Vector competence of northern and southern European *Culex pipiens pipiens* mosquitoes for West Nile virus across a gradient of temperatures

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Abstract. In Europe, West Nile virus (WNV) outbreaks have been limited to southern and central European countries. However, competent mosquito vectors and susceptible bird hosts are present in northern Europe. Differences in temperature and vector competence of mosquito populations may explain the absence of WNV outbreaks in northern Europe. The aim of the present study was to directly compare vector competence of northern and southern European *Culex pipiens* (*Cx. p.*) *pipiens* mosquitoes for WNV across a gradient of temperatures. WNV infection and transmission rates were determined for two *Cx. p. pipiens* populations originating from The Netherlands and Italy, respectively. Mosquitoes were orally exposed by providing an infectious bloodmeal, or by injecting WNV (lineage 2) in the thorax, followed by 14-day incubation at 18, 23, or 28 °C. No differences in infection or transmission rates were found between the *Cx. p. pipiens* populations with both infection methods, but WNV transmission rates were significantly higher at temperatures above 18 °C. The absence of WNV outbreaks in northern Europe cannot be explained by differences in vector competence between *Cx. p. pipiens* populations originating from northern and southern Europe. This study suggests that low temperature is a key limiting factor for WNV transmission.

Key words. *Culex pipiens* complex, arbovirus, infection, northern house mosquito, transmission, Italy, The Netherlands.

Introduction

In Europe, outbreaks of the mosquito-borne West Nile virus (WNV; family: Flaviviridae) among humans and horses are remarkable, because they have been limited to southern and central European countries (Calistri *et al.*, 2010). This is in clear contrast with the ongoing outbreaks throughout North America since its emergence in 1999 (Hayes *et al.*, 2005). Thus far, no outbreaks have occurred in northern Europe, despite serological evidence of WNV circulation in birds in Germany and the United Kingdom (Buckley *et al.*, 2006; Linke *et al.*, 2007).

WNV is transmitted among susceptible bird hosts by mosquitoes, whereas mammals develop lower viraemias and hence are considered dead-end hosts (Kramer *et al.*, 2008).

In North America, *Culex* (*Cx.*) *pipiens* (Diptera, Culicidae) mosquitoes have been identified as one of the main vector species for WNV transmission, because of their vector competence and high abundance (Kilpatrick *et al.*, 2005). The species *Cx. pipiens* consists of two morphologically identical biotypes, *pipiens* (Linnaeus 1758) and *molestus* (Forskål 1775), which differ in physiology and behaviour (Byrne & Nichols, 1999). Particularly the *pipiens* biotype is an important enzootic vector because of its feeding preference for birds (Fritz *et al.*, 2015). Corvid birds (family: Corvidae) are highly susceptible to WNV infection (Wheeler *et al.*, 2009). Differences in WNV transmission between northern and southern Europe may be explained by a lower susceptibility of northern European bird species or lower vector competence of *Cx. pipiens* populations, which may

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be driven by lower temperatures. Recent studies showed that carrion crows (*Corvus corone*) and European jackdaws (*Corvus monedula*) originating from The Netherlands are susceptible to infection with different WNV isolates, with viraemia in crows that could support mosquito infection (Lim *et al.*, 2014, 2015). Thus, it is unlikely that low bird susceptibility can explain the lack of WNV transmission in northern Europe.

Culex pipiens mosquitoes originating from The Netherlands and Germany have been found to be competent vectors for WNV (Fros *et al.*, 2015a; Leggewie *et al.*, 2016; Vogels *et al.*, 2016). However, studies in both Europe and North America have not only shown spatial and temporal variation in vector competence among different mosquito populations (Vaidyanathan & Scott, 2007; Kilpatrick *et al.*, 2010; Leggewie *et al.*, 2016), but also variation in vector competence as a result of environmental factors (Kilpatrick *et al.*, 2008; Fros *et al.*, 2015a; Vogels *et al.*, 2016). This suggests that both genetic and environmental factors play an important role in vector competence, which may explain the differences in WNV transmission across Europe.

Thus far, only a few studies have determined the vector competence of southern European *Cx. pipiens* mosquitoes for WNV (Fortuna *et al.*, 2015; Brustolin *et al.*, 2016). Furthermore, differences in experimental designs hamper the ability to make direct comparisons between vector competence studies on *Cx. pipiens* populations originating from different locations in Europe. The aim of this study was, therefore, to directly compare vector competence of northern (The Netherlands) and southern (Italy) European *Cx. pipiens (p.) pipiens* mosquitoes for WNV, across a gradient of temperatures.

