Comparison of vector competence of Aedes vexans Green River and Culex pipiens biotype pipiens for West Nile virus lineages 1 and 2

Elisabeth Wöhnke1 | Ana Vasic1 | Cristian Raileanu1 | Cora Marielle Holicki2 | Birke Andrea Tews3 | Cornelia Silaghi1,4

1Laboratory of Vector Capacity, Institute of Infectology, Friedrich-Loeffler-Institut, Greifswald, Germany
2Institute for Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institut, Greifswald, Germany
3Laboratory for Molecular Vector-Pathogen-Interaction, Institute of Infectology, Friedrich-Loeffler-Institut, Greifswald, Germany
4University Greifswald, Greifswald, Germany

Abstract
West Nile virus (WNV), a zoonotic arbovirus, has recently established an autochthonous transmission cycle in Germany. In dead-end hosts like humans and horses the WNV infection may cause severe symptoms in the central nervous system. In nature, WNV is maintained in an enzootic transmission cycle between birds and ornithophilic mosquitoes. Bridge vector species, such as members of the Culex pipiens complex and Aedes spp., also widely distributed in Germany, might transmit WNV to other vertebrate host species. This study determined and compared the vector competence of field-collected northern-German Cx. pipiens biotype pipiens and laboratory-reared Ae. vexans Green River (GR) for WNV lineage 1 (strain: Magpie/Italy/203204) and WNV lineage 2 (strain: “Austria”) under temperatures typical for northern Germany in spring/summer and autumn. For assessment of vector competence, 7- to 14-day-old female mosquitoes were offered a WNV containing blood meal via Hemotek membrane feeding system or cotton-stick feeding. After incubation at 18°C respectively 24°C for 14 days engorged female mosquitoes were salivated and dissected for determination of infection, dissemination and transmission rates by reverse transcriptase quantitative real-time PCR (RT-qPCR). Both Ae. vexans GR and Cx. pipiens biotype pipiens were infected with both tested WNV strains and tested 14 days post-inoculation. Disseminated infections were detected only in Ae. vexans GR incubated at 18°C and in Cx. pipiens pipiens incubated at 24°C after infection with WNV lineage 1. Transmission of WNV lineage 1 was detected in Cx. pipiens pipiens incubated at 24°C. These results indicate that Cx. pipiens pipiens from Northern Germany may be involved in the transmission of WNV, also to dead-end hosts like humans and horses.

Keywords
Aedes vexans, Culex pipiens pipiens, vector competence, West Nile virus
1 | INTRODUCTION

West Nile virus (WNV; family Flaviviridae, genus Flavivirus) is a zoonotic arthropod-borne virus that recently successfully established an autochthonous transmission cycle in Germany (Ziegler et al., 2019). In Europe, several outbreaks of WNV lineage 1 have been noted in multiple countries since the 1990s (e.g., Romania 1996, Italy 1998, France 2000–2006; Murgue, Murri, Triki, Deubel, & Zeller, 2001). After the detection of WNV lineage 2 in Hungary in 2004, the virus spread across central Europe (Bakonyi et al., 2006; Wodak et al., 2011). Seasonal reoccurring epidemics in humans and horses and further spread of WNV lineage 2 to Albania (2011), Croatia and Serbia (2012), Austria (2014), France (2015) and Germany (2018) were recorded since 2010 (Barzon et al., 2015; Beck et al., 2013; Di Sabatino et al., 2014; Ziegler et al., 2015) and Germany (2018) were recorded since 2010 (Barzon et al., 2015; Beck et al., 2013; Di Sabatino et al., 2014; Ziegler et al., 2019; https://ecdc.europa.eu/en/west-nile-fever/surveillance-and-disease-data/historical). In 2018, the ECDC reported the highest number of autochthonous acquired human WNV infections since the start of the surveillance programme in 2010. Furthermore, an increasing geographic distribution of WNV infections was noted (https://ecdc.europa.eu/en/west-nile-fever/surveillance-and-disease-data/historical).

The WNV may cause severe neuroinvasive disease in the central nervous system (meningitis, encephalitis) of susceptible animal species and humans with possibly fatal outcome (Chancey, Grinev, Volkova, & Rios, 2015). In nature, WNV circulates in an enzootic transmission cycle between ornithophilic mosquitoes (e.g., Culex spp.) and birds (Chancey et al., 2015). Some mosquito species feeding on both birds and mammals (“bridge vectors”) are capable of transmitting the virus to dead-end hosts like humans and horses via virus-containing saliva (Rochlin, Faraji, Healy, & Andreadis, 2019). Mosquito species, experimentally proven to be vector competent for WNV, include members of the Culex pipiens complex (Farajollahi, Fonseca, Kramer, & Kilpatrick, 2011; Vogels, Fros, Göertz, Pijlman, & Koenraadt, 2016; Vogels, Göertz, Pijlman, & Koenraadt, 2017), Aedes japonicus (Veronesi et al., 2018; Wagner et al., 2018), Aedes albopictus (Fortuna et al., 2015) and Aedes vexans (Goddard, Roth, Reisen, & Scott, 2002; Tiawsirisup et al., 2008). The Cx. pipiens complex mosquitoes are thought to be major vectors and mainly associated with WNV transmission in Europe due to various isolations of virus from field-collected individuals (Farajollahi et al., 2011; Medlock, Snow, & Leach, 2005). Previous studies comparing the vector competence of Ae. vexans and Cx. pipiens, demonstrated that Ae. vexans can be considered a moderately to highly effective WNV vector under laboratory conditions (Tiawsirisup et al., 2008).

