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Culex pipiens and *Culex torrentium* populations from Central Europe are susceptible to West Nile virus infection

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ABSTRACT

West Nile virus (WNV), a *Flavivirus* with an avian primary host, is already widespread in Europe and might also pose an infection risk to Germany, should competent mosquito vectors be present. Therefore, we analysed the ability of WNV to infect German *Culex* mosquitoes with special emphasis on field collected specimens of *Culex torrentium* and *Culex pipiens* biotype *pipiens*. We collected egg rafts of *Culex* mosquitoes over two subsequent seasons at two geographically distinct sampling areas in Germany and differentiated the samples by molecular methods. Adult females, reared from the various egg rafts, were challenged with WNV by feeding of artificial blood meals. WNV infection was confirmed by real-time RT-PCR and virus titration. The results showed that field collected *C. pipiens* biotype *pipiens* and *C. torrentium* mosquitoes native to Germany are susceptible to WNV infection at 25 °C as well as 18 °C incubation temperature. *C. torrentium* mosquitoes, which have not been established as WNV vector so far, were the most permissive species tested with maximum infection rates of 96% at 25 °C. Furthermore, a disseminating infection was found in up to 94% of tested *C. pipiens* biotype *pipiens* and 100% of *C. torrentium*. Considering geographical variation of susceptibility, *C. pipiens* biotype *pipiens* mosquitoes from Southern Germany were more susceptible to WNV infection than corresponding populations from Northern Germany. All in all, we observed high infection and dissemination rates even at a low average ambient temperature of 18 °C. The high susceptibility of German *Culex* populations for WNV indicates that an enzootic transmission cycle in Germany could be possible.

1. Introduction

West Nile virus [(WNV); family *Flaviviridae*, genus *Flavivirus*] infections are a growing concern to Europe as illustrated by repeated outbreaks of West Nile fever (WNF) and West Nile neuroinvasive disease (WNND) in south-eastern parts of Europe [1]. Further, the increase of imported human WNV infections into Germany [2,3] raises concerns that the virus may also become established here. Since the emergence of arboviruses is closely linked to the presence of suitable vectors and susceptible hosts, the knowledge of principle vector species is essential for selection of adequate control measurements [4].

WNV is maintained in nature within an enzootic cycle involving ornithophilic mosquitoes and birds, but it can infect humans, equines and other vertebrates as illustrated by WNF and WNND in humans [5–7]. Since its first isolation in Uganda in 1937 [8], WNV has been isolated from mosquitoes in Eurasia [1] and Australia [9,10]. Moreover, following a single introduction to New York City in 1999, WNV has also spread throughout the Americas [11,12]. Members of the *Culex* (*C.*) *pipiens* complex (Linnaeus 1758), especially *C. pipiens*, *Culex tarsalis* and *Culex quinquefasciatus*, have been described as enzootic and bridge

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vectors for WNV in the United States of America and other WNV endemic regions [4,13,14]. The *C. pipiens* complex members *C. pipiens* biotypes *pipiens* and *molestus* as well as *Culex torrentium* are abundant in Central Europe [15,16]. The *C. pipiens* biotype *pipiens* and *C. torrentium* preferentially take blood meals from birds, rendering them potential enzootic vectors for WNV in Central Europe [17]. Recently, two studies have demonstrated the potential of a Dutch laboratory colony of *C. pipiens* to serve as a vector for WNV [18,19]. Up to now, there are no data available on the vector competence of *C. torrentium* for WNV. In contrast, *C. torrentium* is a proven enzootic vector of another arbovirus, the Sindbis virus, in Sweden [20–22].

Species identification within the *Culex* genus is difficult using classical morphological methods. Differentiation of *C. pipiens* and *C. torrentium* females relies on the occurrence of pre-alar scales, or measurement of the wing veins [23] but both methods are difficult to apply on large number of samples. The difficulties of correct assignment of the *C. pipiens* biotypes and *C. torrentium* might lead to misinterpretations of their vector potential, especially since virus isolation of field collected mosquitoes is a main marker for involvement in transmission.