Materials and methods

Mosquitoes

During June and July 2015, *Culex* egg rafts were collected from aboveground water barrels in Best, in the south of The Netherlands, and from buckets infused with hay and yeast in San Benedetto del Tronto, at the east coast of central Italy. Egg rafts were placed individually in tubes in which larvae could hatch. Approximately 10 larvae from each egg raft were pooled and identified to the biotype level with real-time PCR (Vogels *et al.*, 2015). The Dutch rearing was started by grouping larvae from 120 egg rafts identified as *Cx. p. pipiens*, and the Italian rearing was started by grouping larvae from 114 egg rafts identified as *Cx. p. pipiens*. The F4-6 offspring of both populations were used in experiments. Mosquitoes were reared as described before (Vogels *et al.*, 2016). Briefly, larvae and adults from both colonies were maintained at 23 °C with a LD 16 : 8 h cycle and 60% RH (relative humidity). Larvae were initially fed with Liquifry No. 1 (Interpet Ltd, Dorking, UK) and after 2 days a 1 : 1 : 1 mixture of bovine liver powder (MP Biomedicals, Santa Ana, CA, U.S.A.), ground rabbit food (Pets Place, Ede, The Netherlands) and ground koi food (Tetra, Melle, Germany) was provided. Adults were provided with 6% glucose solution *ad libitum*. Bovine (Carus, Wageningen, The Netherlands) or chicken blood (Kemperkip, Uden, The Netherlands) was provided through Parafilm using the Hemotek® PS5 feeder (Discovery Workshops, Accrington, UK), for egg production.

For both mosquito populations, high reproduction rates were observed for all mosquito generations.

Female mosquitoes were kept together with males for 5–18 days in Bugdorm-1 insect rearing cages (30 cm × 30 cm × 30 cm; Bugdorm, Taiwan, China), before females were transferred in buckets (Ø: 12.2 cm, height: 12.2 cm; Jokey, Wipperfürth, Germany) to the Biological Safety Level (BSL) 3 facility. The age range of female mosquitoes from both The Netherlands and Italy populations was kept similar. Glucose solution was replaced by water 3–4 days before the infectious bloodmeal was offered, to stimulate blood feeding.

Virus

In all experiments, a passage 2 stock of WNV lineage 2 (provided by Erasmus Medical Center, Rotterdam, The Netherlands; GenBank accession no. HQ537483.1) originating from Greece (2010) was used, which was originally isolated from a pool of *Cx. pipiens* mosquitoes (Papa *et al.*, 2011). The WNV stock had a titer of $9.2 \pm 0.9 \times 10^7$ TCID$_{50}$/mL. WNV was grown on C6/36 cells (ATCC CRL-1660) and titrated on Vero E6 cells (ATCC CRL-1586). C6/36 cells were cultured with Leibovitz L-15 medium (Life technologies, Bleiswijk, The Netherlands) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 2% tryptose phosphate broth (Life Technologies) and 1% non-essential amino acids (Life technologies), at 28 °C. Vero E6 cells were cultured with Hepes-buffered DMEM medium (Life Technologies) supplemented with 10% FBS, penicillin (100 IU/mL; Sigma-Aldrich, Zwijndrecht, The Netherlands) and streptomycin (100 µg/mL; Sigma-Aldrich), at 37 °C. The medium was fully supplemented by adding fungizone (2.5 µg/mL; Life Technologies) and gentamycin (50 µg/mL; Life technologies) when samples were tested for presence of virus.

WNV infections

In this study, the same protocols were used as described before (Vogels *et al.*, 2016). To test for the effect of population and temperature on WNV infection and transmission rates, mosquitoes were orally exposed to WNV. *Culex p. pipiens* mosquitoes, originating from The Netherlands and Italy were allowed to feed for 1 h on infectious chicken blood with a WNV titer of 4.2×10^7 TCID$_{50}$/mL, offered through a Parafilm membrane using the Hemotek PS5 feeder in the BSL3 facility. The bloodmeal was offered during the early stage of the dark period in a dark room at 24 °C and 60% RH. After 1 h, mosquitoes were anaesthetized with CO_2 and immobilized on a semi-permeable CO_2-pad connected to 100% CO_2. Engorged females of each group were selected and divided over three separate buckets. The buckets were placed at 18, 23, and 28 °C for 14 days. Oral exposures were replicated four times to reach 50 female mosquitoes for each population per temperature. In addition, directly after the bloodmeal a total of 20 engorged female mosquitoes of both populations were individually placed in 1.5-mL SafeSeal micro tubes (Sarstedt, Nümbrecht, Germany) and stored at −80 °C, to be able to determine the ingested WNV titers.

