native species *C pipiens* biotype *pipiens* and *Culex torrentium* as well as the invasive species *A albopictus*, *Aedes japonicus*, and *A aegypti*.

METHODS

Vector Competence Studies

Five species were investigated: 2 laboratory strains, *A albopictus* (from a population in Heidelberg, Germany, that has been in colony since 2016–2017) and *A aegypti* (as provided by Bayer and established several decades ago with origin unclear), as well as 3 species collected in the field in 2024 from the egg rafts of *C pipiens* biotype *pipiens* and *C torrentium* (Hamburg, Germany) and the eggs of *A japonicus* (Weinheim, Germany). A blood meal was offered via a feeder with a final concentration of 8.9×10^7 plaque-forming units/mL of OROV strain TR 9760, which had been isolated from a febrile patient in 1955 and was currently produced in Vero cells (CCL-81) and titrated via the 50% tissue culture infectious dose (both obtained from ATCC); afterward, a salivation assay was performed as described by Heitmann et al [6]. Engorged females were incubated at 70% humidity at an oscillating temperature of 27 ± 5 °C, mimicking day-night variations (light:dark period of 12:12 hours). At 14 or 21 days postinfection (dpi), the following were determined: infection rate (IR; number of OROV-positive bodies per number of engorged females), transmission rate (TR; number of specimens with OROV-positive saliva per number of specimens with OROV-positive body), and transmission efficiency (TE; number of specimens with OROV-positive saliva per number of engorged specimens). RNA was quantified by quantitative reverse transcription polymerase chain reaction for the L segment as reported by Ciuoderis et al [7] but with VetMAX Xeno (Applied Biosystems, Thermo Fisher Scientific Corporation) as positive control.

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Risk Analysis

The potential risk map for OROV transmission in Europe was estimated by identifying areas presenting the temperature data used in the vector competence studies and the areas already colonized by *A albopictus*. Daily mean temperature data (European Reanalysis and Observations for Monitoring, E-OBS version 31.0e) were obtained from http://www.ecad.eu [8]. These E-OBS data, available on a 0.1° regular latitude-longitude grid, were extracted for a 5-year period from 2020 to 2024. For each grid cell, the number of days per year with the preceding 14 days having a mean daily temperature ≥24 °C or 21 days having a mean daily temperature ≥27 °C, respectively, were calculated. The annual values were then averaged over the 5-year period. The distribution data of *A albopictus* at the regional administrative level (NUTS3), as of July 2024, were obtained from the European Centre for Disease Prevention and Control [9]. All calculations and visualizations were conducted with the program R [10].

RESULTS

OROV Vector Competence Studies for Mosquitoes From Europe

No infection was observed for *A japonicus* (Table 1). Only 1 time point, 21 dpi, was tested for *A japonicus* due to the limited availability of specimens from the field. The 4 other tested species were susceptible for OROV infection. Positive specimens of *A aegypti*, *C pipiens* biotype *pipiens*, and *C torrentium* were exclusively detected at 14 dpi but not 21 dpi, with IRs of 6.5%, 14.3%, and 15.0%, respectively. In contrast, *A albopictus* showed an increasing IR from 6.7% at 14 dpi to 27.6% at 21 dpi at 27 °C. At 24 °C, the IR decreased from 6.4% at 14 dpi to 2.9% at 21 dpi. At 21°, positive specimens of *A albopictus* were also exclusively detected at 14 dpi, with an IR of 6.7%. At the lowest investigated temperature of 18 °C, no positive specimens were detected. Transmission of OROV was observed for *A albopictus* at 27 °C at 21 dpi, with a TR of 12.5% and a TE of 3.5%, and at 24 °C at 14 dpi, with a TR of 25% and a TE of 2.4%. Notably, with 9.5 (27 °C) and 8.4 (24 °C) RNA $\log_{10}$ copies per mosquito, the specimens with the positive saliva had the highest titers among all tested bodies.

Table 1. Infection and Transmission Rates of 5 Mosquito Species Experimentally Infected With OROV

Temperature, °C: Species	No.[a]	IR[b]	Body Titer $\log_{10}$ RNA Copies / Mosquito Specimen, Mean (95% CI)	TR[c]	TE[d]
27 ± 5					
Cx pipiens biotype *pipiens*					
14 dpi	21	14.3	7.4 (6.9–7.9)	0.0	0.0
21 dpi	26	0.0	NA[e]	0.0	0.0
Cx torrentium					
14 dpi	20	15.0	7.1 (3.2–11.0)	0.0	0.0
21 dpi	16	0.0	NA	0.0	0.0
A aegypti					
14 dpi	31	6.5	4.6 (3.7–5.5)	0.0	0.0
21 dpi	29	0.0	NA	0.0	0.0
A japonicus					
21 dpi	30	0.0	NA	0.0	0.0
A albopictus					
14 dpi	31	3.2	8.0 (NA)	0.0	0.0
21 dpi	29	27.6	6.9 (5.5–8.3)	12.5	3.5
24 ± 5					
A albopictus					
14 dpi	41	6.4	7.1 (6.0–8.1)	25.0	2.4
21 dpi	35	2.9	7.0 (NA)	0.0	0.0
21 ± 5					
A albopictus					
14 dpi	30	6.7	5.3 (3.1–7.5)	0.0	0.0
21 dpi	28	0.0	NA	0.0	0.0
18 ± 5					
A albopictus					
14 dpi	30	0.0	NA	0.0	0.0
21 dpi	31	0.0	NA	0.0	0.0

Abbreviations: dpi, days postinfection; IR, infection rate; NA, not applicable; OROV, Oropouche virus; TE, transmission efficiency; TR, transmission rate.

[a]No. of engorged specimens

[b]Infection rate: No. of OROV-positive bodies per number of engorged females

[c]Transmission rate: No. of specimens with OROV-positive saliva per number of specimens with OROV-positive bodies.

[d]Transmission efficiency: No. of specimens with OROV-positive saliva per engorged specimens.

[e]Not applicable for the mean if there were no positive specimens or for the 95% CI if there was only 1 positive body.

Risk Analysis

The average number of days per raster cell (2020–2024) with the preceding 21 days having a mean daily temperature ≥27 °C was observed only for restricted areas within the current distribution of *A albopictus* in Europe (Figure 1). In contrast, 14 days with a mean daily temperature ≥24 °C were observed for a much wider area. Regions directly bordering the Mediterranean Sea were the most suitable risk areas, with the longest periods observed for Spain, Southern Italy, Greece, and Turkey.

DISCUSSION

Our study shows that 2 common European *Culex* species, *C pipiens* biotype *pipiens* and *C torrentium*, and the invasive *A aegypti* are susceptible to OROV infection on a low level. No transmission was detected for the tested *Culex* species and *A aegypti*. This is consistent with results for *C tarsalis* and *C pipiens* biotype *molestus/C pipiens* [3–5]. Interestingly, Payne et al showed transmission of OROV by *C pipiens* but only for the current outbreak OROV strain, not the old OROV strain, which was used in our study [5]. As the number of investigated specimens is low, further studies are needed to interpret these results. Studies on *C quinquefasciatus* are more variable, ranging from no infection to low transmission rates after an artificial blood meal [3, 5]. In line with previous studies, the invasive *A aegypti* and *A japonicus* showed no transmission of OROV [3–5].

Transmission of OROV was observed only for *A albopictus*. Interestingly, transmission was exclusively observed 21 dpi at 27 °C and 14 dpi at 24 °C. This shows that transmission by *A*

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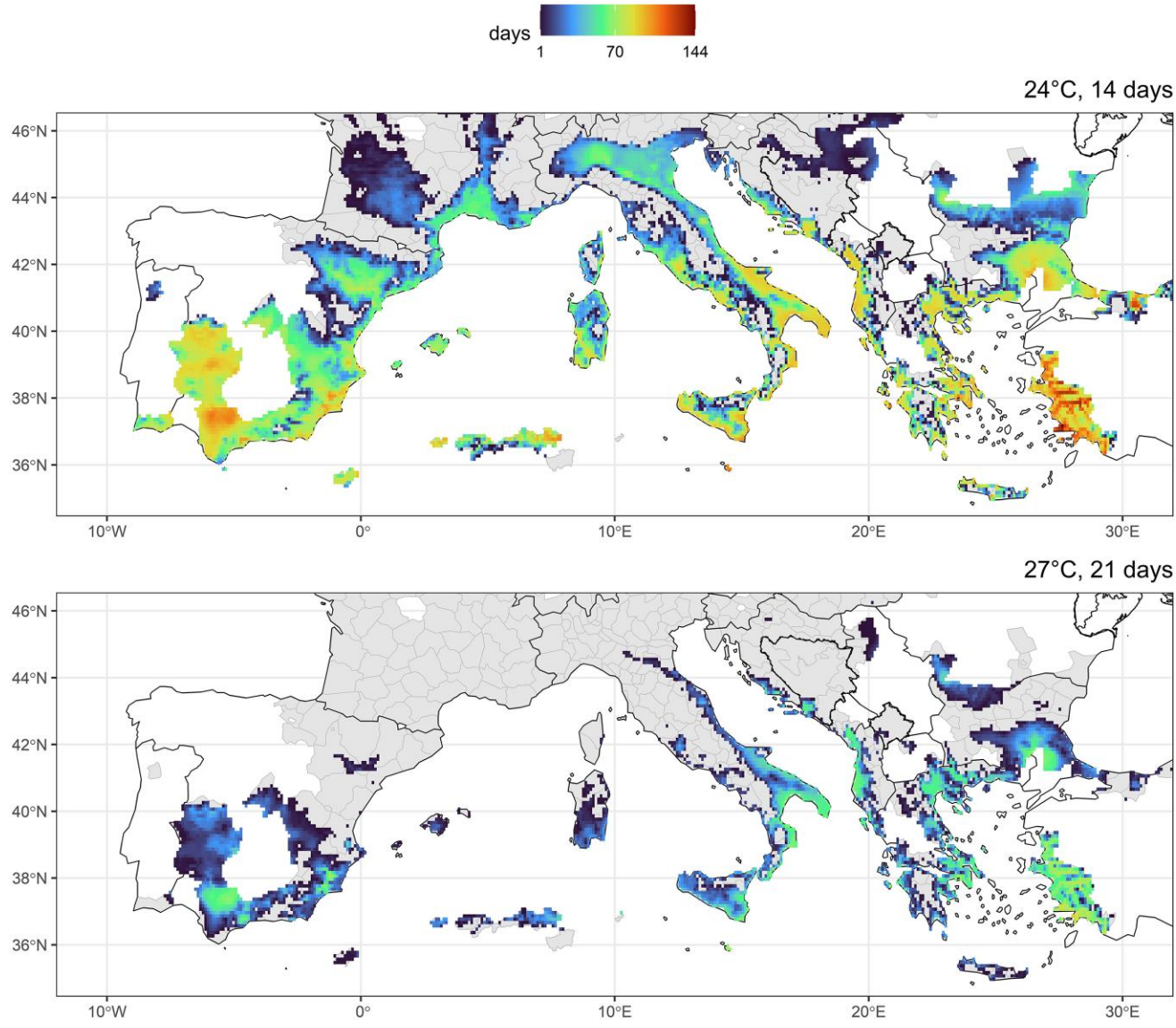

Figure 1. Risk assessment of Oropouche virus transmission in Europe. Average number of days per year with the preceding 14 days having a mean daily temperature ≥24 °C or 21 days having a mean daily temperature ≥27 °C for the 2020–2024 period for the current distribution of *Aedes albopictus* in Europe. Areas with established populations but without reaching this temperature condition are indicated in gray.

albopictus is possible at both temperatures but rarely. Due to the small number of analyzed individuals (n = 30), an exact temporal prediction is not possible. Further experiments on the extrinsic incubation period should be performed. Our results are in contrast to the literature, where no or low OROV infection but no transmission by *A albopictus* was detected after an artificial blood meal [3, 5]. Such differences in vector competence of different *A albopictus* populations are also shown for Zika virus and chikungunya virus [11]. Similarly, different virus strains can result in a different transmission potential of mosquito populations [11]. Additionally, the way of infection (live animal, artificial blood meal, intrathoracic injection) has an important influence on the infection with OROV, whereas the artificial blood meal may be less effective as other methods, as reviewed by Gallichotte et al [3]. The current OROV outbreak lineage is identified as an intraspecies reassortant of 2 OROV lineages [12]. It has been discussed whether the new virus variant has possibly resulted in a worsening of the clinical presentation of human OROV infections and allowed the better transmission by mosquitoes [3, 5]. However, first studies comparing an old OROV strain with the new variant do not show implications for an increased competence of mosquitoes [5]. Further studies are necessary to clarify if the transmission capability of vectors has changed and if the spectrum of potential vectors has expanded.