Here we analyse the ability of Central European populations of *C. pipiens* biotype *pipiens* and *C. torrentium* to become infected with WNV and, in doing so, deliver a proxy for the vector competence estimations of these *Culex* populations for WNV. To avoid culturing effects, we used field collected samples from two geographically distinct regions in Germany and first separated the species and/or biotypes by multiplex qPCR [16]. Secondly, we analysed infection and dissemination rates after experimental feeding of WNV lineage 1 strain NY99 in *C. pipiens* biotype *pipiens* and *C. torrentium* females.

2. Material and methods

2.1. Mosquito strains and field collected mosquito samples

The *C. quinquefasciatus* (Malaysia) laboratory colony was obtained from Bayer (Bayer, Leverkusen, Germany). The *C. pipiens* biotype *molestus* colonies were established in our laboratory and originated from Heidelberg (Mol S), Wendland (Mol W) and Langenlehsten (Mol LL). The Mol S colony was maintained for 3 years in the laboratory prior to infection experiments. The Mol W colony was established from blood fed gravid females collected in 2012 in Wendland/Germany and the Mol LL colony was established from egg rafts collected in 2013 in Langenlehsten/Germany.

C. pipiens biotype *pipiens* and *C. torrentium* mosquitoes were obtained from egg raft collections carried out in Hamburg area/Germany [Altes Land 53°35′N 9°32′E, Langenlehsten 53°30′N 10°44′E and Hamburg City 53°32′N, 9°57′E; hereafter referred to as the North population (N)] and Lake Constance/Germany [Radolfzell-Böhringen 47°44′N, 8°58′E and Mettenau 47°43′N, 8°59′E; hereafter referred to as the South population (S)]. Specimens for infection experiments were collected from August to October in 2012 and 2013. The egg collection was carried out using gravid traps filled with hay infusion placed in proximity to natural breeding sites of *Culex* mosquitoes, i.e. water bodies to attract gravid females and stimulate egg deposition. Traps were checked twice a day for freshly deposited egg rafts, which were retrieved from water surface using a wooden spatula and placed in individual plastic cups for transportation to the laboratory.

2.2. Rearing of larvae and adult mosquitoes

Field collected and laboratory bred mosquitoes were kept at 23 +/− 2 °C with a relative humidity of 80% and a 16 h:8 h light:dark photoperiod. Field collected egg rafts were floated separately in dechlorinated water and hatched larvae were fed on TetraMin flaked fish food (Tetra GmbH, Melle, Germany). From each individual egg raft, 4–5 larvae were used for molecular taxonomic identification as described previously [16]. Once identified, larvae were pooled according to species or biotype and emerging females (4–14 days of age) were distributed into plastic vials at 10–15 females each. Adult mosquitoes were fed on fructose pads (8% D(−)-Fructose, Carl Roth GmbH, Karlsruhe, Germany; 0.02% 4-Aminobenzoic acid, Sigma Aldrich, Seelze, Germany) for maintenance and starved overnight prior to infection. To facilitate egg production of *C. quinquefasciatus* and *C. pipiens* biotype *molestus* laboratory colonies, a blood meal consisting of human erythrocyte concentrate (Blood group 0, Blood bank, University Hospital Hamburg)/50% FCS (PAA/GE Healthcare Life Sciences, Germany)/0.5% fructose (Carl Roth GmbH, Karlsruhe, Germany) was provided weekly.

2.3. Experimental infection and dissemination assays

Mosquitoes used in experiments were kept in incubators at 25 °C or 18 °C/80% humidity. Infection was performed overnight via an artificial blood meal containing 1.0–1.6 × 10^7 PFU WNV lineage 1 strain NY99 [24]/mL blood meal presented on cotton sticks. This method has been shown to lead to efficient WNV infection in *C. pipiens* [25]. Fully engorged mosquitoes were either frozen at −80 °C (day 0) or kept at 25 °C or 18 °C for 14 to 35 days. The two temperatures were chosen to mimic the climatic conditions in Germany with 25 °C representing the mean average temperature in Germany in July/August in the south of Germany and 18 °C representing the maximum average temperature during a minimum of 4 months/year in the north and south of Germany. For WNV RNA purification, mosquitoes were homogenised separately in 500 μl of medium (Schneider's Drosophila Medium, PAN Biotech, Aidenbach, Germany). To calibrate our infection assay, we used laboratory strains of *C. pipiens* biotype *molestus* and *C. quinquefasciatus*. *C. pipiens* biotype *molestus* has been described as a WNV vector in Israel [26], and *C. quinquefasciatus* was described by several studies from North America as a competent vector for WNV [14,27,28]. For the analysis of disseminating infection, frozen mosquitoes were beheaded under a dissection microscope and the body and head were separately homogenized in medium. The virus detection in heads as a method to measure the dissemination rate has been established previously by several other studies [29–32].