To bypass the midgut barrier and test mosquitoes for a known disseminated infection for the presence of a salivary gland barrier, mosquitoes were also infected with WNV through microinjections. *Culex p. pipiens* mosquitoes originating from The Netherlands and Italy were immobilized with CO_2 and injected in the thorax with 69 nL of WNV ($9.2 \pm 0.9 \times 10^7$ $TCID_{50}$/mL) using the Drummond Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, PA, U.S.A.). Again, female mosquitoes from each group were divided over three separate buckets and placed at 18, 23 and 28 °C for 14 days. Infections were replicated four times to reach 50 female mosquitoes for each population per temperature.

Infection and transmission

After the period of incubation, female mosquitoes were immobilized with CO_2 and the legs and wings were removed with forceps. The proboscis of each mosquito was inserted into a 200-μL yellow pipette tip (Greiner bio-one, Alphen aan den Rijn, The Netherlands) containing 5 μL of a 1 : 1 solution of FBS and 50% glucose solution, for a minimum period of 45 min. Each saliva sample was collected in a 1.5 -mL SafeSeal micro tube with 55 μL of fully supplemented Hepes-buffered DMEM medium, whereas bodies were stored in 1.5-mL SafeSeal micro tubes with a small scoop of 0.5 mm Zirconium beads (Bio-Connect BV, Huissen, The Netherlands). All samples were stored at −80 °C until further use.

Frozen mosquito bodies were homogenized in the Bullet blender storm (Next Advance, Averill Park, NY, U.S.A.) for 2 min, and shortly spun down at 14 500 rpm in the Eppendorf minispin plus (Eppendorf, Hamburg, Germany). Next, 100 μL of fully supplemented Hepes-buffered DMEM medium was added and samples were again homogenized for 2 min in the Bullet blender, and spun down for 2 min at 14 100**g**. For each mosquito homogenate or saliva sample, 30 μL was incubated on a monolayer of Vero E6 cells in a 96-well plate (Greiner Bio-one). After 2–3 h the medium was completely removed and replaced with 100 μL of Hepes-buffered DMEM medium. After 3 days, each well was scored for WNV-specific cytopathic effects. WNV titers were determined by endpoint dilution assays on Vero E6 cells, and scored for WNV specific cytopathic effects after 3 days.

Statistical analysis

Generalized linear models (GLM) with a binomial distribution and logit link function were used to test for the effects of the population (The Netherlands vs. Italy) and temperature on the WNV infection and transmission rates. Infection and transmission rates were calculated, respectively, by dividing the number of female mosquitoes with infected bodies or with infected saliva by the total number of female mosquitoes in the respective treatment. Effects of replicate, population, temperature, and the interaction term between population and temperature were included in the model. If the interaction term was not significant, it was removed from the final model. Significant factors were further investigated with likelihood ratio tests
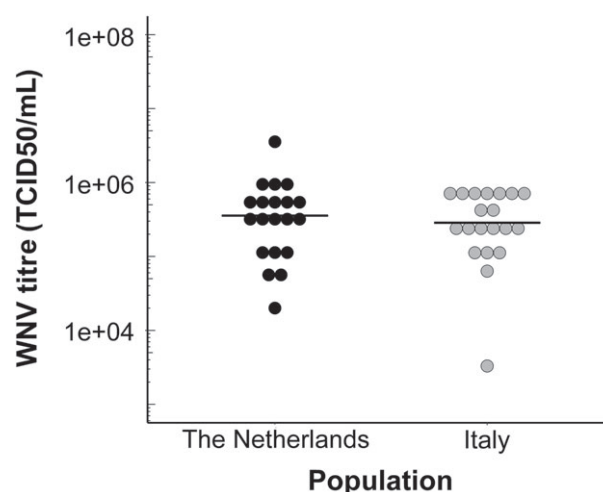
Fig. 1. Ingested WNV titers by Dutch and Italian *Cx. p. pipiens* mosquitoes. Viral titres were determined for engorged females originating from The Netherlands (black) and Italy (grey), which were directly frozen after feeding on the infectious blood. Each data point represents one engorged female mosquito. Horizontal black lines show median titers for each population.

