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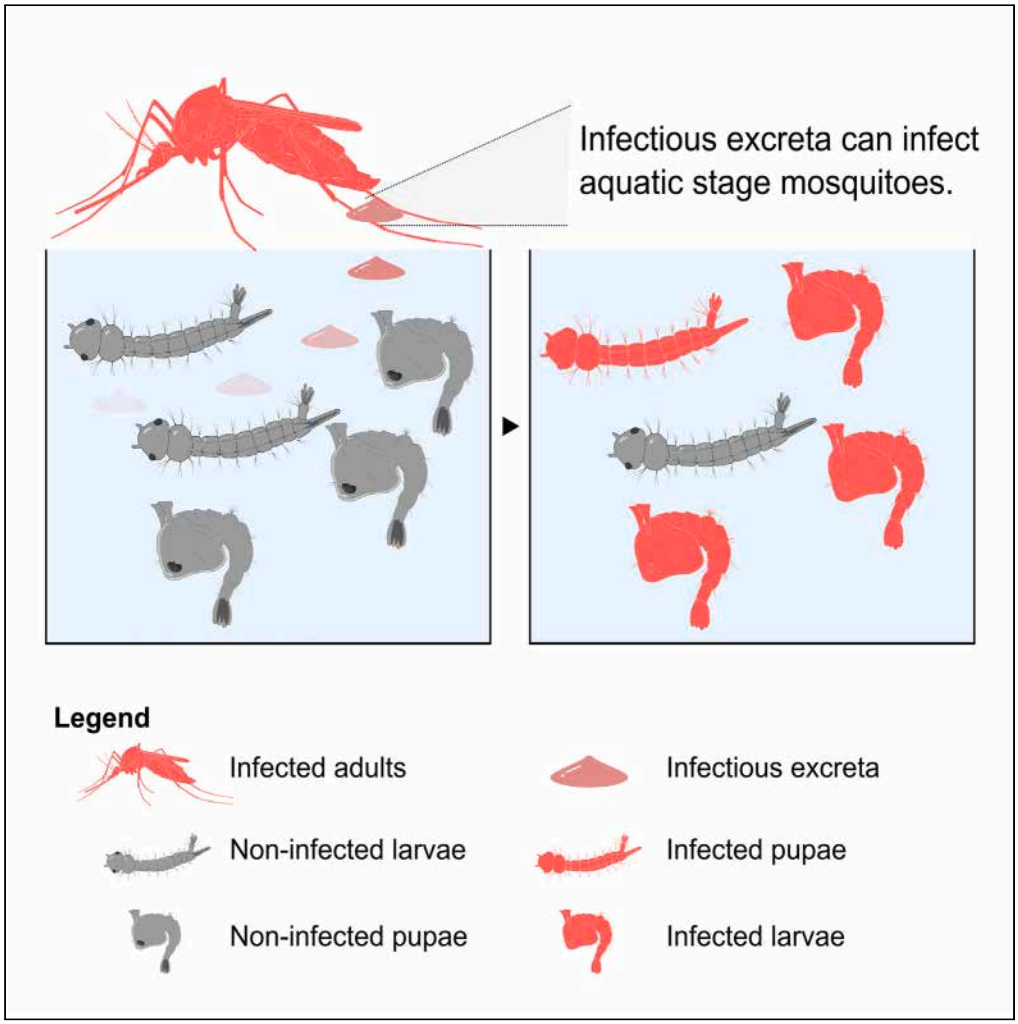

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Highlights

Excreta from WNV-infected mosquitoes contain infectious virions

Virions are excreted early and constantly after mosquito blood feeding

Mosquito aquatic stages are infected by infectious excreta

Modeling shows that excreta-mediated transmission occurs in some breeding sites

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West Nile virus can be transmitted within mosquito populations through infectious mosquito excreta

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SUMMARY

Understanding the transmission routes of arboviruses is key to determining their epidemiology. Here, we tested whether West Nile viruses (WNVs) are transmitted through mosquito excreta. First, we observed a high concentration of infectious units per excreta, although viruses were short lived. Second, we showed that virion excretion starts early after oral infection and remains constant for a long period, regardless of mosquito infection level. These results highlight the infectiousness of excreta from infected mosquitoes. Third, we found that both larvae and pupae were susceptible to infection, while pupae were highly permissive. Fourth, we established the proof-of-concept that immature mosquitoes can be infected by infectious excreta, demonstrating an excreta-mediated mode of transmission. Finally, by mathematically modeling excreta-mediated transmission in the field, we demonstrated that WNV can be transmitted within mosquito populations. Our study uncovers a route of transmission for mosquito-borne arboviruses, unveiling mechanisms of viral maintenance in mosquito reservoirs.