The vector competence of an *A albopictus* population from Central Europe for OROV highlights the need to assess the

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risk of autochthonous OROV transmission in Europe, although the unclear trend across temperatures and days postinfection indicates that the species is probably not an extremely good vector for OROV. However, the widespread establishment of *A albopictus* increases the risk of transmission of tropical viruses, as observed for local outbreaks of dengue, Zika, and chikungunya virus, all linked to infected travelers and established populations of *A albopictus* [13]. Long-term local establishment of these viruses has not been observed so far and is unpredictable for OROV—that is, although several species are considered amplifying and reservoir hosts for OROV, their exact epidemiologic role is not well understood [14]. During the huge OROV outbreak in Central and South America in 2024, travelers infected with OROV were also detected in various European countries where established populations of *A albopictus* exist [1]. A joint analysis of the current vector distribution and temperature data indicates that regions around the Mediterranean Sea are at higher risk of autochthonous OROV transmission, with hotspots in Spain, Italy, and Turkey having longer periods of average daily temperatures >24 °C.

Notes

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Author contributions. S. J., R. L., and A. H. conceived and designed the experiments. S. J., P. H., A. M., U. L., and A. H. performed the laboratory experiments. R. L., H. J., and N. B. performed fieldwork. R. L., P. H., and A. H. analyzed the data. R. L. and A. H. ensured the funding and contributed reagents, materials, and analysis tools. S. J., R. L., and A. H. critically discussed the results and wrote the original draft. P. H., A. M., U. L., H. J., and N. B. critically revised and edited the manuscript. All authors read and approved the final manuscript for publication.

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Paper III

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Vector competence of mosquitoes from Europe for Tahyna virus

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Tahyna virus (TAHV) was the first mosquito-borne virus isolated in Europe, and has since been found throughout Eurasia and Africa. Infections are mostly asymptomatic but can cause "Valtice fever", characterized by influenza-like symptoms, mainly in children, with severe cases occasionally causing neurological symptoms. The virus is maintained in an enzootic cycle between small mammals and mosquitoes. Recent and comprehensive studies of vector competence for TAHV are scarce. To fill this gap, and to better understand the transmission cycle of TAHV, we studied ten taxa (*Ae. aegypti*, *Ae. albopictus*, *Ae. japonicus japonicus*, *Ae. koreicus*, *Ae. rusticus*, *Cx. pipiens* biotype *pipiens*, *Cx. torrentium*, *Cs. morsitans/fumipennis*, *An. daciae*, and *An. stephensi*) by orally infecting them with TAHV. All taxa were susceptible to TAHV infection. Additionally, the invasive species *Ae. albopictus*, and *Ae. rusticus*, a species native to Europe, were able to transmit the virus at 27 ± 5 °C, with transmission efficiencies of 3.3% and 14.3%, respectively. Therefore, it is plausible for TAHV to be transmitted by *Ae. albopictus* and *Ae. rusticus* in natural settings in Europe. At a lower temperature of 24 ± 5 °C, infection and transmission decreased in *Ae. albopictus*. This data will allow future risk models and early warning systems to better predict TAHV transmission.

Keywords *Aedes albopictus*, *Aedes rusticus*, Tahyna virus, Vector competence

In the last decades, the global health significance of arthropod-borne viruses (arboviruses) has increased dramatically. Recent outbreaks of dengue, chikungunya, Rift Valley fever and Zika viruses have highlighted the threat these viruses pose to human and animal health. The health impact of arboviruses is expected to increase in the future due to globalization and urbanization[1,2]. Tahyna virus (TAHV) was the first identified mosquito-borne virus in Europe, isolated from *Aedes vexans* in 1958 in former Czechoslovakia[3]. TAHV is a member of the genus *Orthobunyavirus*, within the *Peribunyaviridae* family[4]. All members of this genus are arthropod-borne, besides biting midges or ticks, different mosquito species are considered the main vector. The genus is further subdivided into 18 different serogroups according to the virus' genetic and antigenic relationship. TAHV belongs to the California serogroup, named after the California encephalitis virus[5]. Like all members of the *Peribunyaviridae* family, TAHV is enveloped and has a tri-segmented negative-sense RNA genome, showcasing remarkable conservation[4,6]. *Orthobunyaviruses* can exchange those genome segments during coinfection, a process called reassortment which can potentially lead to heightened pathogenicity[7]. Reassortment has been observed between La Crosse virus and TAHV in *Aedes triseriatus* in laboratory settings[8]. As TAHV co-circulates with other California serogroup viruses, studying its vector competence is crucial to assess potential reassortment risks.

TAHV is maintained in an enzootic cycle. Based on laboratory infection, and serological studies, the most likely amplifying host is the European hare[9]. However, many different mammals, including non-human primates, have shown viremia and seroconversion in laboratory studies[10]. Antibodies to TAHV have also been detected in different wildlife and livestock species, including deer, wild boars, mouflons, rodents, sheep, horses, cattle, and pigs, but no symptoms have been reported in these species[10,11]. Historically, hedgehogs were thought to be a possible overwintering reservoir for the virus, however, these findings are being questioned, and the true role of hedgehogs in the transmission cycle remains unclear[9,11,12]. TAHV causes a human disease, known as "Valtice fever", first documented in the 1960s[13,14]. The majority of infections are asymptomatic. Symptoms are observed mainly in children in late summer and early autumn. The disease presents with flu-like symptoms, myalgia,

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and other symptoms typical for California serogroup viruses, including headache, nausea, conjunctivitis, stiff neck, and can in rare cases cause meningitis and encephalitis[10]. It has been shown in mice that the degree of neuroinvasiveness varies depending on the strain, however, no data on the medical relevance of different TAHV strains is available[6]. To this day, no fatalities have been recorded[10]. As it is not a notifiable disease and the symptoms are not specific, the number of people affected is thought to be drastically under-reported. While TAHV is predominantly found in Europe, there have also been detections of TAHV in vertebrates and mosquitoes in Asia (including Russia) and Africa[15]. However, reports from African countries primarily rely on serology, therefore, raising the possibility that the findings of TAHV are actually due to cross-reactivity with closely related California serogroup viruses, such as Lumbo virus[10]. Focusing on the situation in Europe, studies from the 1960s in former Czechoslovakia reported high TAHV seroprevalence, reaching 80–90% among elderly residents in endemic areas and up to 30% in regions with mass mosquito occurrence[14,16,17]. More recently, 10% of patients with unsolved neuroinvasive disease in Croatia between 2017 and 2022, and 0.3% of blood donors in the Alps (Austria, Italy) in 2014 tested positive for TAHV antibodies[18,19]. TAHV has not been detected in Germany in recent years, and the reasons for its absence remain unclear. One possible explanation is the lack of surveillance for this arbovirus, meaning the virus could be circulating at undetected levels. The lack of clarity about which mosquito species transmit the virus complicates targeted monitoring efforts.

After its first isolation from *Ae. vexans*, TAHV has since been identified in various species of the genera *Aedes*, *Culex*, *Culiseta*, and *Anopheles*[10,17,19–24]. However, studies for vector competence are rare. Differentiating between the detection of viruses in field-collected specimens and the species' ability to transmit the virus by excreting infectious viral particles in their saliva is crucial. Only the latter can serve as direct evidence for vector competence of a particular mosquito species towards a certain virus. A European study assessed vector competence for *Ae. vexans*, which was shown to be capable of transmitting the virus[25]. In a separate study conducted in China, both *Ae. albopictus* and *Cx. pipiens pallens* were tested, however, only *Ae. albopictus* was found to transmit the virus[26]. Furthermore, it has been proven that *Ae. vexans* can transmit TAHV transovarially to its offsprings[27]. Overwintering of TAHV-infected mosquitoes has been shown for *Cs. annulata* adults as well as *Ae. vexans* eggs[22,27].

To address the gap of knowledge concerning the vector competence of mosquitoes for TAHV we conducted this study with a diverse range of mosquito taxa, including both those native and exotic in Europe. We also investigated the effect of temperature on the risk of transmission by *Ae. albopictus*. The results of this study will help to understand the transmission cycle of TAHV.

Results

Feeding and survival rates

Feeding rates (number of engorged mosquitoes per number of mosquitoes offered the blood meal) varied between 18.7% and 71.4% among all taxa, with no consistent pattern observed across genera (Table 1). Survival rates (number of alive mosquitoes after incubation period per number of engorged mosquitoes) were generally high (Table 1), except for *An. stephensi* and *Cs. morsitans/fumipennis* which exhibited a higher mortality rate. Given the limited availability of *Cs. morsitans/fumipennis*, the incubation period for these specimens was shortened to seven days.

Infection and transmission

TAHV was able to infect all of the tested taxa, with varying infection rates (Table 2). The lowest infection rates were observed in both species of the genus *Culex*, and in the species *Ae. j. japonicus*, with 3.3% of specimens testing positive for TAHV RNA (Table 2). *Aedes albopictus* incubated at 27 °C and *Ae. rusticus* displayed the highest infection rates among all taxa, with 70.0% and 71.4% respectively (Table 2). *Anopheles daciae* was excluded from this comparison due to low sample size. *Aedes albopictus* and *Ae. rusticus* were also the only ones able to transmit TAHV, with transmission efficiencies of 3.3% for *Ae. albopictus* and 14.3% for *Ae. rusticus* at 27 °C. Additionally, these two species showed the highest mean number of viral RNA copies per mosquito body,

Mosquito taxon	Incubation period [days]	Temperature [°C]	Total number of mosquitoes	Feeding rate [%]	Survival rate [%]
Ae. aegypti	14	27 ± 5	187	18.7 (35/187)	91.4 (32/35)
Ae. albopictus	14	24 ± 5	88	54.5 (48/88)	81.3 (39/48)
	14	27 ± 5	85	50.6 (43/85)	79.1 (34/43)
Ae. j. japonicus	14	27 ± 5	190	25.3 (48/190)	70.8 (34/48)
Ae. koreicus	14	27 ± 5	7	71.4 (5/7)	100 (5/5)
Ae. rusticus	14	27 ± 5	12	66.7 (8/12)	87.5 (7/8)
Cx. pipiens biotype *pipiens*	14	27 ± 5	73	63.0 (46/73)	95.7 (44/46)
Cx. torrentium	14	27 ± 5	84	41.7 (35/84)	91.4 (32/35)
Cs. morsitans/fumipennis	7	27 ± 5	31	48.4 (15/31)	33.3 (5/15)
An. daciae	14	27 ± 5	2	50.0 (1/2)	100 (1/1)
An. stephensi	14	27 ± 5	175	29.7 (52/175)	51.9 (27/52)

Table 1. Total input number of mosquitoes offered a blood meal per taxon and temperature. Feeding rate: number of engorged mosquitoes per total input. Survival rate: number of alive mosquitoes after incubation period per number of engorged mosquitoes.

Mosquito taxon	Temperature [°C]	Number of tested mosquitoes	Infection rate [%]	Transmission efficiency [%]	Mean body titer as vRNA copies per mosquito (95% confidence interval)
Ae. aegypti	27 ± 5	30	26.7 (8/30)	0.0 (0/30)	3.09×10^8 (0, 9.95×10^8)
Ae. albopictus	24 ± 5	30	23.3 (7/30)	0.0 (0/30)	2.00×10^8 (0, 5.48×10^8)
	27 ± 5	30	70.0 (21/30)	3.3 (1/30)	9.05×10^8 (3.47×10^7, 1.78×10^9)
Ae. j. japonicus	27 ± 5	30	3.3 (1/30)	0.0 (0/30)	1.12×10^6 (na)
Ae. koreicus	27 ± 5	5	40.0 (2/5)	0.0 (0/5)	2.46×10^7 (8.08×10^6, 4.11×10^7)
Ae. rusticus	27 ± 5	7	71.4 (5/7)	14.3 (1/7)	1.29×10^{10} (0, 4.82×10^{10})
Cx. pipiens biotype *pipiens*	27 ± 5	30	3.3 (1/30)	0.0 (0/30)	7.54×10^6 (na)
Cx. torrentium	27 ± 5	30	3.3 (1/30)	0.0 (0/30)	1.54×10^7 (na)
Cs. morsitans/fumipennis	27 ± 5	5	60.0 (3/5)	0.0 (0/5)	6.05×10^8 (0, 2.90×10^9)
An. daciae	27 ± 5	1	100 (1/1)	0.0 (0/1)	1.26×10^6 (na)
An. stephensi	27 ± 5	25	12.0 (3/25)	0.0 (0/25)	3.54×10^6 (0, 7.23×10^6)

Table 2. Vector competence studies per taxon and temperature. Infection rate: number of positive bodies per total number of tested mosquitoes. Transmission efficiency: number of positive salivas per total number of tested mosquitoes. The mean body titer is given as the mean number of viral RNA copies per mosquito body, with 95% confidence interval. Na: Non applicable, confidence interval could not be calculated due to a singular positve body sample.