(LRT). A Wilcoxon test was used to test for differences in the ingested WNV titers by both populations. Effects of population and temperature on viral titres were tested with Wilcoxon or Kruskal–Wallis (KWT) tests. Multiple comparisons were corrected with the Bonferroni procedure, by reducing the significance level from 0.05 to 0.017. All statistical analyses were done with sas software, version 9.3 (SAS Institute Inc., Cary, NC, U.S.A.), and figures were made using the statistical software package R (R Core Team, 2016).

Results

The effects of population and temperature on WNV vector competence of Dutch and Italian *Cx. p. pipiens* mosquitoes were investigated by orally exposing female mosquitoes to WNV, and incubating them for 14 days at 18, 23, or 28°C. The Dutch mosquito population ingested similar titres of WNV (3.6×10^5 $TCID_{50}$/mL) compared to the Italian mosquito population (2.9×10^5 $TCID_{50}$/mL; Wilcoxon, Z = 0.34, d.f. = 1, P = 0.73; Fig. 1).

After oral exposure, WNV infection rates of Dutch *Cx. p. pipiens* females were 26% at 18 °C, 34% at 23 °C, and 32% at 28 °C, and WNV infection rates of Italian females were 28% at 18 °C, 36% at 23 °C, and 32% at 28 °C (Fig. 2A). No significant effects were found on infection rates of population (GLM, $\chi^2 = 1.07$, d.f. = 1, P = 0.30), temperature (GLM, $\chi^2 = 1.67$, d.f. = 2, P = 0.43), or the interaction between both (GLM, $\chi^2 = 0.03$, d.f. = 2, P = 0.99). Thus, mosquito infection rates were similar for northern and southern European populations at all temperatures.

After oral exposure, WNV transmission rates of Dutch *Cx. p. pipiens* females were 0% at 18 °C, 10% at 23 °C, and 10% at 28 °C, and WNV transmission rates of Italian females were 0%
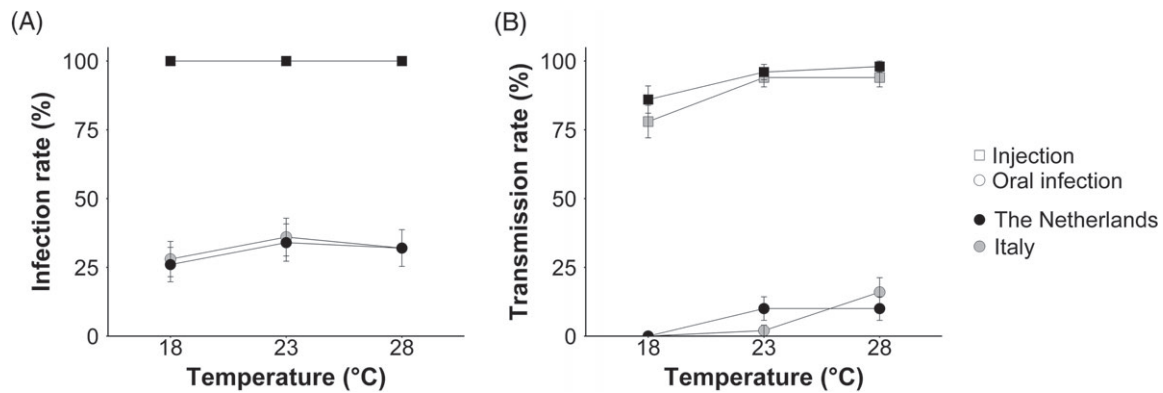
Fig. 2. The effect of temperature on vector competence of Dutch and Italian *Cx. p. pipiens* mosquitoes for WNV. (A) Infection rates and (B) transmission rates of Dutch (black) and Italian (grey) mosquitoes exposed orally (circles) or through intra-thoracic injections (squares). Mosquitoes were incubated for 14 days at 18 , 23 , or 28 °C. Data points of infection rates after injections are overlapping for both populations. Each data point shows average infection rates for 50 female mosquitoes infected either orally or by injection with WNV. Error bars show standard error of the means.