In Germany, six species of the genus Culex and 27 species of the genus Aedes are present and members of the Cx. pipiens complex and Ae. vexans belong to the most widely spread mosquito species (Becker et al., 2014; Börstler et al., 2016; Kilpatrick, Meola, Moudy, & Kramer, 2008; Kronefeld, Kampen, Sassnau, & Werner, 2014; Rudolf et al., 2013). Additionally, Ae. vexans from Germany were found to be infected with Usutu virus (USUV), which is closely related to WNV, showing its involvement in flavivirus circulation (Becker et al., 2014).

Impacts

- Experimentally infected laboratory-reared Aedes vexans GR and northern-German Culex pipiens pipiens were susceptible to WNV lineages 1 and 2 and maintained the infection under realistic German spring/summer and autumn temperatures for 14 days.
- Northern-German Cx. pipiens pipiens were competent vectors for the transmission of WNV lineage 1, while laboratory-reared Ae. vexans GR were not.
- Comparison of viral titres directly after ingestion and after 14 days extrinsic incubation and low dissemination rates compared to infection rates indicates the presence of a midgut barrier for Ae. vexans.

The ability to transmit a pathogen may vary between different mosquito populations of the same species, as was observed for Ae. japonicus from Germany and Switzerland experimentally infected with WNV (Huber et al., 2014; Veronesi et al., 2018). Even though vector competence for WNV of indigenous German mosquitoes has recently been proven for Cx. torrentium, German Cx. pipiens pipiens have only been shown susceptible to infection with WNV without detection of virus in the saliva (Jansen et al., 2019; Leggewie et al., 2016).

The recent introduction of WNV to Germany (Ziegler et al., 2019) and the broad spread of potential mosquito vector species (Börstler et al., 2016; Kronefeld et al., 2014) and susceptible bird hosts (Michel et al., 2018; Seidowski et al., 2010; Ziegler et al., 2015) requires the evaluation of the vector competence of German mosquito populations. For this reason, this study determined and compared the vector competence of northern-German Cx. pipiens pipiens and laboratory-reared Ae. vexans GR under controlled laboratory conditions representing northern German spring/summer and autumn temperatures. The autochthonous transmission cycle of WNV in Germany caused by lineage 2 was discovered while this study was ongoing in 2018, therefore, isolates with high probability of WNV introduction into Germany were previously chosen, but the German isolate was not used in this study.

2 | MATERIALS AND METHODS

2.1 | Identification of field mosquito populations

Egg rafts and larvae of Cx. pipiens pipiens were collected in northeastern Germany during summer 2018 at a local cormorant colony (54.2562N, 13.1958E).

Morphological identification of mosquitoes species in 10% of the adult (F0) population using the identification key of Becker et al. (2010) was performed, and results confirmed through sequencing of the mitochondrial cytochrome C oxidase subunit I gene using...
primers described by Folmer, Black, Hoeh, Lutz, and Vrijenhoek (1994) (Table 1). Resulting sequences were compared with references in GenBank using BLASTn (Altschul, Gish, Miller, Myers, & Lipman, 1990). The biotype of Cx. pipiens was determined by multiplex RT-PCR assay previously described by Rudolf et al. (2013) using GoTaq® G2 Flexi DNA Polymerase Kit (Promega Corporation; Table 1).

Absence of naturally acquired WNV infections in field-acquired mosquitoes was confirmed in eight mosquito pools (2–20 mosquitoes per pool) prior to each experiment using a specific RT-qPCR (Eiden, Vina-Rodriguez, Hoffmann, Ziegler, & Groschup, 2010; Table 1).

### 2.2 | Mosquito breeding

Long-term laboratory colonized Ae. vexans GR were reared at 24–26°C, 65%–80% relative humidity (RH), 16:8 hr light:dark photocycle in mosquito breeding cages “bugdorm” (30 × 30 × 30 cm; MegaView Science Co.) provided with 5% (w/v) glucose solution via soaked cotton sticks, and humidity maintained by biologically cleaned (submerged and washed in water for consecutive 8 days) moist moss for 7–14 days before exposure to an infectious blood meal as previously described (Leggewie et al., 2016). Species confirmed field-acquired mosquitoes (Cx. p. p.) were reared under similar conditions as described for Ae. vexans GR above with the addition of water containments instead of moist moss in breeding cages.

### 2.3 | Vector competence experiment

Female mosquitoes, deprived of glucose solution and water for 24 hr, were offered a WNV containing blood meal via artificial membrane using Hemotek feeding system (Hemotek; Ae. vexans) and a Parafilm membrane (Bemis) or cotton-stick feeding (Cx. p. p.) for 2–3 hr. The blood meal consisted of heparinized bovine (Ae. vexans) or chicken (Cx. p. p.) blood, supplemented with 5 mM ATP (Sigma-Aldrich) and 100 µl virus stock per ml.

Under CO₂ anaesthesia, engorged females (EF) were selected from non-engorged ones under a glovebox (Terra Universal) for incubation. One to 3 EF per feeding group were separated for confirmation of ingested WNV directly after feeding. Remaining EF, provided with 5% glucose solution, were kept in an incubator (MLR-352H-PE, Panasonic) for 14 days at constant temperatures of 18°C respectively 24°C, 80% RH and 16:8 hr (light:dark) photocycle.

