**To be published as an article in Emerging Microbes & Infections**

**Title page**

Succinct title (max. 200 character > 60):

**West Nile virus transmission by *Culex torrentium* mosquitoes from Germany**

Concise running title (max. 50 Characters):

**West Nile Virus transmission by Culex torrentium**

**Culex torrentium as a vector for West Nile Virus**

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**Keywords:** *Culex pipiens pipiens*, *Culex pipiens molestus*, *Culex torrentium*, West Nile Virus, vector competence, salivation assay

**Abstract** (250 words)

**Introduction** (Word limit for articles: 6000 words)

The most commonly detected *Culex* species in Europe are *Culex pipiens* s.l. (L.) and *Culex torrentium* (Martini, 1925) (knight and stone 77, hesson).In Europe, *Cx. pipiens* s.l. is represented by two biotypes: *Culex pipiens pipiens* biotype *pipiens* (*Cx. p. pipiens*) and *Culex pipiens pipiens* biotype *molestus* (*Cx. p. molestus*). Species identification of these three taxa is challenging (Zittra, Rudolf). Both biotypes of *Cx. pipiens* s.l. cannot be identified by morphological features and are traditionally classified by a biotype-specific mating behaviour, breeding sites and hibernation. *Culex p. molestus* is considered stenogamous, aoutogenous, utilize underground breeding sites and is non-diapausing, while *Cx. p. pipiens* is eurygamous, anaoutogenous, breeds above the ground and overwinter in diapause. *Culex torrentium* resembles *Cx. p. pipiens* regarding its breeding ecology and often occurs in sympatry (Becker, Hesson,Brugman, Lühken). *Culex torrentium* males can be differentiated from *Cx. pipiens* s.l. by characters of the hypopygium, but the reliable morphological differentiation of the females is difficult, because pre-alar scales easily fall off and the useage of morphometric wing characters are generally not established (service, onyeka, Hesson,Börstler). Therefore, species differentiation of these *Culex* species often relies on molecular identification assays (e.g. Rudolf et al., Vogels et al. 2015). The members of the *Cx. pipiens* s.l. and *Cx. torrentium* occur in Europe together, with *Cx. torrentium* as the dominant species in northern Europe and *Culex p. pipiens* prevailing south of the alps (Calzolari et al 2016, Hesson et al 2011+2014, Weitzel 2015). In Central Europe, both sister species can be found simultaneously in the same areas, e.g. Germany (Rudolf, Becker, Wetblow) or Austria (Zittra).

Due to the wide distribution and high abundance of three taxa, different studies analysed their relevance as vectors. For a long time, it has been believed, that *Cx pipiens* s.l.as well as *Cx. torrentium* are primarily ornitophilic (hesson), but recent publications proof a high variability concerning their selected hosts (Börstler, osorio2012, Zittra et al 2016). Host feeding patterns of *Cx. p. pipiens* s.l. as well as *Cx. torrentium* include birds and humans for both species, rendering these species into potential vectors for the transmission of zoonotic pathogens to humans (Börstle osorior, Zittra et al 2016). Different mosquito-borne viruses have been detected from field-collected specimens of the three *Culex* taxa: Sindbis Virus (SINV), Usutu Virus (USUV), Batai Virus (BATV) and West Nile virus (WNV) (Cite hanna jöst usw….Brugman). In addition, there is growing evidence from vector competence studies that European populations of both *Cx. pipiens* biotypes and their hybrid are competent vectors for WNV and USUV (Vogels 2017, Vogels 2016, Romo 2018 cite weitere?), but not for Zika virus [CITE]. In comparison to *Cx. pipiens* s.l., there is considerably less information about the vector competence of *Cx. torrentium*. Leggewie et al. demonstrated, that Cx. torrentium mosquitoes from Germany are susceptible to WNV infection, but did not conduct a salivation assay. However, for the *Cx. torrentium* population from Sweden, it was shown that the species is a better vector for SINV compared to *Cx. pipiens* s.l. (Lundström et al 1990, Hesson et al 2015).