at 18 °C, 2% at 23 °C, and 16% at 28 °C (Fig. 2B). Temperature (GLM, $\chi^2 = 19.54$, d.f. = 2, $P < 0.001$) had a significant effect on transmission rates after oral exposure, whereas there was no significant effect of population (GLM, $\chi^2 = 0.01$, d.f. = 1, $P = 0.92$), or the interaction between both (GLM, $\chi^2 = 3.98$, d.f. = 2, $P = 0.14$). As there was no population effect, differences between temperatures could be tested by pooling data from both populations. Pairwise comparisons between temperatures revealed that transmission rates were significantly lower at 18 °C compared to 28 °C (LRT, $P < 0.001$), and at 18 °C compared to 23 °C (LRT, $P = 0.003$), but there was no significant difference between 23 and 28 °C (LRT, $P = 0.07$). Thus, vector competence of northern and southern *Cx. p. pipiens* populations was similar, and no WNV transmission was observed after 14-day incubation at 18 °C.

Mosquitoes were also infected with WNV through intra-thoracic injections to bypass the midgut, and investigate the effect of the salivary glands on virus dissemination. Infection and transmission rates were determined for the Dutch and Italian population at 18, 23, or 28 °C after 14 days of incubation. WNV infection rates were 100% for both populations, at all three temperatures (Fig. 2A). WNV transmission rates of Dutch *Cx. p. pipiens* females were 86% at 18 °C, 96% at 23 °C, and 98% at 28 °C, and WNV transmission rates of Italian females were 78% at 18 °C, 94% at 23 °C, and 94% at 28 °C (Fig. 2B). After injection, transmission rates were significantly different between temperatures (GLM, $\chi^2 = 14.21$, d.f. = 2, $P < 0.001$), but not between populations (GLM, $\chi^2 = 1.00$, d.f. = 1, $P = 0.32$), nor for the interaction between both (GLM, $\chi^2 = 0.31$, d.f. = 2, $P = 0.86$). A similar pattern was observed as was found after oral exposure, with significantly lower transmission rates at 18 °C compared to 28 °C (LRT, $P < 0.001$), and at 18 °C compared to 23 °C (LRT, $P = 0.003$), but no significant difference between 23 °C and 28 °C (LRT, $P = 0.73$). Thus, after injection WNV transmission was lower at 18 °C compared to 23 and 28 °C.

To explain the effect of temperature on vector competence, viral titres of bodies of mosquitoes which had WNV in their saliva after 14 days of incubation were determined for orally exposed (Fig. 3A) and injected female mosquitoes

(Fig. 3B). After 14 days of incubation, none of the orally exposed *Cx. p. pipiens* females incubated at 18 °C were able to transmit WNV. Thus, WNV titers were only determined for females with positive body and saliva which were incubated at 23 and 28 °C. Median WNV titers of orally exposed Dutch *Cx. p. pipiens* females were 2.0×10^5 TCID$_{50}$/mL at 23 °C, and 6.3×10^5 TCID$_{50}$/mL at 28 °C, and median WNV titers of Italian females were 6.3×10^6 TCID$_{50}$/mL at 23 °C, and 2.8×10^3 TCID$_{50}$/mL at 28 °C (Fig. 3A). There was no significant difference in viral titers between both populations (Wilcoxon, $Z = -1.83$, d.f. = 1, $P = 0.07$), nor between temperatures (Wilcoxon, $Z = 1.43$, d.f. = 1, $P = 0.15$) after oral exposure. However, it should be noted that as a result of low transmission rates, viral titers could only be determined from a limited number of positive mosquitoes. In general, WNV replicated to similar levels in the Dutch and Italian *Cx. p. pipiens* populations.

Viral titres of female mosquitoes that were infected with WNV through injections were also compared. The median WNV titres of injected Dutch *Cx. p. pipiens* females were 5.4×10^5 TCID$_{50}$/mL at 18 °C, 6.5×10^6 TCID$_{50}$/mL at 23 °C, and 2.8×10^6 TCID$_{50}$/mL at 28 °C (Fig. 3B). The median WNV titers of injected Italian females were 3.9×10^5 TCID$_{50}$/mL at 18 °C, 5.7×10^6 TCID$_{50}$/mL at 23 °C, and 2.0×10^6 TCID$_{50}$/mL at 28 °C (Fig. 3B). Again, there was no significant difference in viral titers between the two populations (Wilcoxon, $Z = 0.64$, d.f. = 1, $P = 0.52$), but there was a significant effect of temperature (KWT, $\chi^2 = 16.41$, d.f. = 2, $P < 0.001$). Viral titers were significantly lower at 18 °C compared to 28 °C (Wilcoxon, $Z = -3.58$, d.f. = 1, $P < 0.001$), and at 18 °C compared to 23 °C (Wilcoxon, $Z = -3.36$, d.f. = 1, $P < 0.001$), but not between 23 and 28 °C (Wilcoxon, $Z = 0.60$, d.f. = 1, $P = 0.55$). Thus, lower transmission rates at 18 °C after 14 days can be explained by lower viral titres inside mosquito bodies.