INTRODUCTION

West Nile virus (WNV) is currently the most widely distributed mosquito-borne disease.[1] Originally isolated in the West Nile province of Uganda in 1937,[2] circulation of WNV has now been reported on all continents, except Antarctica.[3–6] Although WNV infection in humans remains asymptomatic in most cases, approximately 25% of infected patients develop non-lethal flu-like symptoms and 1% show neurological manifestations such as encephalitis, meningitis, or acute flaccid paralysis, potentially causing death and long-term sequelae.[7] Furthermore, an epidemiologic shift in the 90s resulted in a general increased severity with more frequent neurological symptoms.[8] Initially observed around the Mediterranean basin, the more virulent lineage 1 was introduced in the USA in 1999 and rapidly spread throughout the country and the Americas. Since 2000, WNV has infected an estimated 7 million people and caused more than 2,700 deaths in the USA.[9,10] The disease causes yearly deaths in the EU where more than 100 people died in 2022 and 2023.[11] Despite the alarming situation, there are neither therapeutics nor licensed vaccines for humans.[3,6]

WNV transmission occurs through multiple routes. Primarily, WNV is transmitted between vertebrate hosts through mosquito vectors, mostly from the *Culex* genus; a mode that is referred to as "horizontal" transmission.[1] Successful horizontal transmission occurs when a susceptible mosquito bites an infected host. The virus then multiplies within the vector until it infects the salivary glands, from which it is expectorated into the skin of another susceptible host during subsequent blood feeding, resulting in transmission.[12] WNV circulates in an enzootic cycle between birds, where *Cx. quinquefasciatus*, *Cx. pipiens*, and *Cx. tarsalis* are the main vectors. Occasionally, opportunistic feeding of some *Culex* species results in transmission to humans or other mammals (i.e., horses) outside of the enzootic cycle.[13,14] However, mammals are dead-end hosts as most of them do not develop a sufficiently high viremia to infect mosquitoes during a blood meal. Additionally, WNV can be directly transmitted between vertebrate hosts by contact with or consumption of infectious materials, such as infected birds, mosquitoes, cloacal fluids, blood transfusion, organ transplantation, or even breast milk.[15–17] Finally, WNV as for other flaviviruses can be maintained within mosquito populations by direct transmission from an infected female mosquito to its offspring; a mode referred to as "vertical" transmission.[18–20] However, low vertical transmission rates reported in laboratories imply a moderate epidemiological role,[21] even though vertical transmission efficiency improves with extrinsic incubation duration.[18,22]

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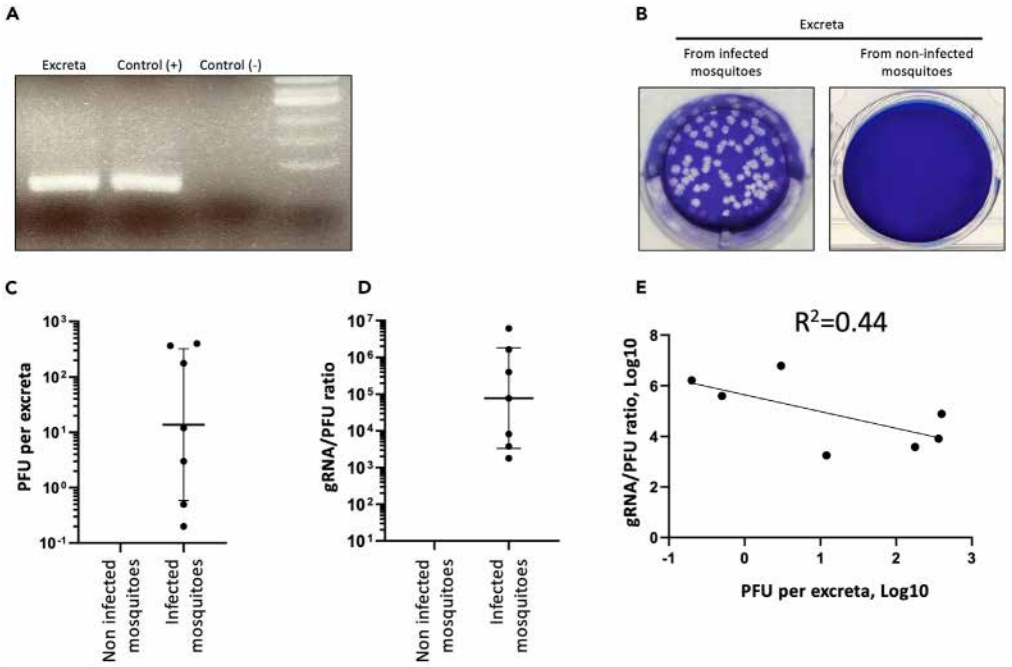