West Nile virus (WNV) belongs to the genus Flavivirus within the family *Flaviviridae* (Calisher 1989). WNV is a zoonoses, with an enzootic cycle between mosquitoes as vectors and birds as the primary host. Humans, equines and other vertebrates are incidental hosts (Gyure 2009). Human WNV infections can range from mild infections to fatal outcomes by neuroinvasive disease (Campbell 2002). No licensed vaccine or specific treatment for humans is available. The high epidemic potential of WNV was illustrated by its fast spread after a single introduction to New York City (United States of America) in 1999 (Nash, Petersen). Subsequently WNV spread rapidly through North America (Kilpatrick 2011, Sambri 2013), resulting into more than 20,000 neuroinvasive cases and more than 2,000 deaths since 1999 (CDC). This changed in 1996, when an outbreak in Romania results in 352 neuroinvasive cases and 17 deaths [cite]. Since then, various outbreaks were recognized in further European countries (e.g. Greece, Hungary, Italy), causing more than 3,000 human WNV cases with at least X deaths (ECDC, x).

The ongoing circulation of WNV in southern and eastern Europe results in an increasing risk of WNV spread to central Europe like Germany, if adequate vector mosquito species are present (Calistri, Gabriel). The here presented work demonstrated that *Cx. p. pipiens* and *Cx. p. molestus* from Germany are competent vectors of WNV. In addition, the study demonstrated a very high vector competence of *Cx. torrentium* for the first time.

**Results** (532 words)

All three investigated *Culex* species were susceptible to WNV (Table), i.e., viral titres of mosquito bodies reached at least the detection limit of the q-RT-PCR of 10.000 RNA copies per mosquito specimen. *Culex p. molestus* showed the lowest infection rates (IRs) between 0 and 6 % (27 °C; 21 dpi). The other two species had higher IRs with 23 % for *Cx. p. pipiens* (24°C; 21 dpi) and 32 % for *Cx. torrentium* (27°C; 14 dpi). The IRs were generally considerably higher at 21 dpi in comparison to 14 dpi. The only exception waswith*Culex p. molestus* infections were only detected 21 dpi. Overall, the titres of *Cx. p. molestus* gave the lowest results between 3 to 6 log10 RNA copies per specimen (Table). The results for *Cx. p. pipiens* and *Cx. torrentium* were higher, ranging between 4 to 8 log10 RNA copies/specimen and 7 - 8 log10 RNA copies/specimen, respectively.

All three tested *Culex* species were able to transmit WNV (Figure 1). No WNV-positive saliva were detected at 18°C or 21°C. Viable WNV particles in the saliva of *Cx. p. molestus* were only detected for 27°C at 21 dpi. For *Cx. p. pipiens* and *Cx. torrentium*, infectious WNV was detected at 27°C, but also at 24°C. At 14 dpi, only *Cx. torrentium* salivated infectious WNV with a transmission rate (TR) of 9 % (1 WNV-positive saliva of 11 infected mosquitoes). The lowest detected TR at 27°C (21 dpi) was 25 % for *Cx. p. molestus* (1/4), followed by 33 % for *Cx. p. pipiens* (1/3) and 90 % for *Cx. torrentium* (9/10). At the lower incubation temperature of 24°C, the TR of *Cx. p. pipiens* dropped to 14 % (1/7) and to 62 % for *Cx. torrentium* (5/8).