Saliva from viable mosquitoes was collected in capillaries (Drummond Scientific Company) filled with 5 µl minimal essential medium (MEM) containing 10% FBS. After salivation for 30–45 min, the saliva-containing capillaries were transferred into 95 µl MEM + 10% FBS (in 2 ml Eppendorf tubes). Saliva samples were processed as described in Jansen et al. (2019). Dissected legs and wings respectively bodies were collected in 300 µl MEM + 10% FBS. Collection tubes contained one (saliva) or three (legs/wings, bodies) 3-mm stainless steel beads (TIS GmbH Wälzkörpertechnologie) for homogenization. For RNA extraction, samples were macerated in Tissue Lyser II (Qiagen) twice for 1 min at 30 Hz. RNA extraction from WNV-exposed samples was performed using TRIzol reagent (Thermo Fisher Scientific) in combination with RNeasy Mini Kit (Qiagen) according to the manufacturers’ recommendations. Infection (% infected/dissected mosquitoes), dissemination (% disseminated/infected mosquitoes) and transmission (% virus-containing saliva/disseminated infections) rates as well as the transmission efficiency (% virus-containing saliva/engorged mosquitoes) were determined by WNV-specific RT-qPCR using iTaq™ Universal Probes One Step Kit (BioRad Laboratory Inc.) and CFX-96 Real-Time system (BioRad Laboratory Inc.).

A 10-fold dilution series of WNV-RNA extracted using TRizol reagent and RNeasy Mini Kit from cell culture reared virus with a defined viral titre (TCID₅₀/ml) were used as standards for quantification of relative viral titres of infection rates (body samples 14 dpi and

### Table 1

| Reference                  | Sequence 5′-3′ | C (µM) |
|----------------------------|---------------|--------|
| Folmer et al. (1994)      | GGTCAACAAATCATAAAGGATA TTGG | 10     |
| HCO2198                   | TAAACCTCAGGGTGACAACTCA       | 10     |
| Rudolf et al. (2013)      | GGGCCCAAAATTAGAGACTT         | 30     |
| Culex pipiens (F)         | C TTCCTAACAATCCAGACA         | 30     |
| Culex pipiens (R)         | Cy55-GGAAACAGTGAGCTCGGG-BHQ1 | 20     |
| Culex pipiens (P)         | JOE-GCTTGGTAGAGTTTG-BHQ1     | 20     |
| Culex pipiens molestus (P)| RoX-TGACCCTCCAGTAAGGTATCAC-T-BHQ2 | 20     |
| Culex torrentium (F)      | GACACAGGCCAGCAA             | 10     |
| Cx. torrentium (R)        | GCCTAGCACTACTAA             | 10     |
| Cx. torrentium (P)        | FAM-CGATGATGCGTCTGCTACGA-BHQ1 | 10     |
| Eiden et al. (2010)       | GGGCTTCTGGTGTGTTC           | 15     |
| FLI_BRC_F                 | GATCTTGGCHTCCAACCT C        | 15     |
| FLI_BRC_R                 | FAM-CCACCCAGGAGTCCTYC6CAA   | 5      |
| FLI_P                     | FLI_P                       |        |

| Reference                  | Sequence 5′-3′ | C (µM) |
|----------------------------|---------------|--------|
| Folmer et al. (1994)      | GGTCAACAAATCATAAAGGATA TTGG | 10     |
| HCO2198                   | TAAACCTCAGGGTGACAACTCA       | 10     |
| Rudolf et al. (2013)      | GGGCCCAAAATTAGAGACTT         | 30     |
| Culex pipiens (F)         | C TTCCTAACAATCCAGACA         | 30     |
| Culex pipiens (R)         | Cy55-GGAAACAGTGAGCTCGGG-BHQ1 | 20     |
| Culex pipiens (P)         | JOE-GCTTGGTAGAGTTTG-BHQ1     | 20     |
| Culex pipiens molestus (P)| RoX-TGACCCTCCAGTAAGGTATCAC-T-BHQ2 | 20     |
| Culex torrentium (F)      | GACACAGGCCAGCAA             | 10     |
| Cx. torrentium (R)        | GCCTAGCACTACTAA             | 10     |
| Cx. torrentium (P)        | FAM-CGATGATGCGTCTGCTACGA-BHQ1 | 10     |
| Eiden et al. (2010)       | GGGCTTCTGGTGTGTTC           | 15     |
| FLI_BRC_F                 | GATCTTGGCHTCCAACCT C        | 15     |
| FLI_BRC_R                 | FAM-CCACCCAGGAGTCCTYC6CAA   | 5      |
| FLI_P                     | FLI_P                       |        |
**TABLE 2** Absolute numbers of mosquitoes used in vector competence experiments, engorged mosquitoes after blood feeding, samples taken for ingestion confirmation and survival rate 14 dpi

| Mosquito species       | Feeding rate (%) | # Samples for ingestion confirmation | Survival rate (%) |
|------------------------|------------------|---------------------------------------|-------------------|
| Ae. vexans GR          | 99/777 (12.7)    | 13                                    | 38/86 (44.2)      |
| Cx. pipiens pipiens    | 49/644 (7.6)     | 8                                     | 12/41 (29.3)      |

Ingestion control and dissemination rates (legs and wing samples). Samples with a detectable relative viral titre higher than zero were considered positive.

### 2.4 Virus stocks

Vero cells (CCLV-RIE #0015), provided by the Biobank of the Friedrich-Loeffler-Institut, were used for generation of virus stocks and virus quantification. Cells were cultivated in MEM containing 10% FBS at 37°C and 2.5% CO₂.

Virus stocks of WNV lineage 1 (Magpie/Italy/2008/203204, GenBank No. JF719066) and WNV lineage 2 (“Austria”, GenBank No. HM015884) were grown to a titre of $10^8$ TCID₅₀/ml.