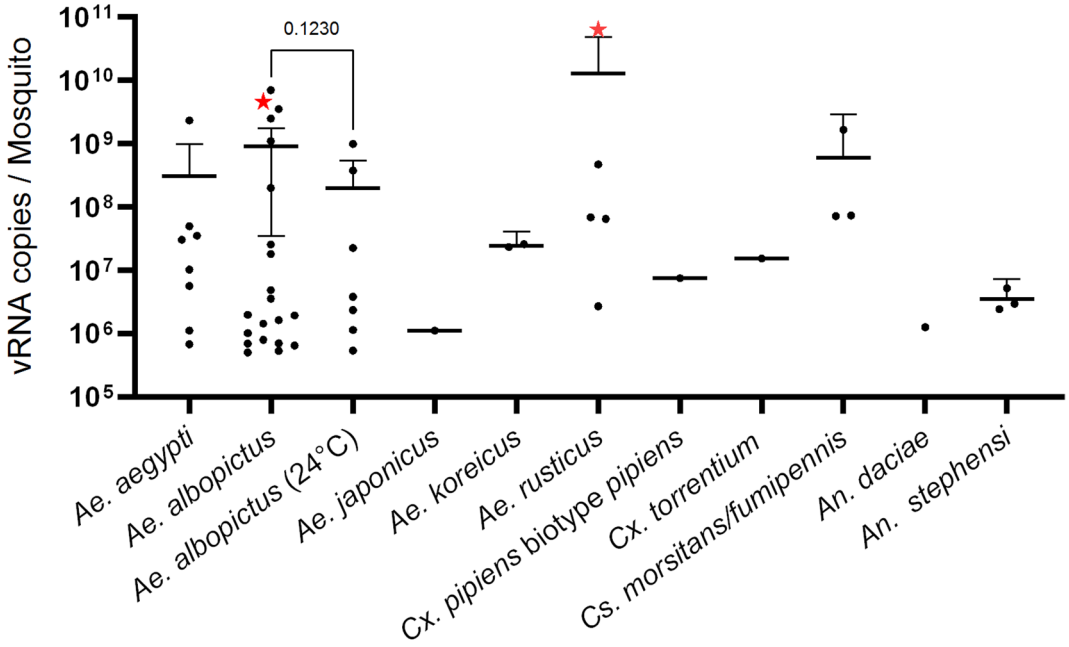

Fig. 1. The concentration of viral RNA copies per mosquito body at 27 °C, unless indicated otherwise. Only data above the limit of detection is shown. Dots represent single mosquito specimens. The mean and 95% confidence interval are shown. Red stars indicate specimens with a corresponding positive saliva.

with 9.05×10^8 and 1.29×10^{10} respectively (Fig. 1; Table 2). Transmission efficiency was assessed by salivation and cell culture followed by RT-qPCR on cell supernatant of positive samples. For *Ae. rusticus*, the specimen with the highest body titer was also the one with the positive saliva sample. In contrast, multiple *Ae. albopictus* specimens had similar or even higher body titers than the one with a positive saliva, yet none of these specimens had viral particles in their saliva (Fig. 1).

To investigate the potential influence of temperature on the infectivity and transmission of TAHV, an additional experiment with *Ae. albopictus* was conducted at an incubation temperature of 24 °C. The results revealed that reducing the mean incubation temperature by 3 °C, significantly decreased the infection rate from 70.0 to 23.3% (Fisher's exact test; $p = 0.0006$) (Table 2). In addition to the reduced infection rate, the transmission efficiency also decreased from 3.3 to 0.0% (Fisher's exact test; $p > 0.9999$), indicating that no transmission occurred at the lower temperature. The mean body titer also decreased from 9.05×10^8 to 2.00×10^8 (Welch's test; $p = 0.1230$) (Fig. 1; Table 2). However, neither of these changes reached statistical significance. During the

salivation assay of *An. stephensi*, two specimens were damaged beyond usability, resulting in a lower number of tested individuals ($n = 25$; Table 2) compared to the number of live mosquitoes initially available ($n = 27$; Table 1).

Discussion

In this study, we found that all ten tested mosquito taxa of the genera *Aedes*, *Culex*, *Culiseta*, and *Anopheles* were susceptible to TAHV infection. *Aedes albopictus* and *Ae. rusticus* were shown to be able to transmit the virus at an efficiency of 3.3% and 14.3%, respectively. Notably, both of the species belong to the *Aedes* genus. This aligns with previous findings on *Ae. vexans*, the only other mosquito species from Europe confirmed as a competent vector for TAHV in laboratory studies[25]. Additionally, several other *Aedes* species, such as *Ae. caspius* and *Ae. sticticus*, are believed to play important roles in the transmission cycle, as TAHV has been detected in field-collected specimens[10,28].

Vector competence is an important but not exclusive factor to determine the capability of a specific vector to transmit a virus in nature known as vector capacity (VC). The vector capacity is calculated using following formula: $VC = ma^2bp^n/-\ln p$, and in addition to vector competence (b) includes number of females (m), daily blood feeding rate (a), probability of daily survival (p), and the extrinsic incubation period (n)[29]. To serve as a bridge vector, a mosquito species must feed on both, the amplifying host and humans. *Aedes albopictus* and *Ae. rusticus* are known to bite humans[30,31]. Besides humans as primary blood source, *Ae. albopictus* also feeds on small mammals, including rabbits, which can act as a host for TAHV. Though less studied, *Ae. rusticus* is also reported to feed on non-human mammals[30,32]. *Aedes vexans*, the only other confirmed vector for TAHV in Europe, on the other hand is known to prefer non-human mammals, but also bites humans[32]. Overall, the feeding behavior of these species makes it plausible for them to transmit TAHV from zoonotic hosts to humans. Vector competence is a crucial part of models predicting the risk of arbovirus transmission[33]. By identifying which mosquito species can transmit TAHV at which temperature and efficiency, the data can help to calculate the basic reproduction number and therefore predict risk areas.

Our study determined that *Ae. albopictus* and *Ae. rusticus* are able to transmit TAHV at a mean temperature of 27 °C. Combined with the virus' short extrinsic incubation period of two days[26], i.e. the time between a mosquito ingesting an infectious blood meal and the appearance of infectious virus in its saliva, it suggests that transmission could occur in Germany. In Southern Europe, mean temperatures of 27 °C are more common, which would increase the possibility of transmission in these regions[34]. *Aedes albopictus* was not able to transmit the virus at the lower tested temperature of 24 °C. Due to the limited availability of field-caught specimens, transmission of TAHV by *Ae. rusticus* could not be assessed at temperatures below 27 °C, however, future studies should prioritize investigating its transmission potential at lower temperatures, as this species is most commonly encountered in early spring, when average temperatures typically fall below 27 °C[35].

Only two studies have investigated the vector competence of *Ae. rusticus*, showing that this species is susceptible to infection by Rift Valley fever virus (order *Bunyavirales*) and infection and transmission of West Nile virus but not Usutu virus (order *Amarillovirales*)[31,36]. Our study identified *Ae. rusticus* as a competent vector for an additional arbovirus, making TAHV only the second virus known to be transmittable by this species. *Aedes rusticus* prefers forested habitat, is widespread and native from Northern Africa through Western, and Central Europe, up to Northern Europe[37].

Mosquitoes with infectious TAHV in their saliva exhibited two of the highest viral RNA titers in their bodies. However, some mosquitoes with similar or even higher body titers were unable to transmit the virus. Body titers of similar orders of magnitude have been reported in other vector competence studies[26,31,38,39]. However, most studies do not stratify body titers based on the infectivity of the mosquito's saliva, limiting direct comparisons of this observation with other research. In this study, the transmission efficiency was assessed by detecting infectious virus in mosquito saliva rather than relying on viral RNA detection. While detection of viral RNA is commonly used, it does not distinguish between infectious and non-infectious viral particles, and might therefore overestimate transmission efficiency. A previous study on other arboviruses showed that viral RNA can be detected earlier and for longer in saliva, compared to infectious virus[40]. To avoid this limitation, we prioritized virus detection through cell culture.

Some limitations must be considered when interpreting the results of this study. The relatively low numbers of investigated specimens of *Ae. koreicus*, *Ae. rusticus*, *Cs. morsitans*, and *An. daciae* limits the precision of the calculated infection rates and transmission efficiencies, potentially leading to under- or overestimation of their vector competence. Statistical statements are therefore not possible for these species, however, the findings remain valuable given the current scarcity of data in this area. Screening for pre-existing TAHV infections in wild-caught mosquitoes was feasible for only two taxa (*Cx. pipiens* biotype *pipiens*, *Cx. torrentium*), therefore an overestimation of infection rate and transmission efficiency for the remaining taxa cannot be fully excluded. However, we consider this risk to be low, as TAHV is not known to circulate widely in mosquito populations in Germany. Additionally, the study was conducted under controlled laboratory conditions, which may not fully replicate the complex environmental and ecological factors influencing virus transmission in natural settings.

Conclusion

TAHV can be transmitted at low efficiency by invasive *Ae. albopictus*, and at higher efficiency by *Ae.rusticus*, a species native to Germany and other European countries. Lower temperature reduces both the infectivity and transmission of TAHV in *Ae. albopictus*. Future studies should investigate other widespread *Aedes* species native to Germany, particularly *Ae. vexans*, *Ae. caspius*, *and Ae. sticticus*, to better understand their potential as TAHV vectors.

Materials and methods

Mosquitoes from established laboratory colonies, field-caught adults, and field-caught eggs were used in this study. Mosquitoes were kept at 26 °C and a 70% relative humidity with a 12:12 light: dark photoperiod including 30 min of twilight. *Aedes aegypti* (Bayer company, Leverkusen, Germany) and *Anopheles stephensi* (strain SxK Nijmegen) are long established laboratory colonies. The *Ae. albopictus* colony was established in 2016/17 from eggs collected in Freiburg, Germany. Eggs of *Ae. koreicus* (Lat: 50.078411, Long: 8.251675) were collected in 2023, *Ae. j. japonicus* (Lat: 49.524, Long: 8.672) in 2023 and 2024 with ovitraps in South-West Germany. Adults of *Aedes rusticus* (Lat: 49.312851, Long: 8.301493) and *Anopheles daciae* (Lat: 49.291527, Long: 8.456074) were collected with CO_2 baited Encephalitis Vector Survey trap (EVS trap) (BioQuip Products, Rancho Domingues, Califonia, USA) in 2024 in Southern Germany. Adults of *Cs. morsitans/fumipennis* were collected using an aspirator in 2024 from a forest near Oldenburg, Lower Saxony, Germany (Lat: 53.159019, Long: 8.125420). Egg rafts of *Culex torrentium* and *Culex pipiens* biotype *pipiens* were collected in the field in northern Germany (Lat: 53.467821, Long: 9.831346) in 2023. The egg rafts were reared individually and 5–10 larvae from each raft were used to identify the *Culex* species by molecular assays as described by Rudolf et al.[41]. To distinguish *An. daciae* within the *An. maculipennis* complex, it was identified to species level by molecular typing following Lühken et al.[42]. *Culiseta morsitans/fumipennis* could not be identified to species level. Therefore, the terms taxon and taxa are used when referring to this classification. All morphological identification was performed according to the key of Becker et al., 2020[43]. To exclude natural arbovirus infections in field-caught specimens, 10 randomly selected adult mosquitoes per taxon were tested using pan-Orthobunya, pan-Flavivirus, and pan-Alphavirus PCRs[44–46]. All tested specimens were confirmed negative. This screening was feasible for *Cx. pipiens* biotype *pipiens* and *Cx. torrentium*, but could not be conducted for the remaining wild-caught taxa due to limited availability of individuals.

Female mosquitoes were deprived of food for 24 to 48 h. Adult mosquitoes from laboratory colonies (*Ae. aegypti*, *Ae. albopictus*, *An. stephensi*) were aged three to 15 days, whereas adults from field-caught eggs (*Ae. koreicus*, *Ae. j. japonicus*, *Cx. torrentium*, *Cx. pipiens* biotype *pipiens*) were aged three to 18 days. The ages of field-caught adults (*Ae. rusticus*, *An. daciae*, *Cs. morsitans/fumipennis*) was unknown, however, they were infected within two to five days after capture. All experiments with live mosquitoes exposed to infectious virus were performed in an insectary of biosafety level 3 (Bernhard Nocht Institute for Tropical Medicine; Hamburg, Germany). Mosquitoes were housed in 50 mm x 100 mm cylindrical plastic tubes with corresponding plugs (Carl Roth; Karlsruhe, Germany). The mosquitoes were orally exposed to an infectious artificial blood meal, containing 50% human blood (type 0; expired blood preservations), 30% of an 8% fructose and 0.02% 4-aminobenzoic acid solution, 10% fetal bovine serum, and 10% virus stock (TAHV; Bardos et al.[3]. ; strain: Bardos92; >22 passage on BHK-J cells; stock produced: 25.07.2022; stored at -80 °C; 1.57×10^8 FFU/mL), via two cotton swabs and two 50 µL drops for two hours. 4-aminobenzoic acid is an amino acid present in plants, a precursor of folic acid, and regularly used in mosquito rearing[47], with no known interaction with viruses. The bloodmeal had a final viral titer of 1.57×10^7 FFU/mL. Fully engorged females were collected and incubated at 70% relative humidity and a temperature profile of 27 °C with a ± 5 °C fluctuation over 24 h, to simulate natural temperature variance. Additionally, *Ae. albopictus* specimens were incubated at 24 ± 5 °C. These temperature profiles followed a 12 h:12 h light: dark photoperiod, with the extreme temperatures in the middle of the respective photoperiod. Hereafter, the temperature profiles 27 ± 5 °C and 24 ± 5 °C will be referred to as 27 °C and 24 °C, respectively. A solution of 8% fructose and 0.02% 4-aminobenzoic acid was available ad libitum. After 14 days of incubation, transmission capability was analyzed via salivation assay, as described by Heitmann et al.[48]. In short, the legs and wings of mosquitoes were removed while anesthetized with CO_2. The proboscis was then inserted into a pipette tip containing phosphate-buffered saline (PBS). After 30 min, the mosquito was removed and stored at -80 °C until further analysis. The PBS/saliva mix was pipetted on BHK-J cells (*Mesocricetus auratus*, CCVL L 0179, Friedrich-Loeffler-Institute, Riems, Germany) and incubated for five days at 37 °C and 5% CO_2. If CPE was detected, the supernatant was subjected to RNA extraction using QIAmp Viral RNA Mini Kit (Qiagen; Hilden, Germany), according to the manufacturer's instructions. If possible, 30 mosquito specimens were tested per experimental condition for infection and transmission; otherwise, all available specimens were included. Examining a minimum of 30 specimens per condition is a widely accepted standard in vector competence studies, enabling the detection of transmission efficiencies (TE) as low as 3%. This sample size allows for the identification of biologically meaningful vector competence (TE ≥ 3%) while keeping experimental effort manageable[39]. The mosquito bodies, excluding wings and legs, were homogenized in 500 µL Dulbecco's Modified Eagle Medium, using a Bio-Vortexer (Biospec Products; Bartlesville, USA). Subsequently, the supernatant was subjected to RNA extraction using MagMAX CORE nucleic acid purification kit (Applied Biosystems, Thermo Fisher Scientific; Waltham, USA), according to the manufacturer's instructions.