2.4. Quantification of viral RNA and infectious particles

WNV RNA purification was performed with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. The quantitative real-time PCR was performed using QuantiTec Probe RT-PCR MasterMix Kit (Qiagen, Hilden, Germany), with Light Cycler 480 II (Roche, Rotkreuz, Switzerland) and using 9 μl reaction mix containing 0.6 μM of the following primers and 0.2 μM of the following probes: OSM_145: GGCAATGGAGTCATAATG; OSM_146: GCATCTCAGG TTCGAATC; OSM_147: -FAM-CCAACGGCTCATACATAAGCG-BHQ1 and 2 μl RNA. For the analysis of virus titres, mosquito organ homogenate was filtered using 0.20 μm filters and inoculated on Vero cells (96-well format) with 10-fold dilutions and indirect immunofluorescent revelation after 3 days. Briefly, inoculated Vero cells were fixed in 4% formaldehyde for 30 min and immunostained using WNV recombinant E protein mouse monoclonal antibody (ABIN782271, antibodies-online GmbH, Germany) diluted 1:100 in PBT for 1–2 h and then with fluorescein (FITC)-conjugated AffiniPure goat anti-mouse IgG (115-095-003, Jackson ImmunoResearch Laboratories, Inc., USA) diluted 1:200 in PBT for 1 h. Infected wells were counted and viral titres were calculated using the Spearman and Kärber algorithm described by Hierholzer and Killington [33].

2.5. Statistics

Fisher's exact test was applied to assess differences of proportions between the species groups. A p value of less than 0.05 is deemed statistically significant. The statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc., CA, USA).

3. Results

3.1. Susceptibility of German mosquitoes for WNV infection

Susceptibility of German mosquitoes for WNV was analysed using 719 egg rafts of *C. pipiens* biotype *pipiens* and 373 egg rafts of *C. torrentium* collected in 2012 and 2013 (Table 1). Laboratory strains of *C. quinquefasciatus* and *C. pipiens* biotype *molestus* served as positive controls to calibrate the infection assay. Analysis of day 0 females of *C. quinquefasciatus* and *C. pipiens* biotype *molestus* for viral RNA content and viral particles revealed that 100% of tested females were positive for WNV viral RNA with viral titres of 1.4×10^1–7.76×10^3 PFU/ml in *C. quinquefasciatus* and 8 to 7.7×10^4 PFU/ml in *C. pipiens* biotype *molestus*. We further tested the correlation between viral RNA and infectious viral particles at different time points after infection to verify the use of virus RNA detection as a proxy for infection in the following experiments and to define the cut-off for qPCR detection. Selected samples from three independent infection experiments of *C. quinquefasciatus* and *C. pipiens* biotype *molestus* sampled at day 14 and day 21 post-infection were used. All samples tested positive in qRT-PCR with Ct-values below 35 were also tested positive for infectious viral particles. Virus titres were 1.2×10^1–1.4×10^2 PFU/ml at day 14 and 4.0–7.7×10^3 PFU/ml at day 21 in *C. quinquefasciatus* and 7.8–2.5×10^1 PFU/ml at day 14 and 7.8–1.4×10^2 PFU/ml at day 21 for *C. pipiens* biotype *molestus*. Next, we analysed the WNV infection rates within the two positive controls. Infection rates were calculated from the percentage of females tested positive for WNV infection with respect to the total number of blood fed females. For *C. quinquefasciatus*,

Table 1
Summary of mosquito samples used for WNV infection assays in 2013 and 2012.
The following lab strains were kept in captivity for 2 month to 2 years prior to the WNV infection assay: *Culex quinquefasciatus* (*C. qui*; Malaysia strain, Bayer Company), *Culex pipiens* biotype *molestus* Heidelberg (*C.* Mol S), *Culex pipiens* biotype *molestus* Wendland (*C.* Mol W) and *Culex pipiens* biotype *molestus* Langenlehsten (*C.* Mol LL).
The following species were reared from field-collected egg rafts: *Culex pipiens* biotype *pipiens* from Hamburg/Langenlehsten/Altes Land (*C. pip* North), *Culex pipiens* biotype *pipiens* from Lake Constance (*C. pip* South), *Culex torrentium* from Hamburg/Langenlehsten/Altes Land (*C. tor* North) and *Culex torrentium* from Lake Constance (*C. tor* South).
For each infection time point, the numbers of individual blood feeding assays performed (experiments) are listed. In addition, the combined number of mosquitoes presented with an infected blood meal during these blood meal assays (individuals) is also depicted.