### 2.5 Statistical analysis

One-tailed test for comparison of viral titres in samples and Fisher’s exact test for statistical comparison of experimental groups were performed using R (version i386 3.6.1) and RStudio. p-Values lower than .05 were considered significant. Confidence intervals with 95% confidence level (95% CI) were calculated in MS Excel.

### 3 RESULTS

#### 3.1 Mosquito identification and WNV negativity

Morphological identification of field-acquired mosquitoes as Cx. pipiens was confirmed by sequencing (identity: 99% compared to GenBank No. MH463059.1). Biotype of Cx. pipiens was identified as *pipiens* by multiplex RT-PCR in all tested samples. None of the 88 tested Cx. *pipiens pipiens* (in eight pools) contained WNV-specific RNA.

#### 3.2 Vector competence trials

Out of 777 *Ae. vexans* GR, 99 were engorged after feeding on infectious blood (feeding rate 12.7%), while 49/644 (7.6%) *Cx. pipiens pipiens* acquired an infectious blood meal. After incubation for 14 days, 38/86 (44.2%) *Ae. vexans* GR were viable, while 12/41 (29.3%) *Cx. pipiens pipiens* were alive (Table 2). However, due to lack of confirmation of ingested virus two 14 dpi sampled *Ae. vexans* GR and two *Cx. pipiens pipiens* were excluded from further analysis.

Average viral titres in EF directly after feeding ranged between $2.35 \times 10^5$ (*Ae. vexans* infected with WNV lineage 1) and $6.57 \times 10^5$ (*Cx. pipiens* infected with WNV lineage 1) TCID₅₀/ml per tested mosquito (Table 3). Feeding groups without confirmation of ingested virus during blood feeding were excluded from further analysis.

Ingestion control and dissemination rates (legs and wing samples) were determined for both mosquito species (*Ae. vexans* GR $C_q$-values 23.87–24.1; titres $4 \times 10^5$ to $4.72 \times 10^5$ TCID₅₀/ml; *Cx. pipiens pipiens* $C_q$-values 24.18–24.69; titres $2.78 \times 10^5$ to $3.86 \times 10^5$ TCID₅₀/ml; one-tailed $t$ test $p = .1164$), while *Ae. vexans* ingested significantly less WNV lineage 1 ($C_q$-values 23.35 to 31.34; titres: $3.96 \times 10^3$ to $6.55 \times 10^5$ TCID₅₀/ml) during blood feeding compared to *Cx. pipiens pipiens* ($C_q$-values 22.34–26.93; titres $6.66 \times 10^5$ to $1.25 \times 10^6$ TCID₅₀/ml; one-tailed $t$ test, $p = .0134$; Table 3).

Results of vector competence trials for *Ae. vexans* GR and *Cx. pipiens pipiens* are summarized in Table 4. After incubation at 18°C, 55.6% (95% CI 23.1; 88.1) *Ae. vexans* GR established an infection with WNV lineage 1, respectively 50.0% (95% CI 10.0; 90.0) with WNV lineage 2. After incubation at 24°C, 91.7% (95% CI 76.1; 107.3) WNV lineage 1 were confirmed, while 77.8% (95% CI 50.6; 105.0) WNV lineage 2 infected mosquitoes. One out of nine incubated *Cx. pipiens pipiens* was viable at day 14 and infected with WNV lineage 2. Leaving temperature aside, *Ae. vexans* GR were comparably susceptible to infection with both tested viral strains WNV lineage 1 and WNV lineage 2 (Fisher’s exact test, $p = .2853$). *Ae. vexans* displayed a significantly higher susceptibility to infection with WNV lineage 1 and incubation at 24°C compared to *Cx. pipiens pipiens* (Fisher’s exact test, $p = .0158$). For *Ae. vexans* GR, no significant difference in the infection rates was observed at higher temperatures, even though a tendency of a higher infection rate at 24°C was observed (95% CI 23.1; 88.1) compared to 18°C (95% CI 50.6; 105.0).

Disseminated infections were detected in *Ae. vexans* GR infected with WNV lineage 1 incubated at 18°C (DR 53.3%; 95% CI 40.2; 66.4; Fisher’s exact test, $p = .141$; Table 4). Disseminated infections were detected in *Ae. vexans* GR infected with WNV lineage 1 incubated at 24°C (DR 60.0%, 95% CI 38.1; 81.9), at 24°C (DR 9.1%, 95% CI −7.9; 26.1) and in *Cx. pipiens* biotype *pipiens* at 24°C after infection with WNV lineage 1 (DR 14.3%, 95% CI −11.6; 40.2) and WNV lineage 2 (DR 100%; Table 4).

WNV lineage 1 RNA was detected in the saliva of *Cx. pipiens pipiens* (TR 100.0%, TE 11.1%). None of the tested saliva samples for *Ae. vexans* GR or *Cx. pipiens pipiens* infected with WNV lineage 2 contained WNV-specific RNA (Table 4).

The recorded relative viral titres is given in Table 5.

