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RESEARCH ARTICLE

Virus interferes with host-seeking behaviour of mosquito

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ABSTRACT

Transmission of vector-borne pathogens is dependent on the host-seeking behaviour of their vector. Pathogen manipulation of the host-seeking behaviour of vectors towards susceptible hosts is supposedly beneficial for transmission. For West Nile virus (WNV), manipulation of the host-seeking behaviour of the main mosquito vector towards birds would be advantageous, because mammals are dead-end hosts. We hypothesised that WNV infection induces a stronger host-seeking response and a shift in host preference towards birds, to enhance its transmission by mosquitoes. However, here we show that WNV infection decreases the host-seeking response, and does not induce a shift in mosquito host preference. Other fitness-related traits are not affected by WNV infection. No effect of WNV infection was found on antennal electrophysiological responsiveness. Thus, the reduced host-seeking response is likely to result from interference in the mosquito's central nervous system. This is the first study that shows changes, specifically in the host-seeking behaviour induced by a pathogen, that do not favour transmission.

KEY WORDS: Manipulation, Host preference, *Culex pipiens*, Virus transmission, Olfaction

INTRODUCTION

The successful transmission of pathogens that are spread by arthropods depends on complex interactions between the pathogen, the arthropod vector, the host and the environment (Gray and Banerjee, 1999; Weaver and Barrett, 2004). The natural transmission cycle can only be completed if an infectious vector is able to find a new susceptible host and transmit the pathogen while feeding. Many examples exist of pathogens that influence host feeding behaviour of their vector in a way that seems to increase their transmission (van Houte et al., 2013). Increased transmission can be achieved through direct manipulation of vector behaviour or through indirect effects of the pathogen on, for instance, host defence or host attractiveness (De Moraes et al., 2014; Hurd, 2003; Targett, 2006; van Houte et al., 2013).

Manipulation can occur during two stages of the vector's host-feeding behaviour: the host-seeking stage and the feeding stage. Manipulation during these two stages has been shown for various medically important pathogens that are transmitted by mosquitoes (Hurd, 2003; Lefèvre and Thomas, 2008). During the first stage, mosquito–host contact can be increased by induction of a stronger mosquito host-seeking response to host odour (Cator et al., 2013; Koella et al., 2002; Rossignol et al., 1986; Smallegange et al., 2013), or a stronger host preference for suitable hosts (Lefèvre et al., 2006). Pathogens can induce such changes by altering the mosquito's odour perception, because host odours, together with carbon dioxide, are important cues for mosquitoes to locate a host (Dekker et al., 2005; Takken, 1991). The second stage starts after the vector has encountered a host. Lowered feeding performance, which leads to increased probing and longer feeding, is a second way by which the pathogen's transmission rates can be increased (Grimstad et al., 1980; Lima-Camara et al., 2011). However, despite this information, evidence that changes in mosquito behaviour are actually due to direct manipulation by the pathogen remains scarce (Ribeiro et al., 1985; Rossignol et al., 1984). Moreover, a recent study showed that stimulation of mosquitoes with heat-killed *Escherichia coli* induced behavioural changes similar to those observed after infection with the malaria parasite *Plasmodium yoelii* (Cator et al., 2013). In addition to evidence of direct manipulation, this suggests that indirect manipulation owing to immune challenge may also induce changes in mosquito behaviour (Cator et al., 2013, 2015). Therefore, more studies on mechanisms of manipulation are needed to fully understand the direct and indirect effects of pathogen infection on vector behaviour.