Both the RNA from the supernatant of the cell cultures inoculated with saliva that showed cytopathic effect, and from the bodies, were tested for the presence of TAHV RNA, M-segment, via qRT-PCR using QuantiTect probe RT-PCR kit (Qiagen; Hilden, Germany), and VetMAX™ Xeno™ Internal PositiveControl (Applied Biosystems, Thermo Fisher Scientific; Waltham, USA). Reactions were performed in 20 µL reaction volume containing 1x reaction buffer, 1x RT-PCR mix, 1 µM forward primer (TGCTGGGAAACAGAATTTACTGAG), 1 µM reverse primer (TGGTGACTGTACATTCTCCTGAG), 0.2 µM probe (CY5-TCCTGCAACTCCTTACCC CATCAC-BBQ650), 1x Xeno™ VIC™ primer probe mix, 20,000 copies Xeno™ IPC RNA, and 2 µL template. The PCR conditions were as follows: 20 min 50 °C, 15 min 95 °C, and 45 cycles of 5 Sect. 95 °C and 60 Sect. 60 °C. A synthetic oligonucleotide (ATGTGCTGGGAAACAGAATTTACTGAGTACATCCAATTTAAACAGAG T GATEGGGTAAGGAGTTG CAGGATGAAAGACTCAGGAGAATGTACAGTCACCATCA) was used as a positive control and for quantification of the body titer. Samples were considered positive if a CY5 signal was detected and the calculated total body titer was above the below-listed limit of detection. Samples were considered negative when no CY5 signal but a positive VIC signal was detected.

The RT-qPCR was validated according to the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" guidelines set by Bustin et al.[49]. A ten-fold dilution series of the TAHV standard, ranging from 0.18 to 1.8×10^8 copies/µL was analyzed in five replicates using the specified RT-qPCR protocol. Following performance indicators were determined: limit of detection: 4.05×10^5 copies/mosquito body, with a standard deviation of 0.759 Cqs; linear dynamic range: detection limit to $\geq 4.05 \times 10^{10}$ copies/mosquito body (i.e. the fluorescence signal is in a linear proportion to the concentration in this range). The calibration curve had a coefficient of determination of 0.9962 with a slope of -3.351, and a y-intercept of 54.86. The PCR efficiency was 0.9906, i.e., 99.1% of the target molecules were amplified per cycle.

To determine statistically significant differences, statistical tests were performed using GraphPad Prism (version 10.3.1 for Windows, GraphPad Software, www.graphpad.com). A p-value less than 0.05 was considered significant.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on request.

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Author contributions

Conceptualization: SJ, AH; Methodology: JSC, SJ, AH; Formal analysis and investigation: PH; Writing - original draft preparation: PH; Writing - review and editing: all authors; Funding acquisition: JSC, AH; Resources: RL, FGS, CVdC, NB, HJ, HPP; Supervision: AH, SJ; All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Paper IV

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Not all West Nile virus lineages behave alike: vector competence and minimum infectious dose differences of lineages 1 and 2

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Abstract

The globally distributed arbovirus West Nile virus (WNV) continues to expand across Europe, with rising numbers of human cases and an increasingly broad geographic distribution. WNV is primarily transmitted by mosquitoes of the genus *Culex*. Out of nine WNV lineages, human pathogenicity has been clearly established for lineages 1 and 2, but differences in their transmission dynamics, such as minimal infectious dose and transmission efficiency, remain poorly understood. In this study, we investigated how viral lineage, mosquito species, and blood meal titer influence the vector competence of two primary WNV vectors from Europe, *Cx. pipiens* biotype *pipiens* and *Cx. torrentium*, as well as the invasive mosquito species *Aedes albopictus*. Female mosquitoes were fed with increasing blood meal titers containing either of the WNV lineages. After an incubation period of 14 days at a mean temperature of 24°C, mosquito body titers were quantified, and the presence of infectious viral particles in the saliva was assessed. Our results revealed clear differences between the two lineages. Lineage 2 resulted in higher transmission efficiencies across all three species and required lower infectious doses to cause transmission. Among the tested species, *Cx. torrentium* proved to be a highly competent vector (max. transmission efficiency = 30%, minimum infectious dose = 10^5 $TCID_{50}$/mL), despite its underrepresentation in research. These findings provide detailed insights into how viral lineage, mosquito species, and blood meal titer might shape WNV transmission, informing future risk assessments and efforts to mitigate WNV transmission in Europe.

Key Words

West Nile virus, lineage 1, lineage 2, transmission, blood meal titer, *Culex pipiens*, *Culex torrentium*, *Aedes albopictus*

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Introduction

West Nile virus (WNV), species *Orthoflavivirus nilense*, within the family *Flaviviridae* and the genus *Orthoflavivirus,* is an emerging mosquito-borne virus of public health concern in Europe [1,2]. Over the last decade, several outbreaks have been recorded, and the affected area has expanded into Central Europe, including Germany. The spread of WNV in Europe can, in large parts, be attributed to higher temperatures due to the ongoing global warming, which, among other effects, shortens the viral extrinsic incubation period. The geographic range of the virus is expected to expand further and could place up to 244 million people across Europe at risk of infection in the future [2,3]. Approximately 20% of WNV infections are symptomatic, causing West Nile fever, characterized by headache, fever, myalgia, and fatigue. Less than 1% develop neuroinvasive disease, such as meningitis or encephalitis, with a case fatality rate of up to 17% [4]. Vaccines for horses have been available since the early 2000s, but no human vaccine has progressed past phase II [5]. The largest epidemic recorded in Europe occurred in 2018, with 1548 confirmed autochthonous human cases and 166 deaths across eleven countries [6]. The most recent data from 2025 reports once again above-average numbers with 989 cases and 63 deaths in 14 countries [7].

WNV is maintained in an enzootic cycle between mosquitoes as vectors and reservoirs, and birds as amplifying hosts. Mosquitoes of the genus *Culex* are considered the main vectors of WNV, with *Cx. pipiens* s.s. being the most abundant in Europe. *Culex pipiens* s.s. can be further subdivided into two distinct biotypes: *pipiens* and *molestus* [4,8,9]. Recent data show that both biotypes, as well as *Cx. torrentium,* feed on avian, human, and nonhuman mammalian hosts [10]. In Central and Northern Europe, *Cx. torrentium* is predominant, but often not distinguished from *Cx. pipiens* s.s., despite its epidemiological importance [11]. Another species of interest is *Ae. albopictus*, the most widespread invasive mosquito in Europe. Although not considered an important WNV vector, the

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69 species is competent for WNV and exhibits an opportunistic feeding behavior, warranting
70 further research into its role in the WNV transmission cycle [12].
71 WNV can be subdivided into nine distinct lineages, six of which have been detected in
72 Europe. WNV lineage 1 (L1) and lineage 2 (L2) are most frequently associated with
73 diagnosed infections in humans and animals [13]. WNV-L1 was first detected in Europe
74 in the 1960s, presumably introduced by migratory birds, and has caused multiple
75 outbreaks since. In contrast, WNV-L2 was first described in Europe in 2004, likely also
76 introduced via migratory birds, and subsequently spread rapidly through Southern and
77 Central Europe, where it has become the dominant cause of human cases [14]. However,
78 WNV-L1 is still co-circulating in Europe, e.g. in Italy, and is the prevailing lineage in
79 other parts of the world [13].
80 The virus can also be transmitted to mammalian hosts, including humans and horses [4].
81 However, humans and horses are considered incidental dead-end hosts, as their viremia
82 is typically too low to further infect mosquitoes [4]. In contrast, viremia levels in avian
83 species vary by several orders of magnitude and can reach levels of more than 10^8 plaque-
84 forming units (PFU) per mL, which influences their potential to be infectious for
85 mosquitoes [15,16]. Therefore, mosquitoes encounter vastly different levels of viremia
86 when feeding on animals, making it important to understand how viremia influences
87 mosquito infection, and which viremia levels are sufficient to enable transmission (i.e.
88 the minimal infectious dose required for transmission, MID). A lower MID implies that
89 mosquitoes can become infectious after feeding from a broader range of hosts, as even
90 closely related birds can have widely different peak viremias [15]. Interestingly, a recent
91 report of a human co-infection with WNV lineages 1 and 3 described an unusually high
92 WNV-L1 RNA concentration of $10^{5.5}$ PFU equivalents/mL, raising the question of
93 whether humans can serve as amplifying hosts under very specific conditions [17].

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94 In this study, we aimed to improve the understanding of the differences between WNV-
95 L1 and L2 in terms of their infection dynamics in mosquitoes. We infected *Cx. pipiens*
96 biotype *pipiens*, *Cx. torrentium*, and *Ae. albopictus* with WNV-L1 or L2 at varying blood
97 meal titers. Using forced salivation assays, we assessed how blood meal titer, mosquito
98 body titer, and mosquito species influence the infection and transmission.

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Methods

Origins of mosquitoes, viruses, and cells

Three mosquito species were tested: *Ae. albopictus*, *Cx. pipiens* biotype *pipiens* and *Cx. torrentium*. The *Ae. albopictus* colony was established in 2016/17 from eggs collected in Freiburg, Germany. For both *Culex* species, egg rafts were field-collected in northern Germany (Lat: 53.467821, Long: 9.831346) in 2024 and 2025. Each egg raft was reared individually at 21°C, and five to ten larvae were used to molecularly determine the species and biotype, as described by Rudolf et al. [18]. Ten adults of each field-caught species were screened for underlying *Orthoflavi*-, *Orthobunya*-, and *Alphavirus* infections using pan-PCRs [19–21] and confirmed negative. All adult mosquitoes were kept at 70% relative humidity, 26°C, and a 12h:12h light:dark photoperiod, including 30 minutes of twilight each. Mosquitoes were fed an 8% fructose solution ad libitum. Two WNV strains were used: WNV strain TOS-09 of L1, isolated in 2008 from a human in Ferrera, Italy (accession no. HM641225, 7th passage, titer: 8.89×10^8 50% tissue culture infectious dose ($TCID_{50}$) /mL), and WNV strain 1382 of L2, isolated in 2018 from a blackbird in Berlin, Germany (accession no. MH986055, 6th passage, titer: 6.67×10^9 $TCID_{50}$/mL) [22]. Virus propagation and detection of infectious viral particles were performed using Vero cells (*Chlorocebus sabaeus*, ATCC, CCL-81).

Infection and salivation of mosquitoes

Female mosquitoes (2-15 days old) were deprived of food and water for 24h (*Aedes*) or 48h (*Culex*) prior to an artificial blood meal (50% human blood preservations (type 0), 30% of an 8% fructose solution, 10% fetal bovine serum, 10% virus suspension) for two hours via two 50 µL drops and two saturated cotton swabs per tube. All mosquito species were fed blood meals with 10^5, 10^6, and 10^7 $TCID_{50}$/mL of either WNV lineage, corresponding to viremia levels commonly found in wild birds [15]. *Culex torrentium*

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was additionally fed with a blood meal containing 10^4 $TCID_{50}$/mL WNV-L2, as there was still transmission observed at 10^5 $TCID_{50}$/mL. Fully engorged mosquitoes were incubated at 70% relative humidity, with a 12h:12h light:dark photoperiod, and at 24°C with a ±5°C fluctuation to simulate the natural daily light and temperature change. The temperature extrema were synchronized with the corresponding light and dark phases. An 8% fructose solution was available ad libitum. After 14 days, a salivation assay was performed as previously described [23]. Briefly, wings and legs were removed, and the proboscis was inserted into a capillary tube for 30 min. The saliva solution was placed on Vero cells and incubated (37°C; 5% CO_2) for seven days. For each experimental condition (viral lineage, mosquito species, blood meal titer), the aim was to analyze 30 mosquito specimens, allowing the identification of transmission efficiencies (TE) of ≥3.33%.