### 4 DISCUSSION

The results showed that under the given temperature regime *Ae. vexans* GR (24 and 18°C) and *Cx. pipiens pipiens* (24°C) are susceptible to
a WNV infection and confirm previous observations that German Cx. pipiens were susceptible to the infection with WNV lineage 1 (Jansen et al., 2019; Leggewie et al., 2016). Compared to another recently published study, the detected IR for Cx. pipiens pipiens in this study (IR 77.8%) was higher than the reported IR for the same mosquito species after a similar infection (different lineage 1 isolate, TOS-09) and incubation conditions (IR 3.1%–3.3%; Jansen et al., 2019), but this might be due to lower sample number in this study. As previously observed for Cx. pipiens, no significant effect of temperature was found on the infection rate of Ae. vexans, even though a slight increase of the IR was noted with increasing temperatures as observed before (Vogels et al., 2017). The observed difference between the amounts of ingested WNV lineage 1 might be due to the different blood types used for the infectious blood meals, since it was previously suggested that the blood source might influence the infection of mosquito midgut cells (Vogels et al., 2017). Blood meal sources for infectious blood meals were selected corresponding to the host preferences of the tested mosquito species (Börstler et al., 2016).

Viral replication, through the detection of disseminated infections, was confirmed for WNV lineage 1 in Ae. vexans (18°C and 24°C) and Cx. pipiens pipiens (24°C). Cx. pipiens pipiens also had saliva containing viral RNA. However, compared to previous reports lower DR

### Table 3
Results of quantification of ingested viral titres for Ae. vexans GR and Cx. pipiens pipiens by qRT-PCR for WNV lineage 1 Magpie/Italy/203204 (WNV1) and WNV lineage 2 “Austria” (WNV2) before incubation

| Mosquito species | Viral strain | Cq-value | Titre TCID<sub>50</sub>/ml |
|------------------|--------------|----------|-----------------------------|
| Ae. vexans GR    | WNV1         | 31.34    | 3.96 × 10<sup>3</sup>       |
|                  |              | 28.15    | 3.05 × 10<sup>4</sup>       |
|                  |              | 27.9     | 3.59 × 10<sup>4</sup>       |
|                  |              | 23.96    | 4.44 × 10<sup>5</sup>       |
|                  |              | 24.04    | 4.22 × 10<sup>5</sup>       |
|                  |              | 27.26    | 5.38 × 10<sup>4</sup>       |
|                  |              | 23.35    | 6.55 × 10<sup>5</sup>       |
|                  |              | 23.89    | 4.63 × 10<sup>5</sup>       |
|                  |              | 25.85    | 1.33 × 10<sup>5</sup>       |
|                  |              | 25.19    | 1.12 × 10<sup>5</sup>       |
|                  | WNV2         | 23.87    | 4.72 × 10<sup>5</sup>       |
|                  |              | 24.1     | 4.06 × 10<sup>5</sup>       |
| Cx. pipiens pipiens | WNV1        | 22.34    | 1.25 × 10<sup>6</sup>       |
|                  |              | 26.93    | 6.66 × 10<sup>4</sup>       |
|                  |              | 23.5     | 5.98 × 10<sup>5</sup>       |
|                  |              | 23.0     | 8.19 × 10<sup>5</sup>       |
|                  |              | 23.61    | 5.54 × 10<sup>5</sup>       |
|                  | WNV2         | 24.69    | 2.78 × 10<sup>5</sup>       |
|                  |              | 24.18    | 3.86 × 10<sup>5</sup>       |

### Table 4
Absolute numbers of positive from tested mosquitoes and resulting infection (IR), dissemination (DR) and transmission (TR) rates and transmission efficiency (TE) in per cent (%) for Ae. vexans GR and Cx. pipiens pipiens 14 dpi with WNV lineage 1 Magpie/Italy/203204 (WNV1) and WNV lineage 2 “Austria” (WNV2) incubated at 18°C and 24°C

| Mosquito species | WNV lineage | Temperature (°C) | IR<sup>a</sup> (%) | DR<sup>b</sup> (%) | TR<sup>c</sup> (%) | TE<sup>d</sup> (%) |
|------------------|-------------|-----------------|---------------------|-------------------|------------------|------------------|
| Ae. vexans GR    | WNV1        | 18              | 5/9 (55.6)          | 3/5 (60.0)        | 0/3 (0)          | NA               |
|                  |             | 24              | 11/12 (91.7)        | 1/11 (9.1)        | 0/1 (0)          | NA               |
|                  | WNV2        | 18              | 3/6 (50.0)          | 0/3 (0)           | 0/0 (0)          | NA               |
|                  |             | 24              | 6/9 (66.7)          | 0/6 (0)           | 0/0 (0)          | NA               |
| Cx. pipiens pipiens | WNV1      | 24              | 7/9 (77.8)          | 1/7 (14.3)        | 1/1 (100.0)     | 1/9 (11.1)      |
|                  | WNV2        | 24              | 1/1 (100.0)         | 1/1 (100.0)       | 0/1 (0)          | NA               |

Abbreviation: NA, not applicable.

<sup>a</sup> Infection rate (infected/dissected mosquitoes).
<sup>b</sup> Dissemination rate (disseminated infections/infected mosquitoes).
<sup>c</sup> Transmission rate (virus-containing saliva/disseminated infections).
<sup>d</sup> Transmission efficiency (virus-containing saliva/engorged mosquitoes).
(11.1% compared to 94% after 28 days) for *Cx. pipiens pipiens* were noted during the present study (Leggewie et al., 2016). This might be due to a varying extrinsic incubation period (EIP) between the studies (14 dpi vs. 21–35 dpi). The tested EIP in this study is within the range of common EIPs (12–15 dpi) used for assessment of vector competence (Goddard et al., 2002; Huber et al., 2014; Tiawsirisup et al., 2008; Veronesi et al., 2018; Vogels et al., 2017). However, taking into consideration the prolonged EIP in other studies with German mosquitoes it might be suggested that German mosquitoes of the *Cx. pipiens* complex could require a longer EIP before being