Several studies have investigated the effect of infection with animal or plant viruses on vector feeding behaviour (Blanc and Michalakis, 2016; Hurd, 2003; van Houte et al., 2013). Studies have mainly focused on host choice (e.g. attraction of aphids to plants infected with Barley yellow dwarf virus; Ingwell et al., 2012) or feeding behaviour (e.g. increased probing behaviour of mosquitoes infected with La Crosse virus; Grimstad et al., 1980). However, no studies have investigated the effect of viruses on changes in vector behaviour during the host-seeking stage. Here, we investigated whether West Nile virus (WNV; family Flaviviridae) infection can induce changes in the host-seeking response and host preference of the mosquito *Culex pipiens* Linnaeus 1758. Mosquitoes in the *C. pipiens* complex maintain WNV in an enzootic cycle with birds, whereas mammals are dead-end hosts (Hayes et al., 2005). From the perspective of WNV, manipulation of the host-seeking response and host preference towards birds is beneficial for its transmission. In particular, *C. pipiens* biotype *pipiens* is an important vector for WNV because of its preference for birds (Fritz et al., 2015; Osório et al., 2012). We hypothesised that WNV-infected *C. pipiens* biotype *pipiens* mosquitoes have a stronger host-seeking response and have a preference shifted towards avian hosts compared with uninfected mosquitoes. In order to test this hypothesis, we investigated the effect of WNV infection on host-seeking behaviour by determining the host-seeking response and preference in an olfactometer. We also investigated mosquito flight activity, blood feeding and survival in order to control for indirect effects of WNV infection on the mosquito's fitness. To understand the underlying mechanisms of changes in the host-seeking

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behaviour, we investigated antennal olfactory responsiveness of uninfected and WNV-infected mosquitoes.

MATERIALS AND METHODS

Mosquitoes

Culex pipiens biotype *pipiens* was established in the laboratory in the summer of 2014. *Culex pipiens* egg rafts were collected from rainwater barrels in Best, The Netherlands. One larva from each egg raft was identified to biotype with a real-time PCR assay (Vogels et al., 2015). Larvae from 162 egg rafts identified as biotype *pipiens* were grouped in trays (25×25×8 cm) as starting material for the rearing. Trays were filled with tap water and a drop of Liquifry No. 1 (Interpet Ltd., UK) was added. Thereafter, larvae were fed daily with a 1:1:1 mixture of bovine liver powder (MP Biomedicals, USA), ground rabbit food (Pets Place, The Netherlands) and ground koi food (Tetra, Germany). Pupae were transferred to Bugdorm cages (30×30×30 cm) and provided *ad libitum* with 6% glucose solution. Bovine or chicken blood was provided through a Hemotek® PS5 (Discovery Workshops, UK) feeder to allow for egg production by the mosquitoes. Larvae and adults were maintained at 23°C with a 16 h:8 h light:dark cycle and 60% relative humidity. The field-collected biotype *pipiens* mosquitoes (F0) successfully produced the next generation of mosquitoes (F1), which were used for experiments. Female mosquitoes were kept together with males for 3 to 6 days before being transferred to the Biological Safety Level 3 (BSL3) facility (Wageningen University & Research) for experiments.

Virus

In all experiments, a passage 2 stock of West Nile virus lineage 2 (GenBank accession no. HQ537483.1) originating from Greece (2010) was used with a 50% tissue culture infective dose ($TCID_{50}$ ml^{-1}) of 1.12×10^9. WNV was grown as described previously (Fros et al., 2015; Vogels et al., 2016).

Mosquito infection with WNV

Three- to six-day-old female mosquitoes were immobilised with CO_2 and injected with 69 nl (46 nl s^{-1}) of either phosphate buffered saline (mock infection) or 1.12×10^9 $TCID_{50}$ ml^{-1} of WNV (WNV infection) using the Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company, USA). Mosquitoes in the control treatment were only immobilised with CO_2. Mosquitoes were provided *ad libitum* with 6% glucose solution and maintained at 23°C with a 16 h:8 h light:dark cycle and 60% relative humidity. Our previous study showed full dissemination of WNV in 100% of *C. pipiens* biotype *pipiens* mosquitoes as soon as 7 days post-injection (Vogels et al., 2016). Based on these results, mosquitoes were used in experiments 8 to 10 days post-injection in order to make sure that all mosquitoes had WNV disseminated to the salivary glands, and thus were able to transmit WNV. To confirm that injections were successfully performed, mosquito bodies were tested for WNV presence by infectivity assays (Fros et al., 2015; Vogels et al., 2016). Shortly, homogenised mosquito bodies were incubated on a layer of Vero E6 cells, and checked for WNV-specific cytopathic effects after 3 days.