Species	Origin	Egg rafts	Temperature [°C]	Infection time (days)	Individuals [#]	Experiments [#]
C. qui	Malaysia	Lab strain	25	14	66 (88)	9 (4)
			25	21	169 (88)	12 (4)
			25	28	41	5
			25	35	103	7
			18	14	29	6
			18	21	64	6
			18	28	38	5
			18	35	36	5
C. pip	North	186 (222)	25	14	22 (94)	5 (3)
			25	21	65 (173)	7 (3)
			25	28	53	6
			25	35	69	5
			18	14	23	5
			18	21	41	5
			18	28	46	5
			18	35	72	5
C. pip	South	311	25	14	8	2
			25	21	24	4
			25	28	11	3
			25	35	10	3
			18	14	8	2
			18	21	26	4
			18	28	12	3
			18	35	14	3
C. mol	S, W, LL	Lab strains	25	14	80	6
			25	21	273	14
			25	28	109	11
			25	35	90	6
			18	14	15	3
			18	21	24	4
			18	28	39	5
			18	35	67	5
C. tor	North	225 (119)	25	14	12 (130)	3 (4)
			25	21	34 (138)	6 (4)
			25	28	9	3
			25	35	9	3
			18	14	12	3
			18	21	15	3
			18	28	16	3
			18	35	19	3
C. tor	South	29	25	14	0	0
			25	21	12	3
			25	28	3	1
			25	35	0	0
			18	14	0	0
			18	21	7	2
			18	28	10	2
			18	35	0	0

The numbers written in parentheses represent the number of individuals/experiments/field-collected egg rafts used for experiments in 2012. All other values are data from 2013.

a maximum infection rate of 82% (n = 38) was found at 18 °C on day 28 post-infection (Fig. S1A). Furthermore, we did not find significant differences in *C. quinquefasciatus* between mosquitoes incubated at 25 °C or 18 °C temperature (Fig. S1A). In the case of *C. pipiens* biotype *molestus* mosquitoes the maximum infection rate of 67% (n = 15) was found at day 14 post-infection and 18 °C incubation temperature (Fig. S1A). Significant differences between infection rates at 25 °C and 18 °C were only observed at day 35 post-infection, where 63% (n = 90) and 42% (n = 67) were infected, respectively (p value = 0.0095, Fig. S1B).

C. pipiens biotype *pipiens* specimens from Germany showed infection rates similar to those of *C. quinquefasciatus* and *C. pipiens* biotype *molestus* with a maximum infection rate of 75% (n = 67) at 18 °C on day 21 (Fig. 1A). Viral titres in *C. pipiens* biotype *pipiens* at 25 °C incubation temperature were 4–1.4 × 10^5 PFU/ml at day 14 and 8–2.5 × 10^3 PFU/ml at day 21 post infection. The percentage of WNV RNA positive females increased over time at both incubation temperatures without significant differences between 25 °C and 18 °C incubation temperature. *C. torrentium* mosquitoes had the highest infection rates amongst all species or *C. pipiens* biotypes tested in this study, with a maximum infection rate of 96% (n = 46) at 25 °C on day 21 post infection (Fig. 1B). Interestingly, significant differences between infection rates between 18 °C and 25 °C at 14, 21 and 35 days post-infection were observed (p-values = 0.0033 (14 dpi), 0.0116 (21 dpi), 0.0346 (35 dpi)). At 25 °C incubation temperature infection rates of 83% (n = 12) were detected as early as 14 days post-infection, whereas only 16% (n = 12) of the tested females were tested positive for WNV RNA at 18 °C incubation temperature on the same day. Infection rates in *C. torrentium* at 18 °C increased over time and were higher compared to 25 °C at 35 days post-infection. Virus titres at 25 °C incubation temperature were 1.4 × 10^1–6.9 × 10^4 PFU/ml at 14 day post-infection and 8–1.4 × 10^3 at 21 days post-infection.