### TABLE 5  Results of RT-qPCR for WNV-specific PCR of infected *Ae. vexans* GR and *Cx. pipiens pipiens* 14 dpi with WNV lineage 1 Magpie/Italy/203204 (WNV1) and WNV lineage 2 "Austria" (WNV2) incubated at 18 and 24°C

| Mosquito species | Viral strain | Temp. (°C) | Sample | Infection rate Cq-value | Titre TCID<sub>50</sub>/ml | Dissemination rate Cq-value | Titre TCID<sub>50</sub>/ml | Transmission rate Saliva | Cq-value | Titre TCID<sub>50</sub>/ml |
|-----------------|--------------|------------|--------|-------------------------|-----------------------------|-----------------------------|-----------------------------|----------------------------|----------|-----------------------------|
| *Ae. vexans* GR | WNV1 18      |            | #1     | 40.36                    | 9.13 × 10<sup>1</sup>       | 36.22                       | 5.04 × 10<sup>1</sup>      | neg. ND                     | ND       | NA                          |
|                 |              |            | #2     | ND                       | NA                          | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #3     | 39.65                    | 3.41 × 10<sup>1</sup>       | 37.51                       | 3.75 × 10<sup>1</sup>      | neg. ND                     | ND       | NA                          |
|                 |              |            | #4     | ND                       | NA                          | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #5     | ND                       | NA                          | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #6     | 37.63                    | 2.03 × 10<sup>1</sup>       | 37.47                       | 5.50 × 10<sup>1</sup>      | neg. ND                     | ND       | NA                          |
|                 |              |            | #7     | ND                       | NA                          | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #8     | 38.01                    | 3.94 × 10<sup>1</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #9     | 40.1                     | 1.08 × 10<sup>1</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              | 24         | #10    | 31.18                    | 1.05 × 10<sup>1</sup>       | ND                          | NA                          | deg. ND                     | ND       | NA                          |
|                 |              |            | #11    | 35.43                    | 7.18 × 10<sup>2</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #12    | 33.95                    | 1.83 × 10<sup>3</sup>       | ND                          | NA                          | deg. ND                     | ND       | NA                          |
|                 |              |            | #13    | 36.39                    | 4.50 × 10<sup>3</sup>       | 39.29                       | 6.93 × 10<sup>1</sup>      | deg. ND                     | ND       | NA                          |
|                 |              |            | #14    | 36.83                    | 4.83 × 10<sup>3</sup>       | ND                          | NA                          | deg. ND                     | ND       | NA                          |
|                 |              |            | #15    | 36.95                    | 4.50 × 10<sup>1</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #16    | 31.26                    | 1.70 × 10<sup>2</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #17    | 42.29<sup>a</sup>        | NA                          | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #18    | 31.74                    | 1.24 × 10<sup>3</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #19    | 38.86                    | 1.33 × 10<sup>3</sup>       | ND                          | NA                          | pos. ND                     | ND       | NA                          |
|                 |              |            | #20    | 32.89                    | 5.80 × 10<sup>2</sup>       | ND                          | NA                          | deg. ND                     | ND       | NA                          |
|                 |              |            | #21    | 35.82                    | 9.23 × 10<sup>1</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 | WNV2 18      |            | #22    | ND                       | NA                          | ND                          | NA                          | deg. ND                     | ND       | NA                          |
|                 |              |            | #23    | 31.32                    | 1.63 × 10<sup>3</sup>       | ND                          | NA                          | pos. ND                     | ND       | NA                          |
|                 |              |            | #24    | 40.3                     | 5.32 × 10<sup>3</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #25    | 39.54                    | 8.63 × 10<sup>0</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #26    | ND                       | NA                          | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #27    | 32.85                    | 6.15 × 10<sup>2</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              | 24         | #28    | 33.49                    | 4.10 × 10<sup>2</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #29    | 32.39                    | 8.30 × 10<sup>2</sup>       | ND                          | NA                          | pos. ND                     | ND       | NA                          |
|                 |              |            | #30    | ND                       | NA                          | ND                          | NA                          | pos. ND                     | ND       | NA                          |
|                 |              |            | #31    | 37.5                     | 3.17 × 10<sup>1</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #32    | ND                       | NA                          | ND                          | NA                          | deg. ND                     | ND       | NA                          |
|                 |              |            | #33    | 34.74                    | 1.84 × 10<sup>3</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #34    | 37.16                    | 3.94 × 10<sup>1</sup>       | ND                          | NA                          | neg. ND                     | ND       | NA                          |
|                 |              |            | #35    | ND                       | NA                          | ND                          | NA                          | incon. ND                   | ND       | NA                          |
|                 |              |            | #36    | 37.85                    | 2.53 × 10<sup>1</sup>       | ND                          | NA                          | incon. ND                   | ND       | NA                          |

(Continues)
able to transmit WNV probably due to their genetic background (Jansen et al., 2019; Leggewie et al., 2016).

Viral titres in established infections determined at 14 dpi were, besides one exception, lower than initially ingested viral titres in Ae. vexans GR and Cx. p. p. after infection with WNV lineage 1 and 2. This might indicate the presence of an infection barrier which prevents the infection of the midgut epithelium or viral replication within the cells and has already been suspected for Ae. vexans (Franz, Kantor, Passarelli, & Clem, 2015; Tiawsirisup et al., 2008). Established infections in 69.4% of tested Ae. vexans and 88.9% Cx. p. p., but low DR (9.1% in Ae. vexans GR and 14.3% in Cx. p. p. infected with lineage 1, incubated at 24°C) after infection with WNV might indicate the presence of a midgut escape barrier as suggested for Cx. p. p. from the Netherlands (Vogels et al., 2016). This escape barrier might be dose-dependent like previously suggested for infection barriers in Ae. vexans (Franz et al., 2015; Tiawsirisup et al., 2008). The level of viral replication in the mosquito midgut determines the efficiency of dissemination to the salivary glands. The salivary gland infection barrier seems to be dose-dependent of the viral titres in haemolymph (Franz et al., 2015; Huber et al., 2014; Veronesi et al., 2018). Therefore, this might be the case for observed differences in WNV circulation during the 2018 transmission season.