Downstream processing and detection of WNV

Mosquito bodies, excluding wings and legs, were homogenized individually in 500 µL medium and subjected to RNA extraction using the MagMAX CORE Nucleic Acid Purification Kit (Applied Biosystems; USA). If cells showed cytopathic effect, RNA of the supernatant was extracted using the QIAmp Viral RNA Mini Kit (Qiagen; Germany). All RNA samples were analyzed using the RealStar WNV RT-PCR Kit 2.0 (altona Diagnostics; Germany). RNA of the cell supernatants was used to confirm WNV as the causative agent of the cytopathic effect, whereas RNA from the mosquito bodies was used to quantify the viral RNA per mosquito.

Data analysis

All calculations, visualizations, and model fitting were performed in R version 4.5.1 [24] using RStudio version 2025.09.1+401 [25] with the packages tidyverse [26] and glmmTMB [27,28]. The infection rate (IR) was calculated as the number of WNV-

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positive bodies divided by the total number of blood-fed mosquitoes per condition. To determine the TE, the number of mosquitoes with WNV-positive saliva was divided by the total number of blood-fed mosquitoes per condition.

To assess the influence of various predictors on the likelihood of positive infection, and on the body titer (viral RNA copies per mosquito), separate hurdle models were fitted for WNV-L1 and WNV-L2. A hurdle model was chosen due to a large proportion of body titers being zero (i.e. no infection), it overcomes this by separating the analysis into two components: (i) the binary component (zero-inflation) models the probability of detecting viral RNA (i.e. positive infection) using logistic regression with binomial distribution, and (ii) the continuous component analyzes the effects on the body titers, which were $\log_{10}$-transformed allowing the use of a linear regression with Gaussian distribution. Mosquito species and blood meal titer ($\log_{10}$-transformed) were chosen as predictors for both components of the hurdle models. To convert the outputs of the binary components from likelihood of non-infection to likelihood of infection, the results were inverted. Model diagnostics were performed and indicated a good fit for both models. Pearson residual-based dispersion of the continuous component was 1.16 for the WNV-L1 model and 1.57 for the WNV-L2 model, demonstrating mild to moderate overdispersion. Multicollinearity was assessed, with variance inflation factors of the (i) zero-inflated component being 1.04 for the WNV-L1 model and 1.07 for the WNV-L2 model, and of the (ii) continuous component being 1.08 for the WNV-L1 model and 1.02 for the WNV-L2 model, indicating no relevant collinearity among predictors in either model.

To assess the influence of different factors on the likelihood of detecting positive transmission, a generalized linear model (GLM) with a binomial distribution and logit link function was fitted for WNV-L2. Transmission outcome was taken as the binary response variable, and mosquito species, blood meal titer ($\log_{10}$-transformed), and body

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titer (log_{10}-transformed) were used as fixed effects. Model diagnostics were performed and indicated a good fit. Pearson residual-based dispersion was 0.92, demonstrating no overdispersion. Multicollinearity was assessed, with variance inflation factors ranging from 1.02 to 1.43, indicating no relevant collinearity among predictors.

For all models, *Ae. albopictus* was set as reference species. The value 1 was added to all body titers to allow log_{10}-transformation of all observations. Model output estimates of the logistic regression models (likelihood of transmission, likelihood of infection) were exponentiated to obtain odds ratios, and 95% confidence intervals were calculated to allow better interpretation of the results.

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Results

Feeding and survival rates

Feeding rates (fully engorged females per females offered a blood meal) ranged from 22.5% to 47.4% (Supplementary Table 1). Mean survival rates (alive females 14 days post infection per fed females) were generally higher in the two *Culex* species (*Cx. pipiens* biotype *pipiens*: 94.07%, *Cx. torrentium*: 93.30%) compared to *Ae. albopictus* (74.60%) (Supplementary Table 1).

Infection rates, transmission efficiencies, and minimum infectious doses

All three investigated mosquito species were susceptible to WNV-L1 and L2 infections at all tested blood meal titers (Table 1). For WNV-L1, IRs in *Ae. albopictus* consistently increased with rising blood meal titers, ranging from 46.7% at a blood meal titer of 10^5 $TCID_{50}$/mL to 60% at 10^7 $TCID_{50}$/mL, while no virus was detected in the saliva at any blood meal titer. In *Cx. pipiens* biotype *pipiens*, IRs rose from 10.0% to 33.3% at 10^5 versus 10^7 $TCID_{50}$/mL, and transmission was only observed at the highest blood meal titer (10^7 $TCID_{50}$/mL), with a TE of 6.7%. For *Cx. torrentium,* 10^5 $TCID_{50}$/mL resulted in a slightly higher IR (16.7%) than 10^6 $TCID_{50}$/mL (13.3%). However, again, the highest blood meal titer of 10^7 $TCID_{50}$/mL produced the highest IR of 63.3%. Similar to *Cx. pipiens* biotype *pipiens*, WNV was detected in the saliva at the highest blood meal titer only, with an efficiency of 6.7%. Overall, increasing blood meal titers generally resulted in increased IR, and positive saliva was observed only at the highest blood meal titer in *Cx. pipiens* biotype *pipiens* and *Cx. torrentium*, but not in *Ae. albopictus*. For WNV-L2, IRs in *Ae. albopictus* increased from 30.0% at 10^5 $TCID_{50}$/mL to 86.7% at 10^7 $TCID_{50}$/mL, with virus detected in the saliva at 10^6 and 10^7 $TCID_{50}$/mL (TE = 6.7% and 33.3%). In *Cx. pipiens* biotype *pipiens*, IRs rose from 24.1% to 60.0% with increasing blood meal

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titers, and transmission was detected at 10^5 (3.4%) and 10^7 $TCID_{50}$/mL (10.0%), but not at 10^6 $TCID_{50}$/mL.

Culex torrentium showed a clear dose-response pattern. For this species, the experiment also included a blood meal titer of 10^4 $TCID_{50}$/mL for WNV-L2. Testing 10^4 $TCID_{50}$/mL of L2 was not possible for *Cx. pipiens* biotype *pipiens*, which also still transmitted WNV at the lowest tested blood meal titer, due to the limited number of field-caught specimens. IRs in *Cx. torrentium* ranged from 20% at 10^4 $TCID_{50}$/mL to 76.7% at 10^7 $TCID_{50}$/mL. Transmission was detected at 10^5 $TCID_{50}$/mL with an efficiency of 3.3% and went up to 30.0% at 10^7 $TCID_{50}$/mL.

The minimum infectious dose (MID, minimum blood meal titer required to cause transmission) was lower for all mosquito species infected with WNV-L2 (Table 1). Both *Culex* species had a MID of 10^7 $TCID_{50}$/mL for L1 and 10^5 $TCID_{50}$/mL for L2. *Aedes albopictus´* MID was 10^6 $TCID_{50}$/mL for L2, while not even the highest blood meal titer tested was sufficient to cause transmission for L1.

Predictors of viral infection, transmission, and body titer

A hurdle model was fitted separately for each viral lineage to assess the factors (mosquito species, blood meal titer) influencing infection. In this model, *Cx. pipiens* biotype *pipiens* had significantly lower odds (odds ratios (ORs): L1 = 0.17, L2 = 0.42) of becoming infected with both lineages compared to *Ae. albopictus*, while *Cx. torrentium* showed the same trend, it was only significant for WNV-L1 (OR = 0.32) (Table 2). In contrast, each log_{10}-fold increase in blood meal titer more than doubled the odds of infection for both lineages (ORs: L1 = 2.04, L2 = 2.63). Furthermore, the influence of the different predictors on the body titer was assessed. Once an infection was established, all predictors showed a significant positive effect on body titers for both lineages, with estimates ranging from 0.51 to 1.77 (Table 2). Notably, body titers visually cluster into two groups,

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231 a low body titer group and a high body titer group (< and >10^6 viral RNA copies/body)
232 (Figure 1). Except for six *Ae. albopictus* specimens, all mosquitoes with positive saliva
233 (n = 26) belonged to the high body titer group (Figure 1). These six specimens were all
234 infected with WNV-L2. One of them showed a negative infection result, despite a positive
235 saliva.
236 In addition, for WNV-L2, a generalized linear model was fitted to evaluate the predictors
237 of transmission. For WNV-L1, no transmission model could be fitted due to the limited
238 number of positive saliva samples (n = 4), therefore, Figures 2 and 3 only show WNV-
239 L2. In this WNV-L2 model, higher body titers significantly increased the likelihood of
240 detecting positive saliva, with each $\log_{10}$-fold increase doubling the odds of transmission
241 (OR = 2.02) (Table 2, Figure 2). Blood meal titer had a similar but non-significant effect
242 (OR = 2.24) (Table 2, Figure 3). *Culex pipiens* biotype *pipiens* significantly reduced the
243 odds of transmission compared to *Ae. albopictus* (OR = 0.12), whereas *Cx. torrentium*
244 also showed reduced odds (OR = 0.32), although this effect was not statistically
245 significant (Table 2). Due to the limited number of positive saliva samples, the same
246 statistical analysis could not be performed for WNV-L1, although it was observed that
247 transmission occurred exclusively at the highest blood meal titer (10^7 $TCID_{50}$/mL), and
248 only in the two *Culex* species, which showed the same TE of 6.67% (Table 1). However,
249 the proportion of mosquitoes with positive saliva in the higher body titer group (>10^6 viral
250 RNA copies) is higher in *Cx. pipiens* biotype *pipiens*, compared to *Cx. torrentium*
251 (Figure 1).

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Discussion

Two native European mosquito species, *Cx. pipiens* biotype *pipiens* and *Cx. torrentium*, and the invasive species *Ae. albopictus* were separately infected with WNV-L1 and L2 with blood meal titers ranging from 10^4 to 10^7 $TCID_{50}$/mL. All species were susceptible to infection by both lineages, and higher blood meal titers were overall significantly associated with increased IRs and higher body titers. The TEs showed a similar trend, however, differences between blood meal titers were not statistically significant, likely due to the small number of positive saliva samples. This dose-response relationship aligns with previous findings for various mosquito species infected with WNV-L1 [29–31], and has been documented for other arboviruses [32,33].

Beyond the general dose-response effect, there were strong differences both between the mosquito species and the viral lineages. Overall, WNV-L2 resulted in higher IRs, higher TEs, and had lower MIDs across all tested mosquito species compared to L1. Particularly notable was the difference between the two *Culex* species, while both showed the same TEs when infected with WNV-L1, *Cx. torrentium* exhibited a three-times higher TE when infected with WNV-L2 compared to *Cx. pipiens* biotype *pipiens* (30% vs. 10%). A previous study on WNV-L2 in *Cx. pipiens* biotype *pipiens* showed higher TEs of up to 53%, however, a blood meal titer of 10^8 $TCID_{50}$/mL was used, which was ten-fold higher than the highest one used in our study [34]. Earlier work on WNV-L1 has also identified *Cx. torrentium* as a more competent vector than *Cx. pipiens* biotype *pipiens* with comparable TEs observed in our study [35].