Test odour blends and individual compounds

For the behavioural assays, odours were collected from three humans by having them wear nylon socks (20 denier sock, Hema, The Netherlands) for 24 h and from five chickens by tying a nylon sock around one leg for 24 h. Chicken odour collections were approved by the Animal Ethics Committee of Wageningen University (DEC protocol 2013113.b), and all methods were performed in accordance with the relevant guidelines and regulations. Each sock was cut into three equal pieces. Three sock pieces, each from different human or chicken individuals, were combined and used as stimulus in the one-port olfactometer. Each set of socks was used during three experimental mornings. Socks were stored in glass jars at −20°C until use.

For the electrophysiological assays, geranylacetone (96%, Aldrich, Germany), hexanoic acid (99%, Sigma, Germany) and nonanal (95%, Aldrich) were selected as volatile chemicals. These volatiles were selected because they are present in odour profiles of humans and chickens, they bind to different olfactory receptors and they elicit high responses in antennae of *Culex quinquefasciatus* (Puri et al., 2006; Syed and Leal, 2009). All three volatiles were diluted to a 1% concentration in dichloromethane (>99.9%, Sigma). Of each diluted volatile or the solvent, 20 µl was applied on a piece of Whatman filter paper (5×40 mm), left for evaporation of the solvent at room temperature, and then inserted in a Pasteur pipette which was sealed at both ends with Parafilm. New Pasteur pipettes with volatiles were prepared every experimental morning.

Behavioural assay

Host-seeking responses of control, mock-infected and WNV-infected female mosquitoes were tested in a one-port olfactometer (1.65×0.65×0.65 m) in the BSL3 facility (Braks and Takken, 1999; Knols et al., 1994). Four to five days prior to experiments, water was provided to female mosquitoes in order to induce the host-seeking response. The last day before being tested in the olfactometer, female mosquitoes were individually transferred to 50 ml tubes with a mesh bottom, without access to water. The olfactometer was specifically designed for safe release of individual mosquitoes from the modified 50 ml tubes. A piece of Velcro was mounted on the lid of the tubes, and a complementary piece of Velcro was mounted on the release mechanism inside the olfactometer. Tubes were inserted in an opening in the middle of one end of the olfactometer, and after closing the olfactometer, they could be safely opened via the release mechanism on the outside of the olfactometer. Individually released mosquitoes were allowed to respond to the odour stimulus by flying through the port at the other end, within a time period of 7 min. For all three odour stimuli (control, human and chicken), 5% CO_2 (450 ml min^{-1}) was released from a circular release point placed directly in front of the port. A trapping device containing a Hemotek PS5 feeder for heat production was placed behind the port. For the control stimulus no sock was added, for the human odour stimulus three pieces of socks worn by humans were wrapped around the feeder, and for the chicken odour stimulus three pieces of socks worn by chickens were wrapped around the feeder. Charcoal-filtered and moistened air was fed through the trapping device at a speed of 0.22±0.02 m s^{-1}. Temperature in the experimental room was set at 24°C and relative humidity at 70%. Experiments were performed during the first hours of the dark phase, with red light and dimmed light turned on. Each of the nine combinations of mosquito treatment and odour stimulus was replicated with 53–57 individual female mosquitoes.

For biosafety reasons (i.e. free-flying WNV-infected mosquitoes in an olfactometer), the order of testing the three mosquito treatments (control, mock-infected and WNV-infected) was kept the same. In addition, the order of odour stimuli was alternated during consecutive experimental mornings according to a Latin square design. Before each stimulus was tested, temperature and relative humidity were measured in the room, the middle of the olfactometer and the port from which the stimulus was released.

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There should be a gradient from room to port in both temperature and relative humidity in order to attract mosquitoes to the port. Mosquitoes that had entered the trapping device were stored in Eppendorf tubes at −80°C, and tested for WNV infection in mosquito bodies (Fros et al., 2015).

Mosquito fitness-related parameters

Flight duration was determined for a selection of control, mock-infected and WNV-infected female mosquitoes tested in the behavioural assay. Flight duration was recorded for 41 control females, 43 mock-infected females and 39 WNV-infected females, which were released in a one-port olfactometer. The fraction of time in flight was calculated as flight duration divided by total time, with total time being the time between the moment of release and the moment the mosquito entered the trapping device containing odour, or 7 min if the mosquito did not respond.