Dissemination rates were measured by separate qPCR testing of heads and bodies and calculated as the number of WNV positive heads with respect to the number of WNV-positive females. We observed maximum dissemination rates of 94% (n = 34) in *C. pipiens* biotype *pipiens* at 25 °C and 28 days of infection (Fig. 2A) and 100% (n = 11) in *C. torrentium* at 25 °C on day 28 of infection (Fig. 2B). For both species no significant difference of dissemination rates between 18 °C and 25 °C were observed.

3.2. Temporal and spatial variation of infection rates

Comparison of WNV infection rates in *C. pipiens* biotype *pipiens* collected in 2012 and 2013 showed significant differences in infection rates at day 14 post-infection (p value = 0.0208), whereas no significant difference was observed at day 21 post-infection (Fig. 3A). For *C. torrentium* we observed higher infection rates in 2013 for both time points, which were only significant at day 21 post-infection (p value = 0.0019). The infection rates were higher in *C. torrentium* compared to *C. pipiens* biotype *pipiens* in both subsequent years (Fig. 3A).

To determine spatial variations of susceptibility to WNV infection, we stratified the infection data obtained with the 2013 *C. pipiens* biotype *pipiens* and *C. torrentium* specimens according to population origin (North (N) and South (S)) and focused on the infection rates at 21 days post-infection. WNV infection rates of *C. pipiens* biotype *pipiens* revealed significant differences between the N and S populations (Fig. 3B). Infection rates of the S population were 22 percentage points higher than N population at 18 °C (p value = 0.0469, N population n = 41; S population n = 26) and 37 percentage points higher at 25 °C (p value = 0.0011, N population n = 65; S population n = 24). The analysis of WNV infection data for *C. torrentium* showed a 44 percentage point difference between S (43%; n = 7) and N (87%; n = 15) at 18 °C, which was not, however, statistically significant (p value = 0.0536, Fig. 3C). At 25 °C comparable proportions were observed.

4. Discussion

We demonstrated that *Culex* mosquitoes native to Germany are susceptible to WNV infection. The infection rates measured for *C. pipiens* biotype *pipiens* within our study largely match infection rates measured in field populations of *C. pipiens* in the US [25]. Furthermore, the high dissemination rates suggest an efficient amplification of the virus in those mosquitoes and a successful escape of the midgut barrier. Although this does not necessarily translate into high transmission rates, dissemination is a prerequisite for successful transmission. Thus, our data can provide evidence for potential vector competence of Central European mosquitoes. However, further transmission studies with larger sample sizes in the respective study strata, are needed to clarify their vector competence. Recent studies with Dutch laboratory colonies of *C. pipiens* and WNV lineage 1 NY99 and WNV lineage 2 also demonstrated the potential of Central European mosquitoes to serve as vectors for WNV [18]. In contrast to the strong correlation of infection rates with incubation temperature in this study, showing significantly decreased infection rates with WNV lineage 2 virus at lower temperature (18 and 23 °C) [18], we did not find significant differences in *C. pipiens* biotype *pipiens* at 18 °C and 25 °C incubation temperature. It is, however, difficult to compare these results and make assumption as to whether the differential temperature dependence is due to the origin of mosquitoes (field collected versus laboratory colony) or due to the virus lineage (lineage 1 versus lineage 2). In this context it is also important to note, that