Figure 1. Detection and quantification of infectious viruses in mosquito excreta

(A and B) Detection of WNV viral RNA (A) and infectious particles (B) in supernatant from cells infected with excreta pools (i.e., amplified excreta inoculum). Control (+) corresponds to RNA extracts from WNV stock. Control (−) corresponds to water.

(C and D) Quantification of PFU per excreta (C) and ratio of viral genomic RNA (gRNA)/PFU in the same excreta pools collected 6 days post mosquito exposure to blood containing 5 × 10^6 PFU/mL. Bars show means ± SEM. Each point indicates one excreta pool, collected from several experiments.

(E) Correlation between PFU per excreta and gRNA/PFU ratio for the previous samples. See also Figure S1.

Several lines of evidence indicate that WNV is maintained within mosquito populations without circulating through vertebrate hosts. WNV has been detected in *Culex* males,[23] in larvae,[24,25] and in pupae,[26] all of which became infected by exposure to inoculum source other than blood. Circulation of the virus between mosquitoes then enables persistence of the virus when conditions are unfavorable for horizontal transmission, and facilitates resurgence of transmission to vertebrate, including humans, when conditions favor mosquito biting of susceptible hosts.[8,27] Understanding the modes of transmission that maintain viruses within mosquito populations is important to promoting innovative interventions and improving epidemiological forecast to adjust interventions.

Here, we establish the proof-of-concept that WNV can be maintained within mosquito populations through excreta-mediated transmission. Our hypothesis is based on the observation that excreta from infected mosquitoes contain detectable amounts of arboviral RNA and for this reason are screened as an innovative surveillance strategy.[28,29] Furthermore, a previous study observed that excreta from *Cx. annulirostris* mosquitoes carry infectious WNV virions but concluded that the amount was too low to infect other mosquitoes.[29] In our study, we used WNV as a flavivirus model and showed that infected *Cx. quinquefasciatus* mosquitoes excrete infectious virions. We then evaluated the possibility of an excreta-mediated transmission to immature mosquitoes by (i) quantifying the inoculum per excreta; (ii) assessing how extrinsic incubation period and mosquito infection intensity influence excreta infectivity; (iii) determining the susceptibility of immature mosquitoes to viral infection; and (iv) demonstrating that infectious excreta can infect immature mosquitoes. Eventually, we combined our multifactorial dataset into a mathematical model to assess the potential for excreta-mediated WNV transmission in breeding sites. Our study uncovers another mode of transmission for mosquito-borne arboviruses.