Blood feeding propensity was determined by offering chicken whole blood through the Hemotek P5 feeder to control, mock-infected and WNV-infected female mosquitoes. Fifteen female mosquitoes of each treatment were placed together in a plastic bucket (Ø=12.5 cm and height=12 cm) and were allowed to blood feed for 1 h. Blood feeding propensity was tested at 7 to 9 days post-injection. The experiment was repeated four times.

Survival was determined by placing 10 control, 10 mock-infected and 10 WNV-infected female mosquitoes in three separate buckets directly after the injection procedure. Mosquitoes were provided with 6% glucose solution and maintained at 23°C with a 16 h:8 h light:dark cycle and 60% relative humidity. The number of surviving female mosquitoes was counted daily for 30 days. The experiment was replicated three times.

Electrophysiology

Electroantennography (EAG) was used to record responses of antennae of control, mock-infected and WNV-infected female mosquitoes to the three volatile compounds and the solvent under BSL3 conditions. One day before EAG recording, female mosquitoes were individually transferred to tubes without access to glucose solution. Female mosquitoes were individually immobilised with CO_2 when being prepared for EAG recordings. EAG recordings were done following the method described by Qiu et al. (2013). For each antenna of an individual female, only the solvent and one test volatile were recorded at 1 min intervals to prevent serial effects. In total, 10 female mosquitoes of each treatment (control, mock-infected and WNV-infected) were tested for each of the three volatile odours (1% geranylacetone, 1% hexanoic acid and 1% nonanal). Standardised responses were calculated by dividing the absolute response amplitude (mV) to the volatile compound by the response amplitude (mV) of the same antennae to the solvent and multiplying by 100.

Statistical analysis

A generalised linear mixed model (GLMM) with a binomial distribution and logit link function was used to test for the effect of WNV infection and odour stimulus on the proportion of responding mosquitoes in the behavioural assay. WNV infection, odour stimulus and the interaction between WNV infection and odour stimulus were included in the model as fixed effects. To account for the experimental design, random effects were included for days, and for blocks of six consecutive measurements within days, in which the stimulus was held fixed. For clarity we present the means and standard errors of the mean obtained from the raw data in Fig. 1, in combination with the statistical output of the GLMM as described above.

A linear mixed model was used to test for the effect of WNV infection and odour stimulus on flight activity. The same fixed and random effects as mentioned above for the behavioural assay were included in the model. Degrees of freedom were calculated according to the method of Kenward and Roger (1997). A GLMM with a binomial distribution and logit link function was used to test for differences in the proportion of blood-fed female mosquitoes over the three treatments (control, mock infection and WNV infection). Random effects were included for replicates. Observation-level random effects were included to handle overdispersion (Harrison, 2014). Kaplan–Meier survival analysis was used to test for differences in survival of mosquitoes exposed to the three treatments.

EAG recordings were analysed with EAG pro software version 1.1 (Syntech, Germany). Kruskal–Wallis tests were used to test for the effect of WNV infection on the standardised median responses for each volatile odour. All data were analysed in the statistical