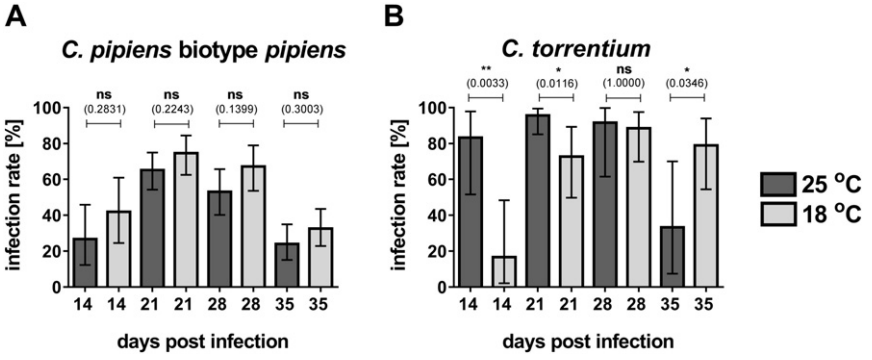

Fig. 1. Temperature dependence of infection rates for field populations *C. pipiens* biotype *pipiens* and *C. torrentium*. (A–B) Adult females were sorted into small plastic containers 5–10 days after emergence and feed over night with human blood containing 1–1.5 × 10^7 PFU WNV lineage 1 strain NY99. Fully engorged females were separated and incubated at 25 °C or 18 °C and 80% humidity in a climate chamber for 14 to 35 days. Graphs represent the infection rates (in percentage) of *C. pipiens* biotype *pipiens* (panel A) and *C. torrentium* (panel B), at 25 °C (dark grey bars) and 18 °C (light grey bars) as determined via WNV-specific qRT-PCR (cut-off = 35 cycles). Detailed numbers of individuals and numbers of independent experiments used for analysis are listed in Table 1. Data presented in these graphs are pooled data from North and South populations collected in 2013. Statistical analyses were performed using the GraphPad Prism software and Fischer's exact test ($p < 0.05$).

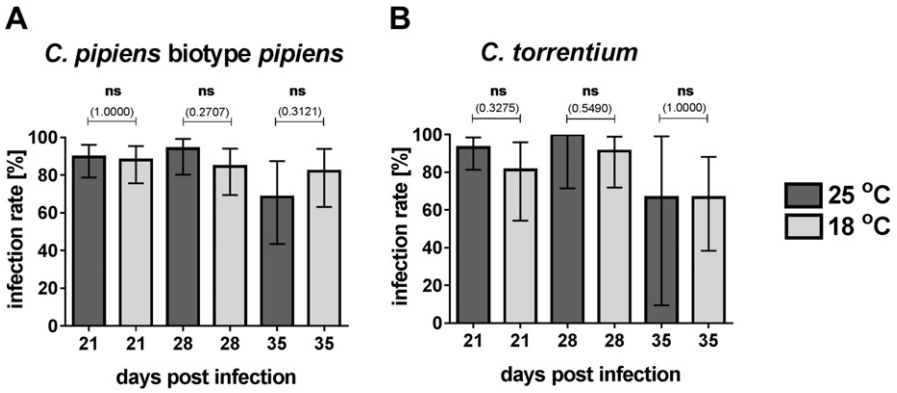

Fig. 2. *C. pipiens* biotype *pipiens* and *C. torrentium* mosquitoes display high dissemination rates at 18 and 25 °C incubation temperature. (A–B) Adult females were sorted into small plastic containers 5–10 days after emergence and feed over night with human blood containing 1–1.5 × 10^7 PFU WNV lineage 1 strain NY99. Fully engorged females were separated and incubated at 25 °C or 18 °C and 80% humidity in a climate chamber for 14 to 35 days. Graphs represent the dissemination rate (in percentage) of *C. pipiens* biotype *pipiens* (panel A) and *C. torrentium* (panel B), at 25 °C (dark grey bars) and 18 °C (light grey bars) as determined via WNV-specific qRT-PCR (cut-off = 35 cycles). Detailed numbers of individuals and numbers of independent experiments used for analysis are listed in Table 1. Data presented in these graphs are pooled data from North and South populations collected in 2013. Statistical analyses were performed using the GraphPad Prism software and Fischer's exact test ($p < 0.05$).

laboratory strains have a different genetic makeup and genetic variability compared to field collected populations due to selection processes during colony establishment. These differences translate into differential susceptibility of laboratory strains and field population for arboviruses as exemplified by experimental infection in *C. tarsalis* with Western equine encephalomyelitis virus (WEEV) and *Aedes albopictus* with dengue virus [34–36]. Thus more comparative transmission experiments using field collected mosquitoes with WNV of both lineages would be