To our knowledge, this study provides the first vector competence assessment of *Cx. torrentium* for WNV-L2. Due to the differences in vector competence between the two tested *Culex* species, it is important to distinguish these morphologically similar species in studies, as it otherwise may obscure key patterns. This is especially important as *Cx.*

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277 *torrentium* is the dominant *Culex* species in Northern Europe, and WNV is predicted to
278 continue its geographic spread towards this area, driven by climate change [2,11].
279 Although *Ae. albopictus* is not a main WNV vector, it is vector competent for the virus,
280 and WNV has been isolated from field-caught specimens on multiple occasions [12].
281 Given its rapid expansion across Europe, understanding its potential role as a bridge
282 vector is important [12]. In this study, *Ae. albopictus* was highly susceptible to infection
283 with both WNV lineages. However, *Ae*. *albopictus* was not competent for WNV-L1,
284 whereas it transmitted WNV-L2 with the highest TEs (max. 33.3%) and at significantly
285 higher odds than *Cx. pipiens* biotype *pipiens*. This contrasts with other European studies
286 that reported transmission of WNV-L1 by *Ae. albopictus* [36]. However, Martinet et al.
287 [36] used a higher mean temperature (28°C) than in this study (24°C), limiting the
288 comparability of results.
289 Independent of the tested species, higher body titers significantly increased the odds of
290 transmission for WNV-L2, and all mosquitoes that transmitted WNV-L1 exhibited a high
291 body titer. In addition, we also observed a clustering of mosquitoes into high and low
292 body titer groups. Large differences in mosquito body titers have also been observed for
293 other arboviruses, such as Sindbis virus, and have potentially been linked to differential
294 gene regulation and variation in viral population structures [37,38]. However, the
295 underlying causes remain incompletely understood and require detailed investigation.
296 In this study, we also determined the MID for each lineage-mosquito-combination. A low
297 MID can not only expand the range of avian hosts [15] but may also broaden the
298 transmission window, allowing mosquitoes to become infected not only at peak viremia,
299 but also during its rise and decline in amplifying hosts. This extended window may
300 enhance WNV transmission and thereby increase the risk of spillover to humans.
301 Remarkably, the MIDs showed a clear lineage-specific difference. The MID was

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302 considerably lower for WNV-L2, with both *Culex* species requiring a 100-fold lower dose
303 to transmit L2 (10^5 $TCID_{50}$/mL) compared to L1 (10^7 $TCID_{50}$/mL). For *Ae. albopictus*,
304 the gap was even clearer, as transmission was only observed with WNV-L2 (MID = 10^6
305 $TCID_{50}$/mL).
306 Previous findings on the MID of WNV-L2 in *Ae. albopictus* are comparable to ours. Only
307 10^7 and 10^5 $TCID_{50}$/mL as blood meal titers were tested, and the former was reported as
308 MID [39]. More studies are available for WNV-L1, with the lowest observed MID of
309 $10^{6.7}$ PFU/mL in *Ae. albopictus* from France [36]. This is a stark difference from our
310 findings, where a similar infectious dose did not result in transmission. But again,
311 Martinet et al. [36] used a higher incubation temperature than the present study. Studies
312 with *Cx. pipiens* biotype *pipiens* and *Cx. torrentium* use a high blood meal titer (often 10^7
313 viral particles/mL) rather than a range of titers, therefore, comparisons are not feasible
314 [35,40,41]. Our data suggest that the combination of WNV-L2 and *Cx. torrentium* may
315 be of particular concern, as this species exhibited both the lowest MID and a high TE.
316 Together with increasingly favorable environmental conditions, this could contribute to
317 the spread of WNV-L2 in Europe.
318 Future work should include multiple viral strains to confirm if differences are truly
319 lineage- or strain-specific, and mosquitoes from different regions to assess the influence
320 of different populations on vector competence and MID. Caution is needed when
321 comparing experimental MIDs to natural viremias, as infection and transmission can
322 differ between live hosts and artificial systems [42]. Additionally, TEs measured via
323 artificial salivation have been shown to potentially underestimate the natural transmission
324 potential [43].
325 In conclusion, this study provides a comprehensive assessment of the infection dynamics
326 of the tested WNV lineages in native European *Cx. pipiens* biotype *pipiens* and *Cx.*

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327 *torrentium*, as well as the invasive *Ae. albopictus*. Distinct viral lineage- and mosquito
328 species-specific differences were observed. WNV-L2 infection consistently resulted in
329 higher TE and lower MIDs across all mosquito species compared to WNV-L1. *Culex*
330 *torrentium* stood out as a particularly efficient vector for WNV-L2, highlighting a need
331 for closer monitoring. Overall, this study strengthens our understanding of how viral
332 lineage, mosquito species, and blood meal titer interact.

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Author Contributions

Conceptualization: SJ, AH; Methodology: PH; Formal analysis: PH, FGS; Original draft preparation: PH; Review and editing: all authors; Funding acquisition: JSC, AH, RL; Resources: RL, NB; Supervision: AH, SJ; All authors have read and agreed to the published version of the manuscript.

Competing Interests

The authors declare no competing interests.

Data availability

The data supporting the findings of this study are available within the article and its supplementary materials. Supplementary Table 1 shows feeding and survival rates. Supplementary Table 2 shows all individual mosquitoes tested, their infection and transmission statuses, and their body titers.

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Table 1: The number of mosquitoes subjected to salivation assay, the IR (positive bodies/fed mosquitoes), and the TE (positive salivas/fed mosquitoes), stratified by WNV lineage, mosquito species, and blood meal titer. Blood meal titers marked in **bold** indicate the MID necessary for transmission. Mosquitoes were incubated for 14 days at 24±5°C.

WNV lineage	Mosquito species	Blood meal titer as $TCID_{50}$/mL	No. of tested mosquitoes	Infection rate (no. of mosquitoes with positive body)	Transmission efficiency (no. of mosquitoes with positive saliva)
Lineage 1	*Ae. albopictus*	10^5	30	46.7% (14)	0.0% (0)
		10^6	30	50.0% (15)	0.0% (0)
		10^7	30	60.0% (18)	0.0% (0)
	Cx. pipiens biotype *pipiens*	10^5	30	10.0% (3)	0.0% (0)
		10^6	30	16.7% (5)	0.0% (0)
		10^7	30	33.3% (10)	6.7% (2)
	Cx. torrentium	10^5	30	16.7% (5)	0.0% (0)
		10^6	30	13.3% (4)	0.0% (0)
		10^7	30	63.3% (19)	6.7% (2)
Lineage 2	*Ae. albopictus*	10^5	30	30.0% (9)	0.0% (0)
		10^6	30	76.7% (23)	6.7% (2)
		10^7	30	86.7% (26)	33.3% (10)
	Cx. pipiens biotype *pipiens*	**10^5**	29	24.1% (7)	3.4% (1)
		10^6	30	50.0% (15)	0.0% (0)
		10^7	30	60.0% (18)	10.0% (3)
	Cx. torrentium	10^4	30	20.0% (6)	0.0% (0)
		10^5	30	33.3% (10)	3.3% (1)
		10^6	33	69.7% (23)	6.1% (2)
		10^7	30	76.7% (23)	30.0% (9)

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Table 2: Results of two models assessing the influence of various predictors on (i) the likelihood of positive infection (ii) the body titer (viral RNA/mosquito body) for WNV-L1 and -L2, and (iii) the likelihood of positive transmission for WNV-L2. No transmission model could be fitted for WNV-L1 due to the very limited number of positive saliva samples (n = 4). The infection and body titer model were fitted as a two-step hurdle model, while the transmission model was fitted as a generalized linear model. Effect sizes from the logistic regression components of the transmission and infection models are expressed as odds ratios, whereas parameter estimates are reported for the linear component of the body titer model (denoted by *). The reference category was *Ae. albopictus* for mosquito species. "n.a." = not available, "n.s." = non-significant, "CI" = confidence interval.

Model	**Predictor**	**Odds ratio / *Estimate (95% CI)**	**p-value**	**Odds ratio / *Estimate (95% CI)**	**p-value**
		WNV Lineage 1		**WNV Lineage 2**	
Positive infection	*Cx. pipiens* biotype *pipiens*	0.17 (0.08 – 0.36)	< 0.001	0.42 (0.18 – 0.70)	0.003
	Cx. torrentium	0.32 (0.16 – 0.64)	0.001	0.76 (0.39 – 1.47)	0.407 (n.s.)
	Log_{10}(Blood meal titer)	2.04 (1.41 – 2.94)	< 0.001	2.63 (1.92 – 3.57)	< 0.001
Body titer	*Cx. pipiens* biotype *pipiens*	*1.38 (0.41 – 2.36)	0.005	*1.04 (0.12 – 1.97)	0.027
	Cx. torrentium	*1.77 (0.92 – 2.62)	< 0.001	*1.24 (0.41 – 2.07)	0.003
	Log_{10}(Blood meal titer)	*0.51 (0.05– 0.96)	0.028	*1.34 (0.91 – 1.77)	< 0.001
Positive transmission	*Cx. pipiens* biotype *pipiens*	n.a.	n.a.	0.12 (0.02 – 0.59)	0.009
	Cx. torrentium	n.a.	n.a.	0.32 (0.08 – 1.24)	0.099 (n.s.)
	Log_{10}(Blood meal titer)	n.a.	n.a.	2.24 (0.92 – 5.45)	0.076 (n.s.)
	Log_{10}(Body titer)	n.a.	n.a.	2.02 (1.55 – 2.65)	< 0.001

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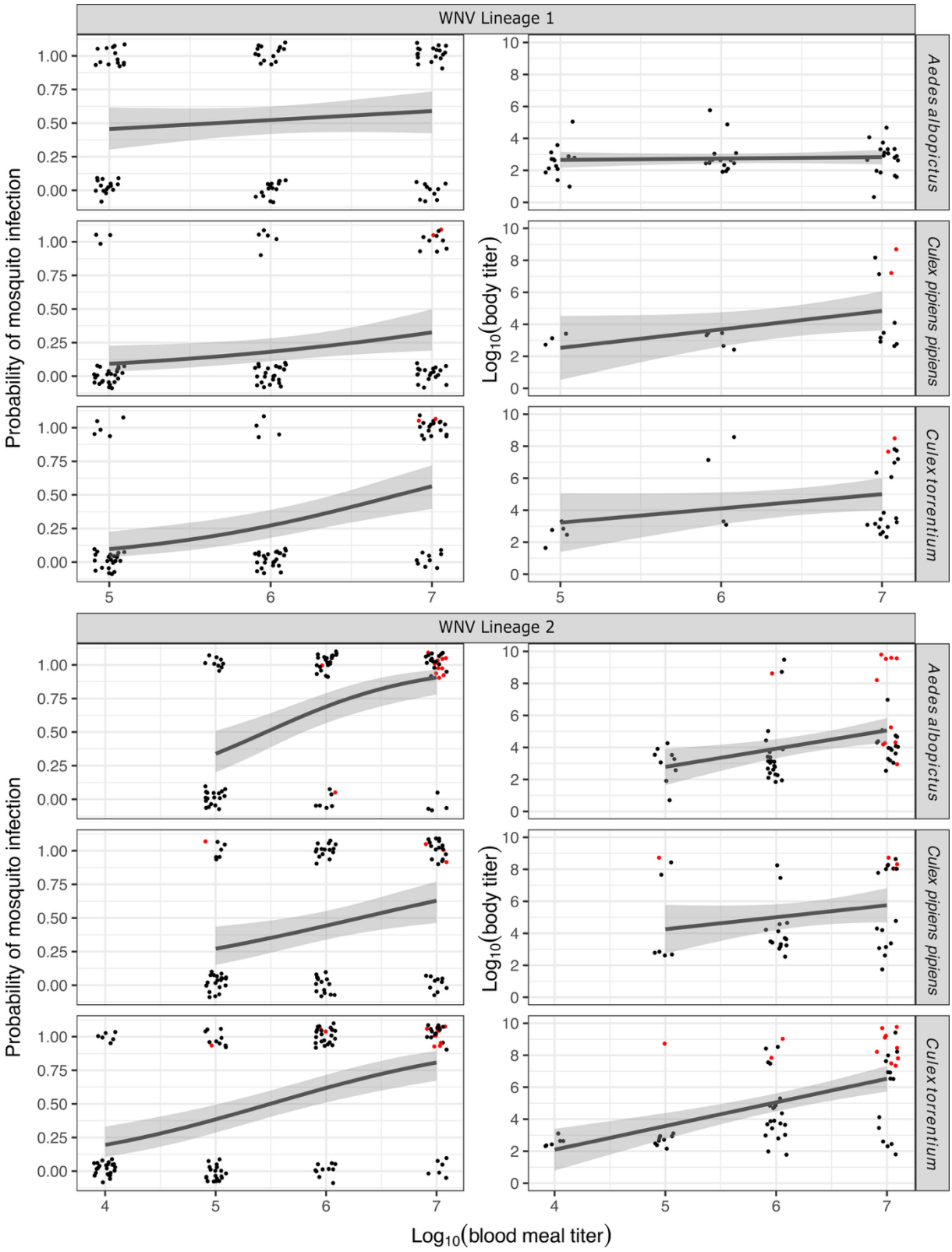

Figure 1: Relationship between the $\log_{10}$-transformed blood meal titer ($TCID_{50}$/mL) and (i) the probability of mosquito infection (left panel, binomial component of the hurdle model) and (ii) the $\log_{10}$-transformed body titer (viral RNA/mosquito body) (right panel, linear part of the hurdle model, including only infected mosquitoes with detectable body

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506 titers) for *Ae. albopictus*, *Cx. pipiens* biotype *pipiens*, and *Cx. torrentium* experimentally
507 exposed to WNV-L1 or L2. For (i), logistic regression curves were fitted, whereas for
508 (ii), linear regression curves were fitted. 95% CI are shown in grey. Black dots indicate
509 mosquitoes with a negative saliva, red dots indicate mosquitoes with a positive saliva,
510 jittered for better visibility.