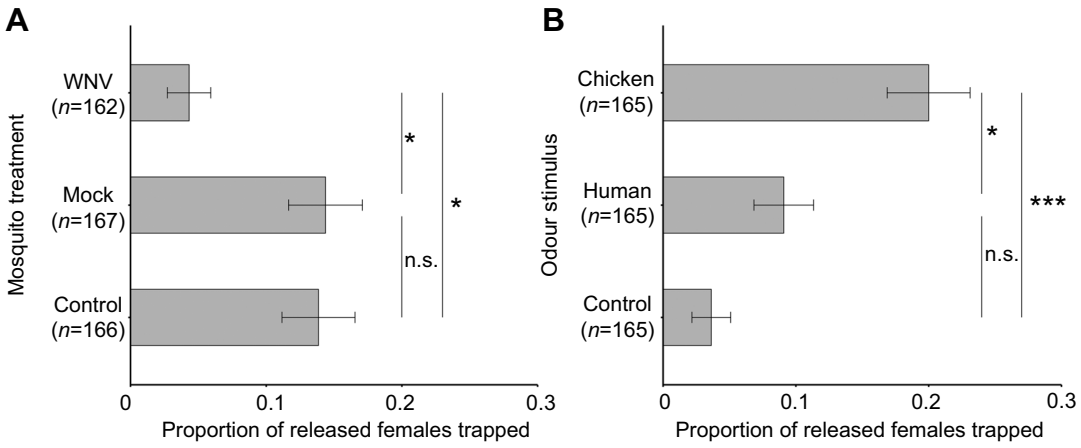

Fig. 1. Host-seeking response of control, mock-infected and West Nile virus (WNV)-infected *Culex pipiens* biotype *pipiens* females to odour stimuli in the one-port olfactometer. (A) Proportion of released female mosquitoes trapped per infection treatment (control, mock-infected and WNV-infected) pooled for all three odour stimuli (control, human odour and chicken odour). (B) Proportion of released female mosquitoes trapped per odour stimulus (chicken, human and control) pooled for infection treatment. Sample size is indicated for each treatment and odour stimulus. Data are mean proportions; error bars show s.e.m. obtained from the raw data. Indicated statistics show the output of the GLMM (n.s., not significant, *P<0.05, ***P<0.001).

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software package R (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

WNV decreases mosquito's host-seeking response

To test whether WNV infection leads to a stronger host-seeking response and a shift in preference towards birds, female mosquitoes were individually exposed to host odours in a one-port olfactometer. There was a significant effect of WNV infection (P=0.003) and odour stimulus (P<0.001) on the host-seeking response, but not of their interaction (P=0.61). Therefore, the interaction term was excluded from the final model. Control, mock-infected and WNV-infected mosquitoes had a similar preference for chicken odour. Thus, WNV infection did not induce a shift in host preference.

Female mosquitoes infected intra-thoracically with WNV showed an overall lower host-seeking response (4.3%), independent of odour stimulus, compared with mock-infected (14.4%; P=0.010) and control females (13.8%; P=0.012; Fig. 1A). The infection procedure did not affect the response, because there was no difference in response between control females and mock-infected females (P=1.00; Fig. 1A). All female mosquitoes injected with WNV were confirmed to be infected with WNV. Thus, independent of the odour stimulus offered, WNV infection decreased the host-seeking response of its mosquito vector.

Chicken odour in combination with CO_2 and heat attracted a significantly higher proportion of female mosquitoes (20.0%), independent of WNV infection, than human odour in combination with CO_2 and heat (9.1%; P=0.026), and CO_2 and heat only (3.6%; P<0.001; Fig. 1B). The addition of human odour did not attract a higher proportion of female mosquitoes than CO_2 and heat alone (P=0.099; Fig. 1B). Thus, both uninfected and infected female mosquitoes had a preference for chicken odour.

No effect of WNV infection on other mosquito fitness-related parameters

Flight activity, blood-feeding propensity and survival were determined in order to control for possible negative effects of WNV infection on mosquito fitness. Flight duration was recorded when mosquitoes were released in the olfactometer (Braks and Takken, 1999; Knols et al., 1994). Median fractions of time in flight were 0.76 for control, 0.75 for mock-infected and 0.75 for WNV-infected female mosquitoes, when pooled over the three odour stimuli (Fig. 2A). No significant effects were found in these fractions of time in flight for treatment ($F_{2,109}$=0.30, P=0.74), odour stimuli ($F_{2,108}$=0.30, P=0.74) or their interaction ($F_{4,109}$=0.50, P=0.74). Thus, WNV infection does not influence flight activity.

We investigated the effect of WNV infection on the mosquito's propensity to take a blood meal. In total, 25.4% of control females, 31.6% of mock-infected females and 31.7% of WNV-infected females took a blood meal (Fig. 2B). These proportions were not significantly different (χ^2=0.65, d.f.=2, P=0.72). The variance among replicates, however, was considerable (χ^2=6.040, 50:50 mixture of χ^2_0 and χ^2_1, P=0.007). Thus, WNV infection does not significantly influence the propensity of mosquitoes to take a blood meal, though this propensity varies over time.