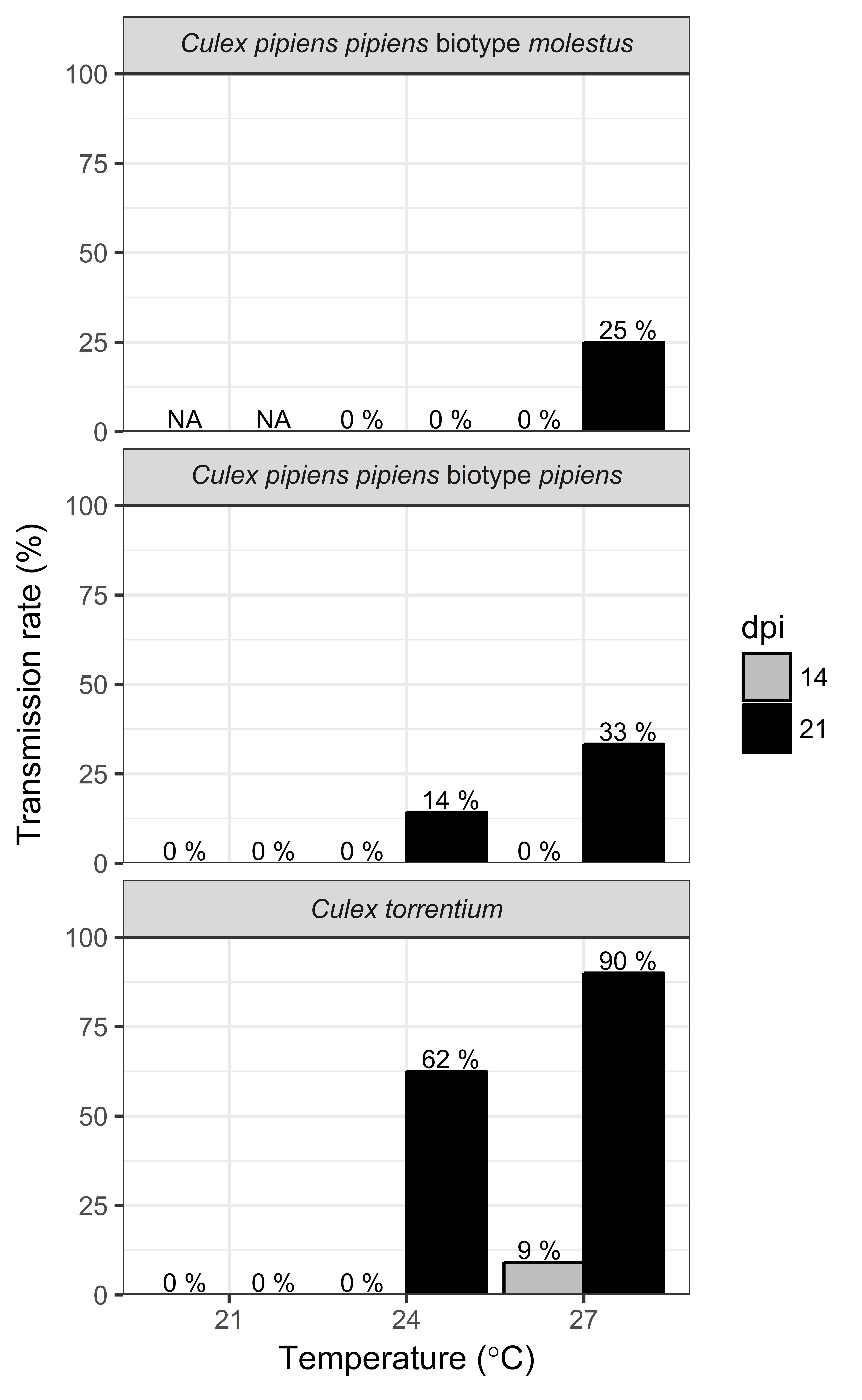
*Culex p. molestus* as well as *Cx. p. pipiens* had a comparable low transmission efficiency (TE) at 27°C (21 dpi): 2 % (1 WNV-positive saliva out of 62 fed females) and 3 % (1/33), respectively (Figure 2). A similar value was found for *Cx. p. pipiens* at 24 °C (21 dpi) with a TE of 3 % (1/31). In contrast, *Cx. torrentium* had a rather high TE (21 dpi) of 24 % (9/38) for 27°C and 17 % for 24°C. With 3 % (1/34), the only TE over zero at 14 dpi was found for *Cx. torrentium* at 27°C.