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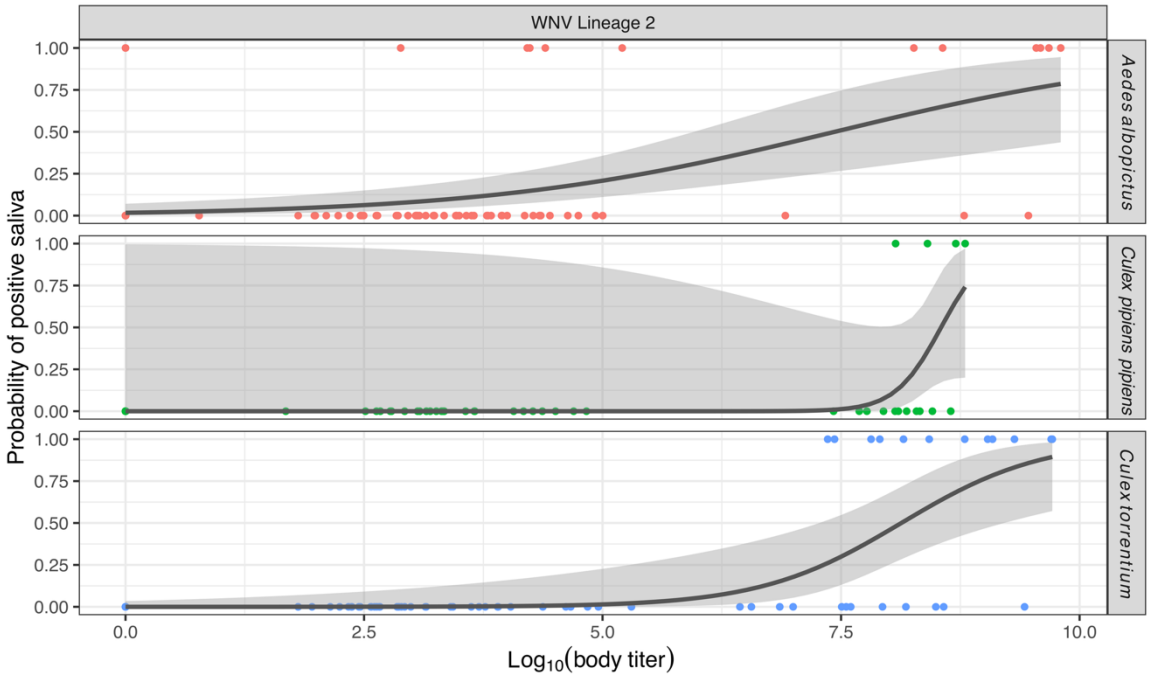

Figure 2: Relationship between the $\log_{10}$-transformed body titer (viral RNA/mosquito body) and the probability of positive saliva for *Ae. albopictus*, *Cx. pipiens* biotype *pipiens*, and *Cx. torrentium* experimentally exposed to WNV-L2. Data points represent observed transmission outcomes (positive = 1; negative = 0), jittered for better visibility. Logistic regression curves with 95% CI in grey were fitted using a binomial generalized linear model.

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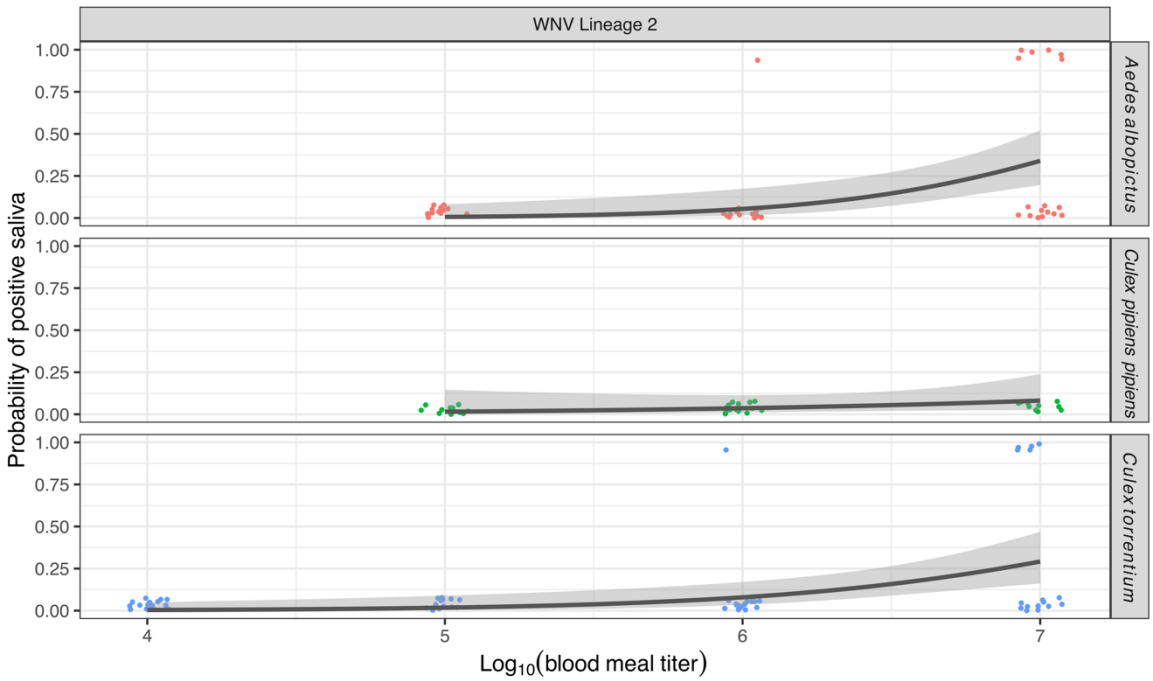

518

519 Figure 3: Relationship between the $\log_{10}$-transformed blood meal titer ($TCID_{50}$/mL) and
520 the probability of positive saliva for *Ae. albopictus*, *Cx. pipiens* biotype *pipiens*, and *Cx.*
521 *torrentium* experimentally exposed to WNV-L2. Data points represent observed
522 transmission outcomes (positive = 1; negative = 0), jittered for better visibility. Logistic
523 regression curves with 95% CI in grey were fitted using a binomial generalized linear
524 model.

Paper V

Vector competence of Aedes and Culex species for Barmah Forest virus

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Abstract

Barmah Forest Virus (BFV), an arbovirus transmitted by mosquitoes, is of significant public health concern in Australia and regions in the Pacific. Recent climate change and global movement patterns raise the potential for BFV to extend its geographic distribution, including the potential introduction into Europe. Despite the rising importance of BFV, its vector dynamics remain poorly understood, particularly concerning different mosquito species and their associated competence in transmitting the virus. This study aims to investigate the vector competence of BFV across various mosquito species, beyond those endemic to the Australasian region.

Key words

Barmah forest virus, *Aedes albopictus*, *Aedes aegypti*, Vector competence

Introduction

Human infections with arboviruses (arthropod-borne viruses) like dengue virus and chikungunya virus (CHIKV) have risen globally in recent decades, driven by climate change, land-use shifts, and globalization, which support the spread of invasive mosquitoes and virus introduction into new regions [1]. One important family of the arboviruses is the family of *Togaviridae*, and more specifically, the genus of *Alphaviruses*, which CHIKV belongs to. Another member of this family is the Barmah Forest virus (BFV), first isolated in 1974 from *Culex annulirostris* mosquitoes collected in the Barmah State forest in Northern Victoria [2] and in the same year from *Cx. annulirostris* and *Ae. normanensis* in Queensland [3], identified as BFV by Dalgarno et al. [4]. It has been associated with human disease since 1988 [5]. Its clinical presentation is similar to other alphavirus infections, such as Ross River virus (RRV) [6], CHIKV, o'nyong-nyong virus, and Mayaro virus [7]. Typical symptoms include fever, arthritis, headache, rash, joint swelling, and pain [6,8]. After RRV, BFV is the second most common arbovirus infection in Australia, causing several 100 cases annually and over 16.000 confirmed cases since 2000 [9]. However, it can be assumed that the actual number of BFV infections is much higher, as misdiagnoses such as CHIKV or RRV are common [10]. Large outbreaks of BFV occurred in the Northern Territory in 1992 [11], in Western Australia in 1993–1994 [12], in New South Wales in 1994–1995 [13], in Victoria in 2002 [14], in Southeastern Queensland in 2002–2003 [15], and in Tasmania in 2019 [16]. Initially, BFV was considered to be limited to Australia, but in 2014, a case was reported in a child from Papua New Guinea with no travel history [17]. Genetic analyses suggest an introduction in the early 1900s by humans, livestock, or mosquitoes from Australia [17].

Host animals of BFV are not well defined, but it is assumed that BFV has a broader range of vertebrate hosts than RRV [18]. Seroprevalence studies revealed BFV antibodies in different marsupials and other nonhuman vertebrates, like horses, brushtail possums, cats, and dogs [19–21], suggesting horses and brushtail possums as amplification hosts [22]. Also, migrating hosts like birds might be part of the transmission cycle [23], and humans might act as reservoir hosts [22]. So far, no symptomatic non-human hosts are known [21].

Based on isolations of BFV from mosquitoes from the field, it seems that BFV has a broad spectrum of vectors, including the five genera of *Aedes*, *Culex*, *Coquillettidia, Anopheles,* and *Verralina*. However, it should be noted that the detection of BFV in mosquitoes does not automatically mean that this species is able to transmit the virus, since even a single blood meal from an infected host can lead to a positive result. Experimental studies are needed to confirm the ability of the species to transmit BFV. Barmah forest virus has been isolated from different field-collected mosquito species, amongst them *Ae. camptorhynchus* [12,24], *Ae. normanensis* [25]*, Ae. vigilax* [13,25,26]*, Cx. annulirostris* [25,27]*, Cx. quinquefasciatus* [27], *Coquillettidia xanthogaster* [27], *Cq. linealis* [26] *Anopheles annulipes* s.l. [28] and the biting midge *Culicoides marksi* [29].

Under laboratory conditions vector competence has been described for *Aedes* species, notably *Ae. notoscriptus , Ae. vigilax* [30–33] and *Ae. procax* [32], but not for *Ae. aegypti* [34]. Transmission of BFV by *Ae. vigilax* has been shown as early as 3 days post-infection and by *Cq. linealis* as early as 6 days post-infection [26,31]. Additionally, the brackish water mosquito *Ve. funerala* was also tested positive for BFV transmission and might have an important role as an

amplifying vector [35]. *Culex annulirostris* is able to transmit BFV on a low level (transmission rates > 10%), whereas *Cx. quinquefasciatus and Cx. sitiens* should not be considered as vectors [34]. It is assumed, that *Ae. camptorhynchus* [24] and *Ae. vigilax* [13,15,25,31,32,36] are key vectors of BFV, which are associated with coastal wetlands. These mosquito species have in common that they are all restricted to the Australasian region.

Understanding which mosquitoes can transmit BFV is crucial. Since travel and trade increase, the risk of spreading BFV to other continents is rising. Climate change may also influence mosquito distribution and virus transmission [22,36]. The related alphavirus CHIKV serves as a cautionary example: it has spread across all continents in the last decades, causing millions of infections annually [37]. This study aims to analyze a wider range of mosquito species for their vector competence for BFV, beyond Australasian species, to better assess the potential risk of introduction and subsequent spread at a global scale. For this, we included *Culex* and *Aedes* species, as Australian mosquito species of both genera show transmission potential for BFV. We included *Cx. quinquefasciatus*, a species widespread in tropical regions, and *Cx. torrentium*, which is native to Europe. We also examined the invasive *Ae. aegypti* and *Ae. albopictus*, two globally distributed species that play a central role in CHIKV transmission. As there have been no laboratory studies on the vector competence of *Anopheles* mosquitoes for BTV, we included *An. stephensi* in our investigations, an Asian mosquito species that is spreading within Asia and into Africa [38].

Materials and Methods

Collecting and rearing of mosquitoes

During summer 2024, egg rafts of *Culex torrentium* were collected in the field in northern Germany (Lon: 53.467821/Lat: 9.831346). Eggs and larvae were kept at room temperature, and were reared under a 12:12 light:dark photoperiod. Species identification was conducted by isolating DNA from a pool of 5 L1/L2 larvae per egg raft (DNeasy blood & tissue kit, Qiagen, Hilden, Germany). Subsequently, a multiplex quantitative real-time PCR (qPCR) was performed following the protocol described by Rudolf et al. (HotStarTaq master mix kit, Qiagen, Hilden, Germany) [39]. Pupae were housed in an insectary with a relative humidity of 70%, 26°C, and a 12:12 light:dark photoperiod, incorporating a 30 min twilight. To eliminate the possibility of natural arbovirus infections, 10 adult mosquitoes were randomly chosen and tested by pan-Orthobunya-, pan-Flavivirus-, and pan-Alphavirus-PCR [40–42]. All screened specimens tested negative. Laboratory colonies of *Cx. quinquefasciatus*, *Ae. aegypti* (both long-established colonies maintained from Bayer, Leverkusen, Germany), and *Ae. albopictus* (established from eggs collected in the field (Heidelberg, Germany) in 2016/2017) were reared under the same insectary conditions. *Anopheles stephensi* (strain SxK Nijmegen) were reared in an insectary with a relative humidity of 70%, 28°C, and a 12:12 light:dark photoperiod, incorporating a 30 min twilight.