The effect of WNV infection on mosquito survival was monitored during 30 days. Thirty days post infection, 100% of the control females, 93% of the mock-infected females and 83% of the WNV-infected females were still alive (Fig. 2C). These differences tended to significance (χ^2=5.70, d.f.=2, P=0.057). Thus, WNV infection had a minor effect on mosquito survival.

WNV does not affect antennal olfactory responsiveness

EAG responses of uninfected and infected female mosquitoes to three host-derived volatile compounds were compared to determine the effect of WNV infection on the sensitivity of antennal olfactory neurons (Qiu et al., 2013). There were no significant differences between the standardised antennal responses of control, mock-infected and WNV-infected female mosquitoes to 1% geranyl acetone (χ^2=0.28, d.f.=2, P=0.87; Fig. 3A), 1% hexanoic acid (χ^2=0.03, d.f.=2, P=0.99; Fig. 3B) and 1% nonanal (χ^2=0.52, d.f.=2, P=0.77; Fig. 3C). Thus, there is no indication that WNV infection interferes with the peripheral olfactory system.

DISCUSSION

WNV infection decreased the host-seeking response of its mosquito vector, and did not induce a shift in host preference. As other fitness-related parameters (flight activity, blood feeding propensity and survival) were not affected, we investigated the effect of WNV infection on the mosquito's olfactory response. No effect was found of WNV infection on electrophysiological responses in the peripheral olfactory system. The reduced host-seeking response is, thus, likely due to interference of WNV infection with the function of the mosquito's central nervous system.

Infection of nervous tissues occurs 8 days after *C. quinquefasciatus* becomes infected with WNV through an infectious blood meal (Girard et al., 2004). WNV has been isolated from all nervous tissues of the mosquito including the brain,

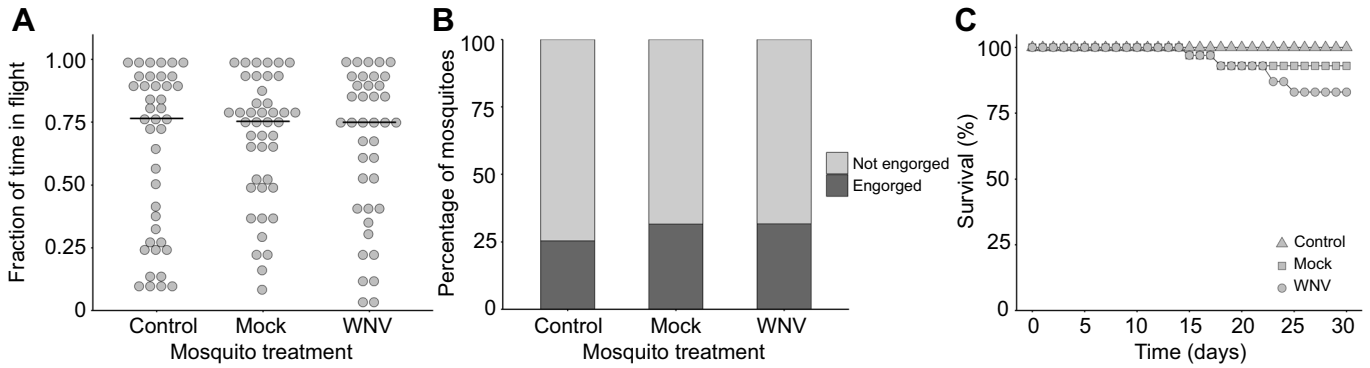

Fig. 2. Mosquito fitness parameters flight activity, blood feeding propensity and survival of control, mock-infected and WNV-infected *Culex pipiens* biotype *pipiens* females. (A) Fraction of time in flight in the behavioural assay, pooled for the three odour stimuli. Each circle represents one mosquito. Horizontal black lines indicate the median fraction of time in flight. (B) Percentage of blood-fed female mosquitoes based on four replicates of 15 females per infection treatment. (C) Percentage survival over a time period of 30 days post-infection. Each treatment consisted of three replicates of 10 female mosquitoes.