**Figures** (Max. 8)

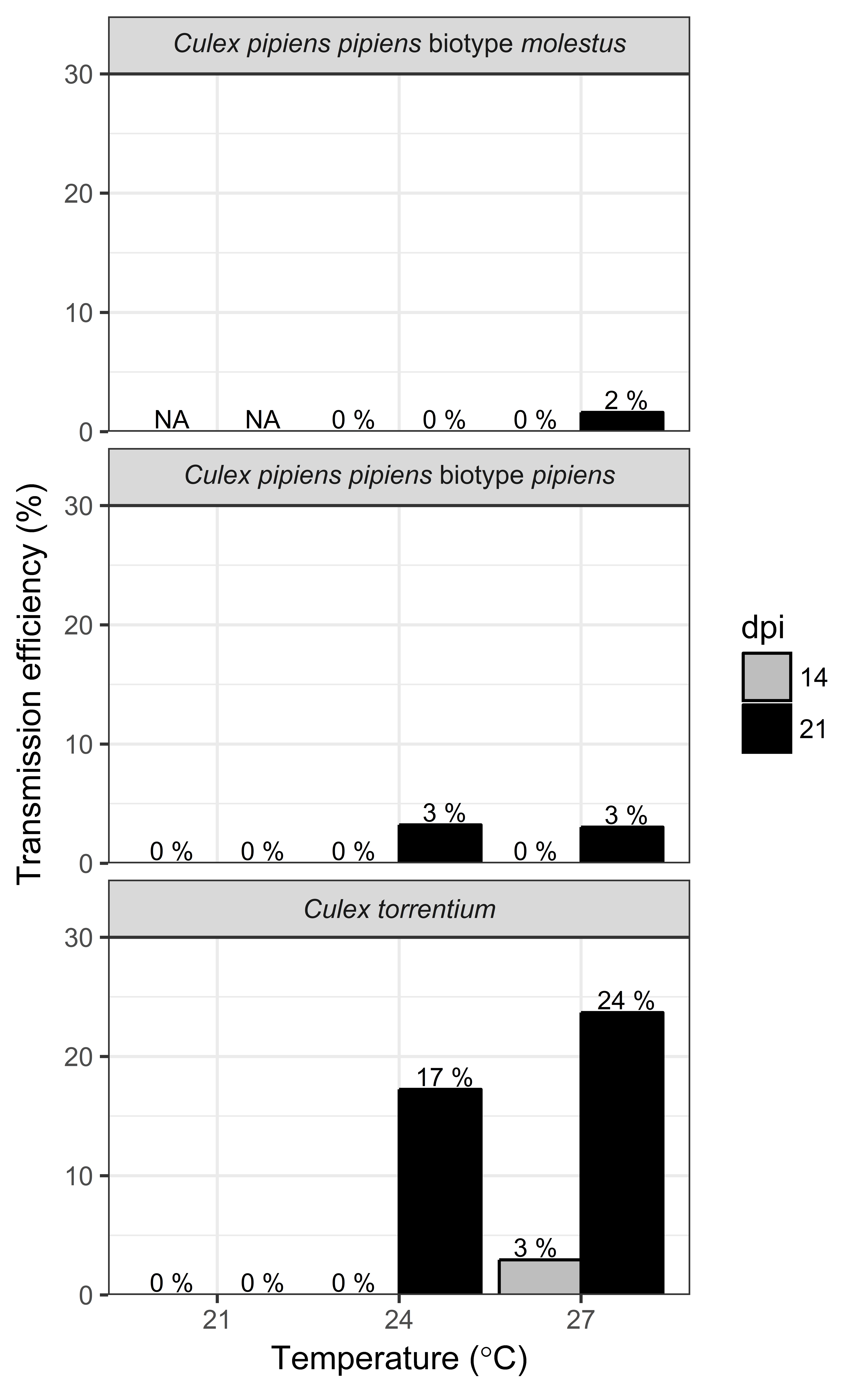
**of three *Culex* species experimentally infected with West Nile virus**