The presented results are in line with previous observations that Ae. vexans and Cx. p. p. might the involved in WNV transmission since both species were susceptible to viral infection (Tiawsirisup et al., 2008). However, the present results also stand in contrast to previous results since the tested laboratory reared population of Ae. vexans GR could not transmit WNV under the given conditions (Goddard et al., 2002; Tiawsirisup et al., 2008). From all mosquito species, Cx. p. p. contribute to 80% of WNV transmission in humans, while 20% is due to other Culex and Aedes species (Koenraadt, Möhlmann, Verhulst, Spitzen, & Vogels, 2019). Vector competence for German Aedes species has not been shown until now. Due to the weather conditions during summer 2018 (driest summer since 1881; https://www.dwd.de/DE/klima_ueberwachung/deutschland/deutschland_node.html, Imbery et al., 2018) in Mecklenburg-Pomerania, it was not possible to obtain field Ae. vexans. Influences of genetic difference between mosquito populations on vector competence were already suspected for Cx. p. p. and Ae. japonicus (Fros, Geertsema, et al., 2015; Huber et al., 2014; Veronesi et al., 2018). Therefore, this might be the case for observed differences between previous reports of vector competence for Ae. vexans and tested Ae. vexans GR laboratory colony. This emphasizes the importance of different mosquito populations and viral strains to be tested for vector competence, as previously reported for Cx. p. p. (Farajollahi et al., 2011). For this reason, ongoing evaluation of vector competence of varying mosquito species and circulating viruses is required not only in Germany but worldwide in order to obtain relevant WNV risk assessments, plan future mosquito control measures.

### TABLE 5 (Continued)

| Mosquito species | Viral strain | Temp. (°C) | Sample | Cq-value | Titre TCID<sub>50</sub>/ml | Cq-value | Titre TCID<sub>50</sub>/ml | Saliva assay | Cq-value | Titre TCID<sub>50</sub>/ml |
|------------------|--------------|------------|--------|----------|---------------------------|----------|---------------------------|-------------|----------|---------------------------|
| Cx. pipiens      | WNV1         | 24         | #37    | ND       | NA                        | ND       | NA                        | pos.        | ND       | NA                        |
|                  |              |            | #38    | 36.32    | 6.70 × 10<sup>1</sup>     | ND       | NA                        | neg.        | ND       | NA                        |
|                  |              |            | #39    | 32.85    | 6.15 × 10<sup>2</sup>     | ND       | NA                        | neg.        | ND       | NA                        |
|                  |              |            | #40    | 33.62    | 3.76 × 10<sup>1</sup>     | ND       | NA                        | neg.        | ND       | NA                        |
|                  |              |            | #41    | 35.68    | 1.01 × 10<sup>2</sup>     | ND       | NA                        | neg.        | ND       | NA                        |
|                  |              |            | #42    | 33.41    | 4.30 × 10<sup>2</sup>     | ND       | NA                        | neg.        | ND       | NA                        |
|                  |              |            | #43    | 37.76    | 2.69 × 10<sup>1</sup>     | ND       | NA                        | neg.        | ND       | NA                        |
|                  |              |            | #44    | 17.9     | 8.54 × 10<sup>3</sup>     | 20.2     | 1.96 × 10<sup>6</sup>    | incon.       | 37.11    | 3.47 × 10<sup>3</sup>    |
|                  |              |            | #45    | ND       | NA                        | ND       | NA                        | pos.        | ND       | NA                        |
|                  |              |            | #46    | 33.08    | 5.29 × 10<sup>2</sup>     | ND       | NA                        | pos.        | ND       | NA                        |

Abbreviations: incon., inconclusive; NA, not assessed; ND, not detected; neg., negative; pos., positive.
*Evaluated as negative.
and make action plans regarding WNV control and prevention of cases in humans and animals.

5 | CONCLUSION

This study showed that Ae. vexans GR and northern-German Cx. pipiens pipiens can be infected with WNV lineages 1 and 2 at 18°C and 24°C. Only Cx. pipiens pipiens was able to transmit WNV lineage 1. Spread of WNV to Germany in 2018 requires knowledge on the vector competence of indigenous mosquito species for WNV to provide information for WNV control and prevention.

ACKNOWLEDGEMENTS

The authors would like to thank Prof. Dr. Martin Pfeffer (University of Leipzig) for providing the Ae. vexans GR laboratory colony. For WNV strain “Austria” (acc. no. HM015884), we thank Dr. Ute Ziegler and Prof. Dr. Martin H. Groschup (both Institute of Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institut). The WNV strain “Austria” was initially kindly provided by Sandra Revilla-Fernandez (AGES Mödlingen, Austria). The authors thank Dr. Davide Lelli (Istituto zooprofittatico sperimentale della Lombardia e dell’Emilia Romagna) for providing WNV lineage 1. Furthermore, the authors thank Kersten Biebl (Institute of Diagnostic Virology, Friedrich-Loeffler-Institut) for performing the sequencing, PD. Dr. Helge Kampen (Institute of Infectology, Friedrich-Loeffler-Institut) for support during identification of field breeding sites and Lisa Tippelt for assisting in mosquito identification. Furthermore, we would like to thank Marlene Hausner, Oliver Tauchmann and Ulrike Neumann for their excellent technical assistance.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

Not applicable for the study subjects. Blood for mosquito feeding was collected from the animals kept at the Friedrich-Loeffler-Institute. Animals were held and sampled according to the national and European legislation (Directive 2010/63/EU). The procedures were approved by the competent authority of the Federal State of Mecklenburg-Western Pomerania, Germany (reference number: 7221.3-2-041/17).