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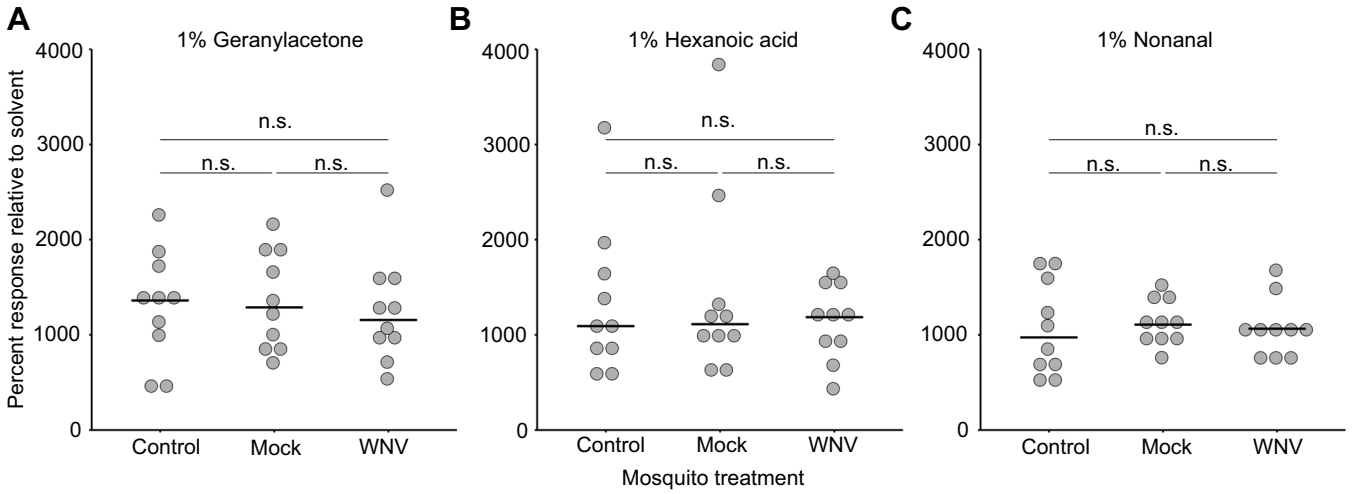

Fig. 3. Standardised antennal responses of control, mock-infected and WNV-infected *Culex pipiens* biotype *pipiens* females for three different host-derived volatiles. Percent responses of control, mock-infected and WNV-infected female mosquitoes to (A) 1% geranyl acetone, (B) 1% hexanoic acid and (C) 1% nonanal, relative to the solvent dichloromethane. Each odour was tested on antennae of 10 female mosquitoes per infection treatment. Antennal responses were standardised by dividing the absolute response (mV) of an antenna to the volatile compound by the response (mV) to the solvent. Each circle represents one tested antenna. Horizontal black lines indicate the median percent response. n.s., not significant.

thoracic ganglia, abdominal ganglia, cephalic ganglion and Johnston's organ (Girard et al., 2004, 2005). In our study, behavioural assays were performed with female mosquitoes at 8 to 10 days post WNV injections. At this moment, WNV must have already infected the mosquito brain. Therefore, we hypothesise that WNV interferes with the processing of signals from the olfactory neurons in the brain. Further investigation of WNV pathogenesis in the antennal lobes, the regions in the brain in which olfactory responses from antennae are processed at different time points, is needed to confirm the potential role of the mosquito brain in the observed behavioural changes after WNV infection.

An alternative explanation for the observed decrease in host-seeking response may be found in the mosquito's immune system. Studies with the malaria vector *Anopheles stephensi* showed that an increase of the host-seeking response could also be induced by stimulation with heat-killed bacteria (Cator et al., 2013). Thus, manipulation may be an indirect result of immune challenge, or the result of direct manipulation due to infection with the malaria parasite. It would therefore be interesting to further investigate the effect of immune challenge on other mosquito species such as *C. pipiens*. Of particular interest would be the effects of inoculation with non-infectious WNV on the mosquito's host-seeking response.