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| --- | --- | --- | --- | --- |
| **species** | **temperature** | **days post infection** | **infection rate\*** | **titre\*\*** |
| *Culex pipiens pipiens* biotype *molestus* | 18 | 14 | 0.0 (0/29) | NA (NA) |
| 21 | 3.4 (1/29) | 4.9 (NA) |
| 21 | 14 | NA | NA |
| 21 | NA | NA |
| 24 | 14 | 0.0 (0/31) | NA (NA) |
| 21 | 3.2 (1/31) | 5.0 (NA) |
| 27 | 14 | 0.0 (0/31) | NA (NA) |
| 21 | 6.4 (4/62) | 5.8 (2.2) |
| *Culex pipiens pipiens* biotype *pipiens* | 18 | 14 | 3.1 (1/32) | 4.5 (NA) |
| 21 | 6.1 (2/33) | 4.0 (0.0) |
| 21 | 14 | 3.3 (1/30) | 6.4 (NA) |
| 21 | 9.7 (3/31) | 6.3 (1.3) |
| 24 | 14 | 3.3 (1/30) | 8.1 (NA) |
| 21 | 22.6 (7/31) | 7.2 (1.1) |
| 27 | 14 | 0.0 (0/35) | NA (NA) |
| 21 | 9.1 (3/33) | 4.7 (0.4) |
| *Culex torrentium* | 18 | 14 | 6.2 (2/32) | 5.4 (0.1) |
| 21 | 15.2 (5/33) | 4.9 (0.5) |
| 21 | 14 | 0.0 (0/31) | NA (NA) |
| 21 | 12.5 (4/32) | 6.9 (1.5) |
| 24 | 14 | 6.4 (2/31) | 7.3 (0.8) |
| 21 | 27.6 (8/29) | 7.8 (1.1) |
| 27 | 14 | 32.4 (11/34) | 5.9 (1.1) |
| 21 | 26.3 (10/38) | 5.6 (1.1) |



**Figure 1**: **Transmission rate of three *Culex* species experimentally infected with West Nile virus** Defined as the number of mosquitoes with WNV-positive saliva per number of WNV-positive mosquito bodies.



**Figure 2: Transmission efficiency of three *Culex* species experimentally infected with West Nile virus**

Defined as the number of WNV-positive saliva per the total number of fed females.

**Discussion**

Six European mosquito species were previously known to be susceptible to WNV infections and able to transmit infectious WNV particles in the laboratory: *Aedes albopictus*, *Aedes detritus*, *Aedes japonicus*, *Culex modestus*, *Cx. p. pipiens* and *Cx. p. molestus* [Fortuna et al 2015, Blagrove et al 2016, Veronesi E et al 2018, Balenghien et al 2007, Vogels et al 2016, Vogels et al 2017B].This is in line with the here presented results. All three investigated *Culex* taxa were susceptible to WNV infections and able to transmit infectious WNV particles, while the WNV vector competence of *Cx. torrentium* was shown for the first time. Our results confirm the vector competence for the two biotypes of *Cx. pipiens* s.l., with a maximal TR of 25% for *Cx. p.* *molestus* and a TR of 33% for *Cx.* *p.* *pipiens* at an incubation temperature of 27°C (21 dpi). This corresponds to previous findings of vector competence studies with other European populations of both biotypes, showing TRs up to 10% for *Cx*. *p.* *molestus* and a TR up to 30% for *Cx*. *p.* *pipiens* [Figure 3]. Higher TRs between 30% and 75% were only found for not further differentiated *Cx. pipiens* s.l. mosquitoes.

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**Figure 3 A: Transmission rate of three *Culex* species experimentally infected with West Nile virus in comparison to previous published vector competence studies with European populations of *Culex pipiens* s.l./*torrentium* [**Vogels et al 2016, Vogels et al 2017B] B: Average temperature (July/August) in the year of each detected human WNV case in Europe between 2011 and 2017 [ECDC WNV].

The susceptibility of WNVfor *Cx. torrentium* no salivation assay was conductedexplicit of the species wasmissing The TRs of *Cx. torrentium* were substantially higher compared to the other two tested *Culex* taxa with 90% (27°C, 21 dpi) and 62% (24°C, 21 dpi), and the only species with infectious saliva at 14 dpi (27°C). Comparative studies regarding the infection rate of *Cx. p.* *pipiens* and *Cx. torrentium* also showed higher infection rates for *Cx. torrentium* [Leggewie et al 2016]. With 90% at 27°C (21 dpi), *Cx. torrentium* had the highest transmission rate detected for any of the *Cx. pipiens* s.l./*torrentium* taxa so far (Figure 3). Accordingly, the TE of *Cx. torrentium* with 17% (24°C) and 24% 27°C (21 dpi) was also notable higher in comparison to 3% for *Cx. p. pipiens* and 2% for *Cx. p. molestus*.