Discussion

The aim of this study was to directly compare the vector competence of a northern and southern European *Cx. p. pipiens*
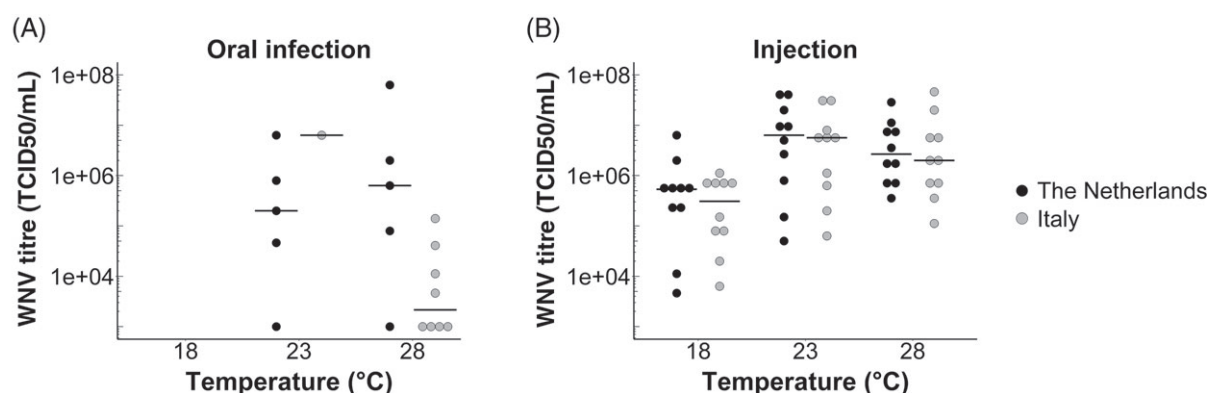
Fig. 3. The effect of temperature on WNV titers of Dutch and Italian *Cx. p. pipiens* mosquitoes with positive body and saliva. Viral titres of (A) orally exposed and (B) injected *Cx. p. pipiens* mosquitoes originating from The Netherlands (black) or Italy (grey), determined after 14 days incubation at 18, 23, or 28 °C. Each data point represents one female mosquito with WNV positive body and saliva. Horizontal black lines show median titres for each population per temperature.

population for WNV across a gradient of temperatures. Overall, no difference in vector competence (infection and transmission rates) for WNV between the Dutch and Italian *Cx. p. pipiens* populations was found. However, a positive effect of higher temperature on vector competence for both populations was detected. Transmission of WNV after an infectious bloodmeal was only observed at 23 and 28 °C, whereas no transmission was observed after 14 days of incubation at 18 °C.

Recently, several European studies determined vector competence of *Cx. pipiens* populations originating from The Netherlands (Fros *et al.*, 2015a, 2015b; Vogels *et al.*, 2016), Germany (Leggewie *et al.*, 2016), Spain (Brustolin *et al.*, 2016) and Italy (Fortuna *et al.*, 2015). However, due to the large diversity in experimental designs, it is hard to make a direct comparison between outcomes of these studies. For instance, only two studies used molecularly identified biotype *pipiens* mosquitoes (Leggewie *et al.*, 2016; Vogels *et al.*, 2016), whereas all other studies used the other biotype or did not differentiate between biotypes. Infection rates of the Dutch population at 18 °C were similar compared to previous studies on Dutch and German biotype *pipiens* populations, whereas infection rates at 23 and 28 °C were slightly lower in the present study (Leggewie *et al.*, 2016; Vogels *et al.*, 2016). Transmission rates could not be compared with the German mosquito population, because no transmission rates were reported for this study (Leggewie *et al.*, 2016). Transmission rates were similar in the present and a previous study on Dutch biotype *pipiens* mosquitoes, with no transmission at 18 °C after 14 days, and low transmission rates at 23 °C. Interestingly, previously recorded transmission rates were about three-fold higher at 28 °C compared with the present study, but the ratio between mosquitoes with infected saliva and mosquitoes with infected bodies is comparable. This indicates an overall lower success of initial infection in the present study, which may be due to the midgut infection barrier. Such differences are probably as a result of variation between populations or temporal variation in vector competence (Vaidyanathan & Scott, 2007; Kilpatrick *et al.*, 2010). Both infection and transmission rates of the Italian population in this study were lower compared with a previous study with a *Cx. pipiens* population in Italy (Fortuna