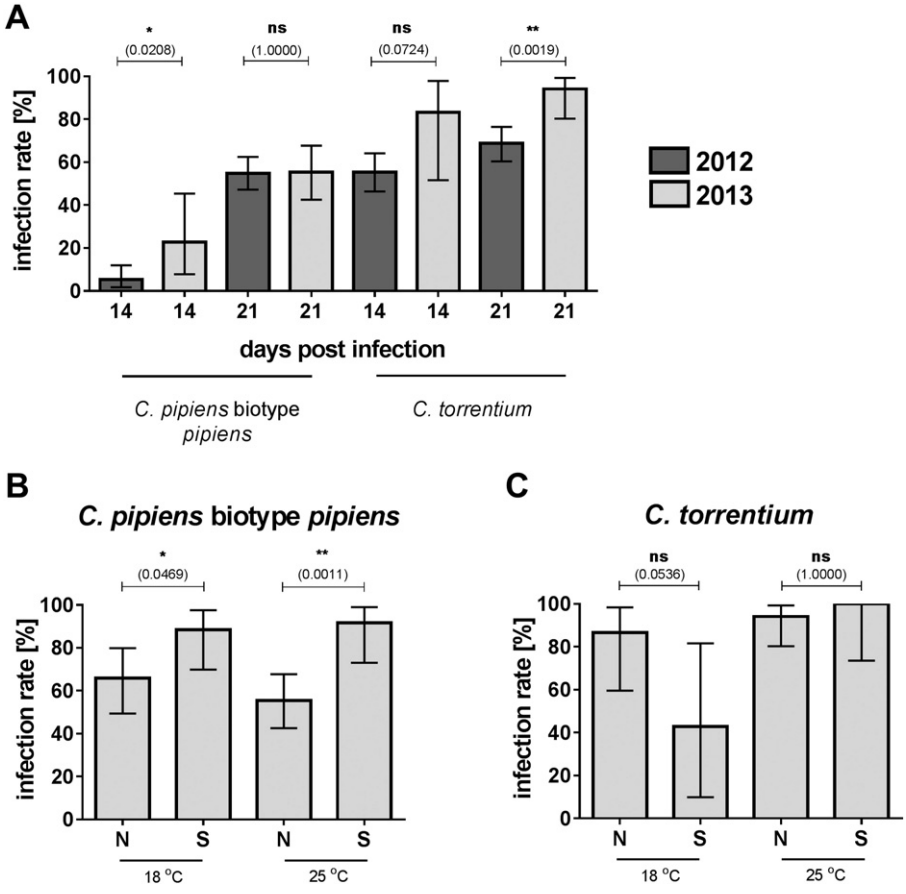

Fig. 3. Spatial and temporal variation in vector competence of wild *C. pipiens* biotype *pipiens* and *C. torrentium* populations. (A–C) For comparison of infection rates between two years and two geographically distinct mosquito populations of the same species, the data obtained through infection experiments described in Table 1 and Fig. 1 of 2013 were analysed together with data obtained in 2012. (A) Infection rates for 2012 are indicated by dark grey bars and infection rates for 2013 are represented by light grey bars. Statistical analysis was performed using GraphPad Prism and Fisher's exact test ($p < 0.05$). Detailed numbers of individuals and numbers of independent experiments used for analysis are listed in Table 1. (B and C) Infection rates are determined via WNV-specific qRT-PCR (cut-off = 35 cycles). Statistical analysis was performed using GraphPad Prism and Fisher's exact test ($p < 0.05$). Detailed numbers of individuals and numbers of independent experiments used for analysis are listed in Table 1. (B) Infection rates for *C. pipiens* biotype *pipiens* at 21 days post-infection and two temperatures, 18 °C and 25 °C, for the North population (N) and South population (S) respectively. (C) Infection rates for *C. torrentium* at 21 days post-infection and two temperatures, 18 °C and 25 °C, for the North population (N) and South population (S) respectively.

needed to answer this question. Nevertheless, our infection data suggest that susceptibility to WNV infection as well as efficient dissemination of the virus within the mosquitoes are not affected by lower ambient temperatures in field population of *C. pipiens* biotype *pipiens*.