RESULTS

Quantification of infectious virions in mosquito excreta

To test whether excreta from infected mosquitoes carry infectious virions, we orally infected *Cx. quinquefasciatus* with 10^5 plaque-forming unit (PFU)/mL of WNV. Mosquitoes were then offered a sucrose solution, and we collected pools of excreta across different days post exposure (DPE) using a specific device (Figure S1, see STAR Methods). Excreta solutions were used to inoculate virus-susceptible Vero cells. Cells were extensively washed after inoculation and at 6 days post inoculation, we detected viral genomic RNA (gRNA) in the resulting cell supernatant (Figure 1A), demonstrating active viral infection. To confirm that excreta induced a productive infection, we performed a cell-based titration assay and showed that the supernatant of cells inoculated with mosquito excreta contained infectious virions as indicated by the presence of many lytic plaques on the cell monolayer (Figure 1B). In contrast, cells inoculated with excreta of non-infected mosquitoes did not show any

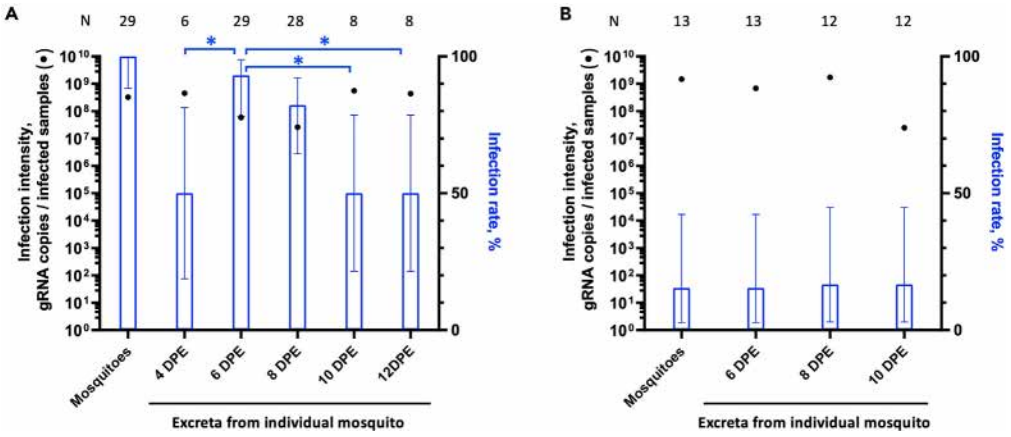

Figure 2. The effect of oral inoculum and days post exposure (DPE) on virus excretion

(A and B) Infection intensity and infection rate in mosquitoes exposed to blood containing 10^7 (A) or 10^5 (B) PFU/ml of WNV and in their excreta collected every two days. Black dots show geometric mean ± SD for infection intensity. Blue bars show percentage ±95% C.I. for infection rate. *N*, number of samples from at least two experiments. Mixed-effects one-way ANOVA was used to compare infection intensities, but no statistical difference was found. Chi^2 was used to compare infection rates. *, $p < 0.05$.

plaque. As observed in one previous study,[29] our results confirm that excreta of infected mosquitoes, in our case *Cx. quinquefasciatus* mosquitoes infected with WNV, carry infectious viral particles.

We next quantified the number of infectious particles per excreta. To enable excreta counting, we offered mosquitoes a sugar solution supplemented with food colorant that resulted in blue-colored excreta and counted the blue dots on the plastic walls as a proxy for excreta. To maximize the number of collected excreta, we grouped mosquitoes in one container and regularly collected excreta by washing the plastic containers with cell culture media used to perform viral titration. However, we could not detect infectious particles when the collection was conducted every 24 h or more. We reasoned that viruses may not be stable for a long time in dried excreta and thus collected excreta at shorter intervals of 1 h–1 h 30 min to limit virus degradation. Under these conditions, we detected infectious particles in pools of excreta and were able to quantify the number of PFU, which we divided by the estimated number of excreta to obtain an average PFU/excreta. We observed a large variation in PFU/excreta between the different samples ranging from 0.2 to 400 PFU/excreta with a mean of 136.7 PFU/excreta (Figure 1C). As a control, we did not detect any plaque in excreta from mosquitoes that were not exposed to an infectious blood meal.