ORCID

Ana Vasić https://orcid.org/0000-0002-0653-2935

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Bakonyi, T., Ivancics, E., Erdélyi, K., Ursu, K., Ferenczi, E., Weissenböck, H., & Nowotny, N. (2006). Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. *Emerging Infectious Diseases*, 12(4), 619–623.

Barzon, L., Papa, A., Lavezzo, E., Franchin, E., Pacenti, M., Sinigaglia, A., ... Palù, G. (2015). Phylogenetic characterization of Central/Southern European Lineage 2 West Nile virus: Analysis of human outbreaks in Italy and Greece, 2013–2014. *Clinical Microbiology & Infection*, 21, e1121–e1122.e10.

Beck, C., Jimenez-Clavero, M. A., Leblond, A., Durand, B., Nowotny, N., Lepare-Goffart, I., ... Lecollinet, S. (2013). Flaviviruses in Europe: Complex circulation patterns and their consequences for the diagnosis and control of West Nile disease. *International Journal of Environmental Research and Public Health*, 10, 6049–6083. https://doi.org/10.3390/ijerph10116049

Becker, N., Krüger, A., Kuhn, C., Plenge-Bönig, A., Thomas, S. M., Schmidt-Chanasit, J., & Tannich, E. (2014). Stechmücken als Überträger exotischer Krankheitserreger in Deutschland. *Bundesgesundheitsbl*, 57, 531–540 (article in German). https://doi.org/10.1007/s00103-013-1918-8

Becker, N., Petric, D., Zgomba, M., Bosse, C., Madon, M. B., Dahl, C., & Kaiser, A. (2010). *Mosquitoes and their control*. Berlin-Heidelberg, Germany: Springer.

Börstler, J., Jöst, H., Garms, R., Krüger, A., Tannich, E., Becker, N., ... Lübben, R. (2016). Host feeding patterns of mosquito species in Germany. *Parasites and Vectors*, 9, 318. https://doi.org/10.1186/s13071-016-1597-z

Chancey, C., Grinev, A., Volkova, E., & Rios, M. (2015). The global ecology and epidemiology of West Nile virus. *BioMed Research International*, 2015, 1–20. https://doi.org/10.1155/2015/376230

Di Sabatino, D., Bruno, R., Sauro, F., Danzetta, M. L., Cito, F., Iannetti, S., ... Calistrì, P. (2014). Epidemiology of West Nile Disease in Europe and in the Mediterranean Basin from 2009 to 2013. *BioMed Research International*, 2014, 907852. https://doi.org/10.1155/2014/907852

Eiden, M., Vina-Rodriguez, A., Hoffmann, B., Ziegler, U., & Groschup, M. H. (2010). Two novel real-time quantitative reverse transcription polymerase chain reaction assays with unique target sites for the specific and sensitive detection of lineages 1 and 2 West Nile virus strains. *Journal of Veterinary Diagnostic Investigation*, 22, 748–753.

Farajollahi, A., Fonesca, D. M., Kramer, L. D., & Kilpatrick, A. M. (2011). “Bird biting” mosquitoes and human disease: A Review of the Role of *Culex pipiens* complex mosquitoes in Epidemiology. *Infection, Genetics and Evolution*, 11(7), 1577–1585. https://doi.org/10.1016/j.meegid.2011.08.13

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I form diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3(5), 294–299.

Fortuna, C., Remoli, M. E., Severini, F., Di Luca, M., Toma, L., Fois, F., ... Ciufolini, M. G. (2015). Evaluation of vector competence for West Nile virus in Italian *Steagomyia albopicta* (=Aedes albopic tus) mosquitoes. *Medical and Veterinary Entomology*, 29(4), 430–433. https://doi.org/10.1111/mve.12133

Franz, A. W. E., Kantor, A. M., Passarella, A. L., & Clem, R. J. (2015). Tissue barriers to arbovirus infection in mosquitoes. *Viruses*, 7, 3741–3767. https://doi.org/10.3390/v7072795

Fros, J. J., Geertsema, C., Vogels, C. B., Roosjen, P. P., Gailloux, A.-B., Valk, J. M., ... Pijlman, G. P. (2015). West Nile Virus: High transmission rate in North-Western European mosquitoes indicates its epidemics potential and warrants increased surveillance. *PLoS Neglected Tropical Diseases*, 9(7), e0003956. https://doi.org/10.1371/journal.pntd.0003956

Fros, J. J., Miesen, P., Vogels, C. B., Gaibani, P., Sambri, V., Martina, B. E., ... Pijlman, G. P. (2015). Comparative Usutu and West Nile virus transmission potential by local Culex pipiens mosquitoes in north-western Europe. *One Health*, 1, 31–36.

Goddard, L. B., Roth, A. E., Reisen, W. K., & Scott, T. W. (2002). Vector competence of California mosquitoes for West Nile virus. *Emerging Infectious Diseases*, 8(12), 1285–1391.