It is of particular interest that we found the highest infection rates in *C. torrentium* mosquitoes, a species that has not yet been described in the context of WNV transmission. The high prevalence of *C. torrentium* in northern and Central Europe has just come to general attention due to the recent revision of the *Culex* complex distribution in Europe by several groups using molecular taxonomy methods [15,16]. Thus, the contribution of *C. torrentium* to WNV circulation might be underestimated due to poor identification rate of the species among trapped mosquito females in WNV surveillance studies. It is to note, that the distribution of *C. torrentium* [15] seems to be restricted to the North and Central Europe, while most WNV cases have been reported from the South-eastern part of Europe. Consequently an overlap of WNV endemic area with the *C. torrentium* distribution is currently restricted to northern Italy and Austria. Nevertheless, *C. torrentium* has already been identified as a major vector for other relevant viruses, for example, Sindbis virus [20–22]. Our infection data indicate that *C. torrentium* should be taken into consideration as a potential WNV vector, especially in northern and Central Europe.

Analysing the spatial variation in susceptibility to WNV, we found population-based differences between *C. pipiens* biotype *pipiens* mosquitoes from northern and southern Germany at both incubation temperatures, which indicates that intrinsic factors in different *C. pipiens* populations contribute to a differential susceptibility to WNV infection. This hypothesis is supported by reports of spatial variation in vector competence of North American *C. pipiens* mosquitoes for WNV [25]. Furthermore, experimental infection studies with *C. tarsalis* and WEEV and *Ae. albopictus* and dengue virus highlight the significance of (i) a population based analyses of vector competence and (ii) the use of field collected populations in addition to laboratory colonies to estimate vector competence of resident mosquito species. However, experimental infection with Italian laboratory strains and field collected populations of *C. pipiens* did not show differences between these distinct populations [37]. There are several possible explanations for these contradicting observations. Firstly, the laboratory colonies used in the Italian study were only cultivated for approximately 1 year (F7-F11) so the discrepancy between laboratory colony and field collected samples might not be very high [38]. Secondly, it might be possible that Italian *C. pipiens* populations are more genetically uniform than German populations, which would account for the differing observations. However, recent studies of genetic diversity of *C. pipiens* in Italy and Germany, although not conducted at the sites used for sample acquisition in the infection experiments, hint to a high genetic diversity of population in Italy and low genetic diversity in Germany [39]. A third possible hypothesis is that different *Wolbachia* strains within distinct populations may change the genetic diversity of these populations [40] and also influence their vector competence [41].

Interestingly, WNV infection rates in *C. torrentium* mosquitoes were not significantly different between North and South populations at 25 °C, whereas a visible but not statistically different difference was found at 18 °C. Additionally, the pooled infection rates from northern and southern populations showed a significantly reduced infection rate at 18 °C in *C. torrentium* and delayed infection compared to 25 °C resulting in significant differences in infection rates at early and late time points after infection. Furthermore, similar dissemination rates at 18 and 25 °C 35 days post-infection, despite the significant higher infection rates at 18 °C at this time point, hint to delayed virus replication at lower temperatures in this species These results might point to a temperature dependent difference in susceptibility to WNV of *C. torrentium* populations from Germany. But the lack of experimental WNV infection data in *C. torrentium* makes it difficult to draw conclusions on spatial variation and temperature-based variation in this species at the moment. In contrast to our WNV infection data, infection studies with Sindbis virus in this species showed temperature independent high infection rates in *C. torrentium* [21]. Thus, there might be also virus specific variation in different mosquito populations.

We found temporal variation in infection rates for both species with generally higher infection rates in 2013 compared to 2012, which might be due to variation in population in these two years. For example, experimental studies with C. *quinquefasciatus* colonies have shown the influence of colony age, specific age on the female included into the experiment and temperature [27], and experimental infection using US field collected samples of *C. pipiens* have revealed similar variations [25].

Taken together, our experimental infection data with field populations of *C. pipiens* biotype *pipiens* and *C. torrentium* mosquitoes show that both are permissive to WNV infection and that there are spatial, temporal and species-based variation in susceptibility to this virus. These variations are of interest for further studies of the mechanisms of virus–mosquito interactions as well as for the development of targeted control programmes.

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Competing interest

The authors declare that they have no competing interest.

Author's contribution

MB collected the mosquito egg rafts for vector competence assays. ML, MB, SJ and KH carried out the vector competence assay experiments and participated in data analysis. MR, JB and AK did the taxonomic classification of mosquito specimens. ML and RK did the statistical analysis. JSC designed the WNV real-time PCR. ET, ML and JSC participated in study design and manuscript writing. SCB directed and conceived the study, analysed data and wrote the first draught of the manuscript. All authors read and approved the final manuscript.

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