To evaluate the infectivity of excreted virions, we calculated the ratio of gRNA/PFU, which estimates the number of infectious particles among all particles.[30] For this, we assumed that each particle contained one gRNA copy and each PFU resulted from one infectious unit. In excreta, the gRNA/PFU ratio exhibited variability, ranging from 1.8×10^3 to 6.1×10^6, with a mean of 1.2×10^6 (Figure 1D). In comparison to a gRNA/PFU ratio of 100 for dengue virus, another flavivirus, secreted from mosquito cells,[31] the higher gRNA/PFU ratio for excreted WNV indicates a high proportion of non-infectious particles, which may have undergone degradation before excreta collection. We reasoned that the elevated gRNA/PFU ratio might be attributed to virion degradation in certain samples. Indeed, the collection times varied as it was contingent on mosquito excretion dynamics even though we collected excreta at short intervals. Supporting this hypothesis, we observed a negative correlation ($R^2 = 0.44$) between excreta infection load, measured by the PFU/excreta ratio, and virion infectivity, estimated by the gRNA/PFU ratio (Figure 1E). This observation underscores the lability of excreted virions in our conditions, implying an underestimation of PFU per excreta. Altogether, our findings demonstrate that WNV-infected mosquitoes excrete infectious virions, which quantification at an average of 136.7 PFU per excreta was probably underestimated due to virus lability.

Virions are excreted early and continuously after exposure to an infectious blood meal

To deepen our comprehension of virion excretion, we assessed the kinetics of virion excretion and how mosquito infection level influences virion excretion. To monitor the time period of excretion, we collected excreta from single mosquitoes every 2 days from 4 to 12 DPE to a WNV blood inoculum of 10^7 PFU/mL, which is within the high end of bird viremia.[32,33] Excreta collected at each time point corresponded to all excreta from the past 2 days. For instance, the samples at 4 DPE included excreta from 2 to 4 DPE. We did not collect excreta earlier than 2 DPE to avoid collecting viruses from the blood inoculum.[34] We then quantified viral gRNA and calculated both the infection rate, as the percentage of samples with detectable amounts of gRNA among collected samples, and the infection intensity, as gRNA copies per infected sample.

First, we quantified infection in the orally exposed mosquitoes from which we collected excreta at the end of the experiment (12 DPE). The high blood inoculum resulted in 100% of mosquitoes infected with a geometric mean of 3.2×10^8 gRNA copies per mosquito (Figure 2A). Second, we observed that about 50% of excreta carried viruses as early as 4 DPE and that excreta infection rate peaked at 93% at 6 DPE before gradually decreasing to 50% at 10 and 12 DPE (Figure 2A). In contrast, the infection intensity (i.e., gRNA copies per infected sample) did not

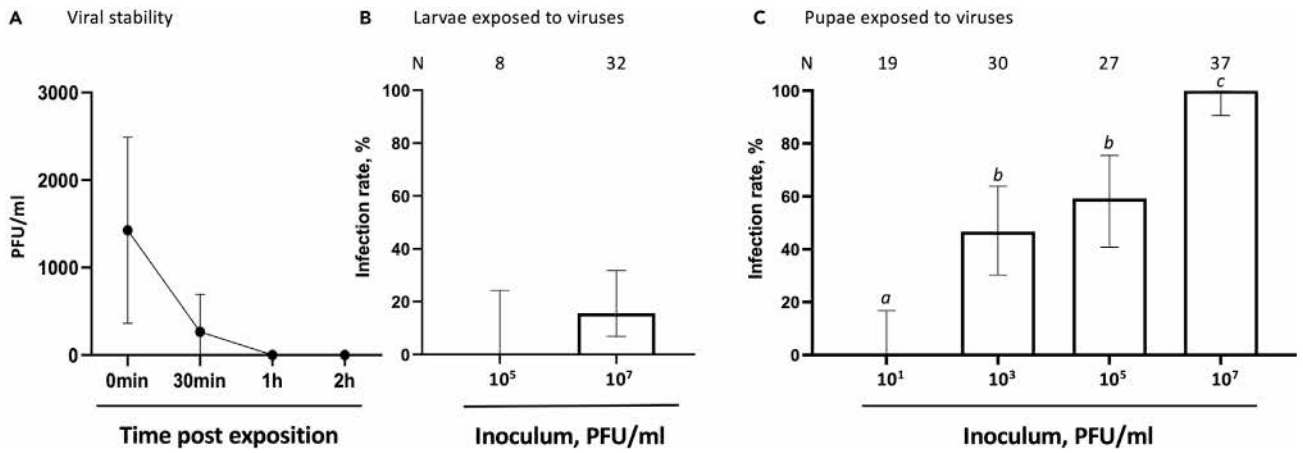

Figure 3. Susceptibility of aquatic stages to WNV exposure

(A) Stability of WNV in rearing water. Points indicate mean ± SEM of PFU/ml in water at different time post inoculation. *N*, 4.