There was only one vector competence study performed with *Cx. torrentium* before, which revealed the species as a potent vector of SINV. SINV is a mosquito-borne virus of the genus alphavirus, with a similar ecology as WNV, i.e. enzootic cycle with birds and spill-overs to mammalian species. Experimental infection of birds by either *Cx. torrentium* or *Cx. pipiens* s.l. revealed a clearly higher vector competence by *Cx. torrentium* [Lundström et al 1990]. In addition, field studies found highest SINV infection rates for naturally infected *Cx. torrentium* in comparison to *Cx. pipiens* s.l. and *Culiseta morsitans* [Hesson et al 2015]. Therefore, *Cx. torrentium* is considered the main vector of SINV in Northern Europe. Our study demonstrated that *Cx. torrentium* may be able to also play a major role in WNV transmission in area where the species is present in Central and Northern Europe, if the local environmental conditions would be suitable [Hesson et al. 2013].

Two different lineages of WNV are circulating in Europe [QUELLE]. Our infection experiments were performed with WNV lineage 1, whereas the studies of Vogels *et al*. [Vogels et al 2016] were performed with WNV lineage 2. The results suggest that both WNV lineages do not show a big difference in the TRs for the studied *Culex* taxa. Thus, in general, the introduction of WNV to Germany by infected birds seems to be a realistic scenario, since the virus is sporadically even circulating in neighbouring countries (e.g. France, Czech and Austria) [ECDC 2016]. However, although intensive surveillance studies were conducted, no WNV cases were detected in Germany. A screening of birds and mammals in Germany could verify WNV neutralizing antibodies in migratory birds, but none of the mammals and birds was positive for WNV RNA [Ziegler et al 2012, Ziegler et al 2015, Michel et al 2018]. A relative new method for the surveillance of maternal WNV virus in chicken eggs confirmed this negative results [Börstler et al. 2016B]. Finally, although an intensive surveillance of mosquitoes over the last decade confirmed the circulation of different moboviruses in Germany [USUV, SINV, BATV, Jöst et al 2010, Jöst et al 2011], these studies did not detect WNV.

An obvious explanation is the local temperature conditions. The temperature dependency of WNV replication in the vector was discussed before [Kenney et al 2014, Vogels et al 2017B]. Tropical temperatures around 27/28°C support transmission of WNV, while moderate temperatures of 23/24°C lead to considerably reduced TRs (Figure 3). In concordance with the presented experiments, most studies did not show any transmission at lower temperatures (≤ 21°C) or only TRs below 10%. As shown before (VOGELS), this is concordance with the distribution of human WNV cases in Europe between 2011 and 2017. Most cases were observed for area around the Mediterranean Sea and Eastern Europe, which had an average temperature between 21 and 26°C in July/August. Thus, although *Cx. torrentium* is a highly competent vector of WNV, the species’ main distribution in Central and Northern Europe with relative moderate temperature conditions probably prevent the transmission of WNV North of Alps.In conclusion, due to the continuing circulation of WNV in Europe and the prevalence of potent vectors for WNV like *Cx. torrentium* and *Cx. pipiens* s.l., a surveillance system that includes birds/eggs, mosquitoes and humans should be established or maintained in all European countries to increase the chance of early detection and subsequent interventions. This should also include areas in Northern and Central Europe, where *Cx. torrentium* is the predominant *Culex* species [Hesson 2013, Rudolph]. In addition, due to the high vector competence of *Cx. torrentium* for WNV and SINV [Lundström 1980], future studies should further evaluate the species’ susceptibility to other moboviruses, e.g. the widely circulating USUV [x].