The reduced host-seeking response implies that WNV-infected *C. pipiens* biotype *pipiens* mosquitoes would be less likely to localise a host in nature, which could consequently reduce the chances of successful enzootic WNV transmission. To our knowledge, this is the first study that shows changes in host-seeking behaviour of a vector induced by a pathogen that do not favour transmission, but which seem to be detrimental for both pathogen transmission and fitness of the vector. However, given the repeated outbreaks of WNV, WNV transmission in nature is not reduced to the extent that it halts transmission. To explain the ongoing transmission of WNV, we hypothesise a dose-dependent effect of WNV on the mosquito's host-seeking behaviour. High WNV titers result in a high chance of dissemination to the saliva, but transmission may be counteracted by a reduced host-seeking response. In contrast, at low WNV titers there is a lower chance of dissemination to the saliva, but we hypothesise that there is a relatively high host-seeking response. In nature, this dose-dependent effect is expected to reach an equilibrium because of stabilising natural selection on intermediate WNV replication levels, which may explain the ongoing repeated outbreaks of WNV.

In contrast to other mosquito species that readily respond to host odours in an olfactometer setup (Allan et al., 2010; Pates et al., 2001), *C. pipiens* biotype *pipiens* mosquitoes were less motivated to respond. Based on pilot studies in which we varied several parameters such as response time, air speed and period of glucose deprivation, we selected the best combination of parameters that resulted in the highest overall response. Therefore, we deprived mosquitoes from glucose solution for 4 to 5 days to stimulate host-seeking behaviour. Although this deprivation period was longer in comparison with earlier studies (Cornet et al., 2013; Simpson et al., 2009), it did not negatively affect the mosquito's survival. This deprivation may interact with pathogen infection, because pathogens use host resources to develop and replicate (Cressler et al., 2014). However, WNV-infected mosquitoes showed similar flight activity compared with both control and mock-infected mosquitoes. Thus, glucose deprivation does not seem to result in a higher energy depletion of WNV-infected mosquitoes.

In this study, mosquitoes were infected with WNV through intra-thoracic injections. Injections are needed to guarantee that WNV disseminates to the salivary glands of all mosquitoes at 8–10 days post-infection (Vogels et al., 2016). In addition, use of naive females, which have no prior blood-feeding experience, allows for recording of unbiased responses towards host odours in the olfactometer. Our previous study showed a similar range of viral titers in mosquito bodies after an infectious blood meal or injections, with maximum titers of approximately 10^8 $TCID_{50}$ ml^{-1} (Vogels et al., 2017). Results obtained with mock-infected mosquitoes showed that the injection procedure itself did not have any effects on mosquito behaviour or fitness. Thus, although injections are not a natural way to infect mosquitoes, we believe that this method does provide results that are relevant for the natural situation.

Future research will focus on the effect of other arboviruses on the host-seeking behaviour of mosquitoes, in order to investigate whether this phenomenon is specific for WNV or more broadly occurring with other arbovirus–vector systems. Understanding the influence of arbovirus infection on vector behaviour is necessary to

fully understand the dynamics of arbovirus transmission, which is needed for proper risk assessment and tailored control strategies.

Conclusions

WNV infection decreases the mosquito's host-seeking response, and does not induce a shift in host preference towards avian hosts. Other mosquito fitness-related traits (flight activity, blood feeding propensity and survival) are not affected by WNV infection. The reduced host-seeking response cannot be explained by inhibition of peripheral odour perception via the antennae, but might be due to interference of WNV with neural processing in the mosquito's brain.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.B.F.V., C.J.M.K.; Methodology: C.B.F.V., J.J.F., G.P.P., J.J.A.v.L., C.J.M.K.; Validation: C.B.F.V.; Formal analysis: C.B.F.V., G.G.; Investigation: C.B.V.; Writing - original draft: C.B.F.V.; Writing - review & editing: C.B.F.V., J.J.F., G.P.P., J.J.A.v.L., G.G., C.J.M.K.; Visualization: C.B.F.V.; Supervision: C.J.M.K.

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