Infection of mosquitoes

Female mosquitoes aged between 4 and 14 days were deprived of food prior to the infection experiments. While most species were starved for 24 hours, *Cx. torrentium* underwent an

extended starvation period of 48 hours. An artificial blood meal was offered for two hours at 24°C, containing 50% human blood (expired transfusion reserves), 30% of an 8% fructose solution, 10% filtrated bovine serum (FBS), and 10% virus stock, final virus concentration was 6.88 x 10^7 FFU/mL. The BFV stock (strain BH2193 / U73745) was propagated in Vero cells (*Chlorocebus sabaeus*, CVCL_0059, obtained from ATCC, cat # CCL-81). Artificial blood meal was offered either via cotton stick (*Culex*) or via two 50 µL drops on the bottom of the vial (*Aedes, Anopheles*). Feeding rates (FR, number of engorged females per number of fed females) are shown in Table 1. Only fully engorged specimens were incubated for 7 days at 70% humidity and fluctuating temperature profiles of 18 °C, 21 °C, 24 °C, or 27 °C with variations of +/−5 °C within 24h. To simulate natural day-night temperature cycles, peak temperatures were programmed to occur midway through the light phase, while minimum temperatures coincided with the midpoint of the dark phase. Mosquitoes had continuous access to fructose solution provided on a cotton pad, fructose was refreshed every 2-3 days. The survival rates (SR, number of alive mosquitoes 7 days post-infection per number of fed females) of all mosquito species are shown in Table 1. All experiments were performed in 2 replicates.

Table 1. Feeding- and survival rates of all investigated species. Total input = Number of mosquitoes per experiment condition, FR = feeding rate, number of engorged females per number of fed females), SR = survival rate, number of alive mosquitoes seven days post-infection per number of fed females

Species	Temperature	Total input	FR (%)	SR (%)
Cx. quinquefasciatus	27°C +/-5°C	59	92	56
Cx. torrentium	27°C +/-5°C	68	72	88
An. stephensi	27°C +/-5°C	97	45	91
Ae. aegypti	27°C +/-5°C	100	56	68
	24°C +/-5°C	78	53	93
	21°C +/-5°C	170	25	95
	18°C +/-5°C	252	17	84
Ae. albopictus	27°C +/-5°C	100	66	95
	24°C +/-5°C	100	56	100
	21°C +/-5°C	91	66	86
	18°C +/-5°C	99	61	94

Analysis

To evaluate the transmission rate (TR, number of BFV-positive saliva per number of positive bodies) and the transmission efficiency (TE, number of BFV-positive saliva per number of fed females), a salivation assay was conducted as previously described [43]. In short, saliva was collected for 30 minutes in a tip containing 10 µL phosphate-buffered saline (PBS). Saliva solution was applied on Vero cells and incubated at 37 °C and 5% CO_2 for five days. If cells showed a cytopathic effect, supernatant was harvested, RNA was extracted, and a qPCR was performed to confirm BFV infection. Reverse transcription qPCR was performed after the protocol of Inglis et al. [27] using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden,

Germany), with an addition of the VetMAX™ Xeno™ Internal Positive Control (Applied Biosystems, Thermo Fisher Scientific Corporation, Waltham, MA, USA). For quantification, a standard was designed (ATGCCTCAGGGCCACTCACCATACTTGTGGTAGCTATTATAGTCGTTGTTG TAGTATCCATTGTAGTATGTGCAAGACACTAGCAGAACTA).

The RT-qPCR was validated following the guidelines outlined by Bustin *et al.* in the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments." [44]. Ten-fold serial dilutions of the BFV standard, ranging from 1.09 to 1.09×10^{11} copies/µL of the above-mentioned standard were analyzed in five replicates using the previously described RT-qPCR protocol. The limit of detection was identified as 1.09×10^{2} copies/reaction, or 1.23×10^{4} copies/mosquito body, with a standard deviation of 0.527 C_qs at this concentration. The linear dynamic range extended from the detection limit to ≥ 1.23×10^{13} copies/mosquito body, proving a proportional relationship between concentration and PCR signal within this range, ensuring reliable results. The calibration curves resulted in a coefficient of determination of 0.993, a slope of -3.772, a y-intercept of 45.112, and a PCR efficiency of 0.841. In essence, 84.1% of the target molecules were successfully amplified in each cycle.

Additionally, RNA of mosquito bodies (including the head) was extracted, and qPCR was performed to calculate the infection rate (IR, number of BFV-positive bodies per fed females) and the mean body virus titer.

Results

At 7 days post-infection, testing of the *Culex* species (*Cx. quinquefasciatus*, *Cx. torrentium*) revealed no BFV-positive saliva (Table 2). The infection rate (IR) in these species ranged from 12.9% to 23.3%, with a mean body titer of 4.6 and 4.8 log10 BFV RNA copies per specimen. In a similar range, *An. stephensi* showed an IR of 16.7%, with a mean body titer of 5.0 log10 BFV RNA copies per specimen (Table 2). Like for the *Culex* species, no transmission was observed for *An. stephensi*. These results indicate successful infection of some *Culex*/*Anopheles* individuals, but no evidence of possible transmission.

In contrast, both *Ae. aegypti* and *Ae. albopictus* showed positive saliva at all four investigated temperature profiles (Table 2). In *Ae. aegypti*, we detected IRs from 60% to 80% with a mean RNA body titer between 5.2 and 6.5 log10 BFV copies per specimen (Figure 1). Transmission rates (TR) for *Ae. aegypti* ranged from 16.7% to 28.6%, with transmission efficiencies (TE) between 10% and 20% (Table 2), with no major differences between the analyzed temperature profiles. *Aedes albopictus* showed comparable IRs, ranging from 65.6% to 75.9%, with slightly lower mean viral loads, ranging from 5.3 to 5.8 log10 BFV copies per specimen (Table 2, Figure 1). Transmission rates in *Ae. albopictus* were notably higher than in *Ae. aegypti* at 27°C, showing a TR of 47.6% and a TE of 31.3% (Table 2). At 24°C, with a TR of 25% and a TE of 16.7%, *Ae. albopictus* showed values in the same range than *Ae. aegypti*. In contrast to *Ae. aegypti*, a clear trend of decreased transmission at lower temperatures of 21°C and 18°C could be identified for *Ae. albopictus*, showing TRs of 4.8% and 4.5% and TEs of 3.5% and 3.4%.

Table 2. Infection rate, transmission rate, and transmission efficiency of different mosquito species for Barmah Forest virus. dpi = days post infection, n = Number of fed and investigated

specimens, IR = infection rate, number of BFV-positive specimens per engorged, TR = transmission rate, number of BFV-positive saliva per BFV-positive bodies, TE = transmission efficiency, number of BFV-positive saliva per number of fed females

Species	dpi	Temperature	n	IR (%)	TR (%)	TE (%)
Cx. quinquefasciatus	7	27°C +/-5°C	30	23.3	0.0	0.0
Cx torrentium	7	27°C +/-5°C	31	12.9	0.0	0.0
An. stephensi	7	27°C +/-5°C	30	16.7	0.0	0.0
Ae. aegypti	7	27°C +/-5°C	30	70.0	28.6	20.0
	7	24°C +/-5°C	30	60.0	16.7	10.0
	7	21°C +/-5°C	30	80.0	25.0	20.0
	7	18°C +/-5°C	33	63.6	28.6	18.2
Ae. albopictus	7	27°C +/-5°C	32	65.6	47.6	31.3
	7	24°C +/-5°C	30	66.7	25.0	16.7
	7	21°C +/-5°C	29	72.4	4.8	3.5
	7	18°C +/-5°C	29	75.9	4.5	3.4

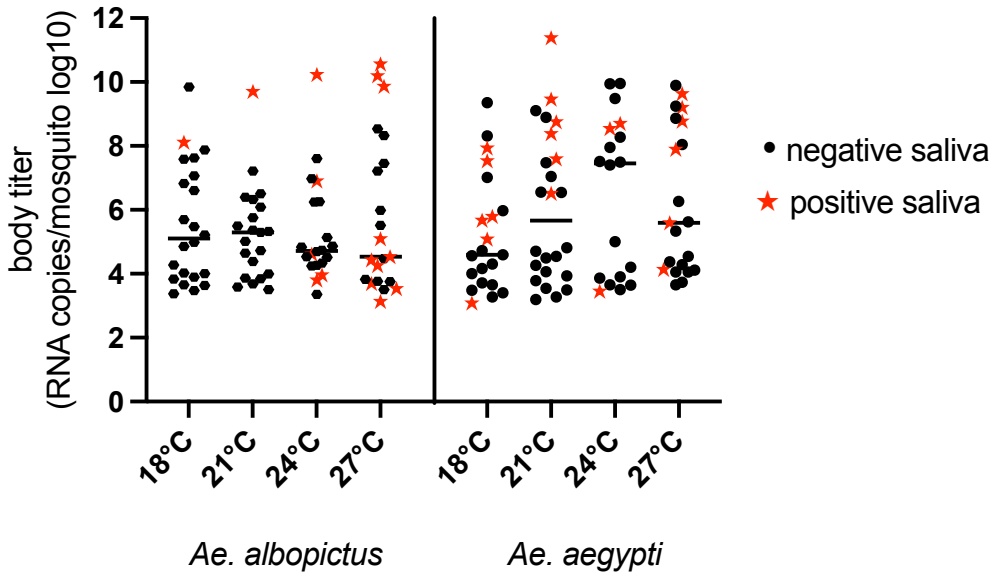

Figure 1. BFV RNA body titer of *Ae. aegypti* and *Ae. albopictus* at different temperature profiles. Each dot/star represents a single mosquito specimen. The mean body titer per condition is indicated by a black line. Specimens that showed no transmission are indicated as a black dot, specimens with positive saliva are shown as a red star.

Discussion

Our results demonstrate that *Culex*, *Anopheles*, and *Aedes* species are susceptible to BFV infection under laboratory conditions. However, only the two tested *Aedes* species were capable of transmitting the virus, as indicated by the presence of infectious virus particles in their saliva. To our knowledge, this is the first time transmission of BFV by *Ae. aegypti* and *Ae. albopictus* has been shown. The observations for *Ae. aegypti* are in contrast to studies by Boyd

et al., who did not observe transmission of BFV by this species, even though infection was detected [34]. This discrepancy can likely be explained by the lower input titer used for the infectious blood meal in that study. Further studies are needed to determine the minimal infectious dose required for BFV transmission. So far, no studies on the vector competence of *Ae. albopictus*, *An. stephensi*, and *Cx. torrentium* for BFV have been published. *Culex quinquefasciatus* has been investigated previously, and in line with our results, no transmission of BFV was observed [34]. In fact, the only investigated *Culex* species that showed low transmission potential is *Cx. annulirostris*; however, there are also studies that did not detect any transmission of BFV in this species [32,34]. This suggests that vector competence for BFV may be largely restricted to *Aedes* species, despite a broader infection susceptibility across other mosquito genera.

These findings are particularly relevant for risk assessment of BFV transmission in Australia, where *Ae. aegypti* is present in some regions but has not been considered a potential vector so far [34]. BFV transmission has mainly been linked to coastal Australian *Aedes* species such as *Ae. camptorhynchus* and *Ae. vigilax* [24,36]. As *Ae. aegypti* is distributed across all continents except Antarctica, BFV transmission could potentially occur outside Australia. *Aedes albopictus* is not yet established on the Australian mainland, but repeated introductions have been reported in the Torres Strait, and BFV transmission has already occurred in neighbouring Papua New Guinea [45]. Given its demonstrated potential to transmit BFV, the establishment of *Ae. albopictus* in Australia would increase the risk of transmission. More broadly, this species is one of the most invasive mosquito species globally and continues to expand its range into temperate regions such as Central Europe. Our findings, therefore, highlight not only a potential threat for Australia but also for BFV emergence in other parts of the world.

In Europe, *Ae. albopictus* has been linked to all known autochthonous outbreaks of CHIKV, as well as dengue and Zika virus infections [46]. In 2025, a significant increase in autochthonous CHIKV infections in Europe was reported, reaching case numbers never observed before. This highlights the particular relevance of *Ae. albopictus* as a competent vector. Interestingly, both CHIKV and BFV can be transmitted at relatively low temperatures (21 °C and 18 °C, respectively) [47], which are typical for temperate regions such as Central Europe. Given the expanding global distribution of *Ae. albopictus* and its established role in CHIKV transmission, the potential for BFV to follow a similar path cannot be dismissed.

Compared to other alphaviruses such as CHIKV and RRV, BFV most likely has a broader spectrum of potential hosts [18,34], although recent studies suggest a broader host range for CHIKV and RRV as well [48,49]. While RRV and CHIKV transmission is largely associated with specific marsupial or primate hosts, respectively [50], BFV has been detected in multiple vertebrate species, including birds and various mammals [19–21,23]. This broader host range may increase the likelihood of BFV establishing sustained enzootic transmission cycles in new environments and thereby facilitating geographic expansion. As already mentioned, one important driver of increasing arbovirus transmission is climate change. Studies show that extreme weather events like heavy rainfall and tidal flooding favour transmission of BFV as well as RRV [36,51,52]. With more frequent extreme weather events due to climate change, the risk of BFV transmission increases.

Conclusion

This study provides novel insights into the mosquito vector of BFV, a topic that remains underexplored, especially for mosquito species not native to Australia. We could show that the two invasive mosquito species *Ae. albopictus* and *Ae. aegypti*, which play a major role in the global transmission of arboviruses, are potent vectors for BFV. Given the potential for arbovirus emergence in new regions due to globalization and climate-driven shifts in vector distributions, understanding whether BFV can be transmitted by mosquitoes outside of its endemic area is of increasing importance.

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