et al., 2015). However, the same study showed significant differences in infection rates among different *Cx. pipiens* populations in Italy, which again suggests that vector competence is a variable trait.

Increased vector competence at higher temperatures has been described for several arthropod-borne viruses and their respective mosquito vectors (Reisen *et al.*, 1993; Kilpatrick *et al.*, 2008; Zouache *et al.*, 2014; Fros *et al.*, 2015b). Higher temperatures increase viral replication, which can result in shorter extrinsic incubation periods and higher infection and transmission rates (Chamberlain & Sudia, 1961; Hardy *et al.*, 1983). However, the temperature may also affect the midgut barrier or antiviral responses such as RNAi (Adelman *et al.*, 2013; Alto & Bettinardi, 2013). Thus, complex interactions between virus and mosquito may shape the relation between temperature and vector competence.

In the present study, no transmission after 14 days of incubation at 18 °C was demonstrated. However, it is possible that mosquitoes required longer extrinsic incubation periods before WNV could be transmitted at 18 °C. Previous studies showed that at relatively low temperatures the extrinsic incubation period increases to 15–22 days, depending on the specific combination of mosquito species and WNV strain (Reisen *et al.*, 2006; Kilpatrick *et al.*, 2008). Thus, European *Cx. p. pipiens* might be competent vectors for WNV when incubated for longer periods at 18 °C. Future studies should determine the minimum extrinsic incubation periods of European *Cx. pipiens* populations for WNV at different temperatures.

In the present study, mosquitoes were reared and kept at constant temperatures. However, daily fluctuations in temperature may have consequences for virus transmission. Previous studies on dengue virus showed that daily fluctuations in temperature could both result in increased as well as decreased virus transmission, depending on the temperature range and mean temperature that virus-exposed mosquitoes experience (Lambrechts *et al.*, 2011). In contrast, two studies on the effect of temperature fluctuations on WNV transmission showed no difference between mean and fluctuating temperatures (Cornel *et al.*, 1993; Danforth *et al.*, 2016). Thus, results from experiments on

WNV with constant temperature seem to provide reliable estimates for vector competence but should be interpreted with care nevertheless.

Here, it was shown that temperature, but not population origin, had an effect on vector competence of *Cx. p. pipiens* mosquitoes for WNV. Despite variation in vector competence of different European populations (Fortuna *et al.*, 2015; Leggewie *et al.*, 2016; Vogels *et al.*, 2016), the present findings suggest that temperature is the main factor that can explain differences in WNV outbreaks between northern and southern Europe. Interestingly, no transmission of WNV by both populations after 14 days of incubation at a temperature of 18 °C was observed, which is representative for an average northern European summer (Haylock *et al.*, 2008). Although this does not exclude possible transmission after longer incubation periods, it shows that transmission is inefficient at low temperature. This can explain the absence of WNV outbreaks in northern European countries (Chancey *et al.*, 2015). Although the current risk for WNV transmission in northern Europe seems low, circulation is possible under favourable climatic conditions due to the presence of competent mosquito vectors (Vogels *et al.*, 2015, 2016) and susceptible bird hosts (Lim *et al.*, 2014, 2015). Models are needed to assess future risks of WNV transmission in northern Europe, especially in the light of more frequent and longer periods of intense heat due to global warming.

Conclusion

Differences in WNV outbreaks between northern and southern Europe cannot be explained by intrinsic differences in vector competence between *Cx. p. pipiens* populations from both regions. In addition, no transmission was observed after 14 days of incubation at the average summer temperature of 18 °C of northern Europe, whereas both the Dutch and Italian *Cx. p. pipiens* population were able to transmit WNV at similar rates at 23 and 28 °C. This suggests that temperature is a key limiting factor for WNV transmission in northern Europe.

Acknowledgements

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