**Material and methods** (491 words)

**Collection and rearing of mosquitoes**

Field-caught *Cx. torrentium* and *Cx. p. pipiens* specimens were obtained from egg raft collections in the summers of 2016 and 2017 as previously described [Leggewie et al 2016]. The collection site was close to Hamburg, Germany (Langenlehsten 53°30´N 10°44´E). The labstrain of *Cx. p. molestus* where collected in the field in 2011 in Heidelberg, Germany (08°39´E 49°11´N). Field collected eggs were reared to adults at room temperature. The *Cx. p. molestus* colony and all adult mosquitoes were incubated at 26°C, with a relative humidity of 80% and a 12:12 light:dark photoperiod. For differentiation of the three *Culex* taxa, DNA was extracted from x-x larvae (EXTRAKTIOSNKIT) and molecular identification was performed by multiplex qPCR as previously described [Rudolf et al 2013, Vogels et al 2015]. To exclude other virus infections, 10 randomly selected adult mosquitoes per species were tested by pan-flavi-, pan-alpha- and pan-Orthobunyavirus PCRs, but all individuals revealed negative results.

**Experimental infection and analysis**

4-14 days-old female mosquitoes were starved 24 hours before challenged with blood meals, containing West Nile virus (WNV isolate TOS-09, Genbank HM991273/HM641225) [Rossini et al 2011] at a final concentration of 107 plaque forming units per millilitre (PFU/ml). This concentration is recommended for artificial blood meals [Vogels et al.], because it corresponds to natural bird viremia [Vogels et al 2017]. Artificial blood meals were provided overnight, using cotton sticks bloated with infectious blood. Engorged females were incubated at 80% humidity at 18°C, 21°C, 24°C or 27°C, respectively.

Mosquitoes were analysed for infection, transmission and transmission efficiency 14 and 21 days post infection (dpi), respectively. Infection was investigated by analyses of the extracted DNA (KIT) from the bodies (excluding legs and wings) via a previously established qRT-PCR assay for WNV RNA [Leggewie et al 2016]. These results were used to calculate the infection rate (IR, number of WNV-positive mosquito bodies per number of fed females). The endpoint dilution the limit of detection for the qRT-PCR was analysed according the protocol of Caraguel et al. [Caraguel et al 2011], following the guidelines of the World Organisation of Animal Health. The limit of detection was defined as 100 copies/µL, corresponding to about 10,000 copies per mosquito specimens.

Transmission was analysed by performing a salivation assay for the detection infectious virus particles as described in detail previously [Heitmann et al 2017]. Thereby, transmission rate (TR) was defined as the number of mosquitoes with WNV-positive saliva per number of WNV-positive mosquito bodies [x]. In short, mosquitoes were anesthetised by CO2 to remove legs and wings and forced salivation was performed by placing the proboscis into a filter tip filled with 10 µL phosphate buffered saline (PBS) with pH 7.4 for 30 min. PBS containing saliva was incubated on Vero cells for 7 days and checked for cytopathic effect (CPE). For verification of a WNV infection, the supernatant was analysed for WNV RNA by qRT-PCR as described above. Transmission efficiency was calculated as described by Chouin-Carneiro et al. [Chouin-Carneiro et al. 2016], defined as the number of specimens with WNV-positive saliva per total number of fed females.

Literature research:

In order to evaluate the study results, data on the transmission rate of European mosquito populations with corresponding information on the mosquito species, dpi and incubation temperature were extracted from the existing literature [CITE]. In addition, to assess the results of these vector competence studies, the temperature data corresponding to human case data in Europe (2011-2017) were analysed [ECDC]. For all human WNV case, average temperature data in the corresponding NUTS3 region were extracted for the year when the case was reported. Human cases of WNV in Europe, during 2011, 2012 or 2013 were projected on the location where they were reported [11].

**Data availability**

All relevant data are provided within the paper.

**Acknowledgement**

We thank Branka Žibrat for excellent technical assistance. This work was financially supported by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE) with the grant number 28-1-91.048-15.

Conflict of interest (from each contributing author)

None declared.

Authors’ contributions

Conceived and designed the study: SJ, AH, RL, JSC, ET. Performed the data collection: SJ, AH, ML, MH. Analysed the data: SJ, AH, RL. Provided the ZIKA virus strain: GR. Provided mosquito specimens: MB. Wrote the paper: SJ, AH, RL, ET. All authors read and approved the final version of the manuscript.

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