(B and C) Infection rate for L4 larvae exposed to WNV at L1 stage (B) and for adult mosquitoes exposed at the pupal stage (C). Bars show percentage ±95% C.I. Chi^2 was used to compare infection rates between different virus concentrations. Different letters indicate significant differences, $p < 0.05$. *N*, number of individual mosquitoes from at least two experiments. See also Figure S2.

significantly change with time and remained relatively constant between 2.6×10^7 and 5.5×10^8 gRNA copies per excreta sample across the different time points (Figure 2A).

To evaluate the influence of mosquito infection level, we repeated the excreta collection kinetics with mosquitoes orally exposed to a lower inoculum (i.e., 10^5 PFU/mL) of WNV, resulting in 15% of infected mosquitoes with a mean of 1.5×10^9 gRNA copies per mosquito collected at 10 DPE (Figure 2B). Excreta infection rate from 6 to 10 DPE was stable between 15 and 17% (Figure 2B), and gRNA was mostly detected in excreta from infected mosquitoes. Additionally, we found that each infected excreta sample contained 6.8×10^8 and 1.7×10^9 gRNA copies at 6 and 8 DPE, respectively, before diminishing to 2.5×10^7 gRNA copies at 10 DPE (Figure 2B). Altogether, the kinetic study from mosquitoes infected with a high and low inoculum shows that virions are excreted early after oral exposure to infectious blood and for a long period at a relatively constant intensity level.

Pupae are highly susceptible to infection

To determine whether infectious excreta can infect mosquito aquatic stages, we first monitored the virus stability in mosquito rearing water. At the initial collection time (0 min), just after diluting the virus stock, the number of infectious particles was 1,425 PFU/mL (Figure 3A). Infectious particles then rapidly diminished to reach zero at 1 h post inoculation, indicating a high lability of the virus in the rearing water.

We evaluated the susceptibility of L1 larvae and pupae to different concentrations of WNV (derived from cell culture) in rearing water. Our experimental design included several precautions to avoid confounding effects. Mosquito aquatic stages were exposed for only 1 h to minimize effects due to exposure to the viral stock solution. Viral gRNA was quantified in extensively washed L4 larvae resulting from the exposed L1 larvae in order to avoid detecting viral remnants from the inoculum. For the same reason, viral gRNA was quantified in adult mosquitoes resulting from the exposed pupae, as gut content is expelled and the cuticle renewed during morphogenesis.[35] None of the larvae were infected after exposure to 10^5 PFU/mL and only 15% after incubation with 10^7 PFU/mL (Figure 3B). In contrast, pupae were more susceptible to infection with 46% infected with 10^3, 59% with 10^5 and 100% with 10^7 PFU/mL (Figure 3C). We also evaluated survival after inoculum exposure. Larvae were not affected, whereas pupae exhibited a slightly reduced survival (Figure S2). Altogether, our results show that the short duration stability of WNV in rearing solution is sufficient to infect larvae and pupae, albeit pupae are more susceptible to infection. These observations imply that mosquito excretion in rearing water pools may lead to infection of aquatic stages.

Infectious mosquito excreta infect pupae

While our previous experiments separately determined the excreta infectivity and the infection susceptibility of immature mosquitoes, we then tested the proof of concept that infectious excreta can infect mosquito pupae. We collected pools of excreta every 1 h from mosquitoes at 6 DPE to a high blood inoculum to ensure maximum excreta infectivity. The excreta pools were quantified and diluted in rearing water at 4.6×10^3 PFU/mL. Pupae were reared in the excreta-containing solution and the infection rate was assessed in adult mosquitoes. We found that 17% of pupae-exposed adults were infected (Figure 4), thereby establishing the proof of concept of excreta-mediated transmission for WNV.

Excreta-mediated infection can maintain WNV infection within certain field mosquito populations

To determine the contribution of excreta-mediated infection in WNV maintenance within mosquito reservoir, we built and examined a compartmental model (Figure 5A). In a given breeding site, we modeled egg laying, mortality, hatching, and emergence to calculate the

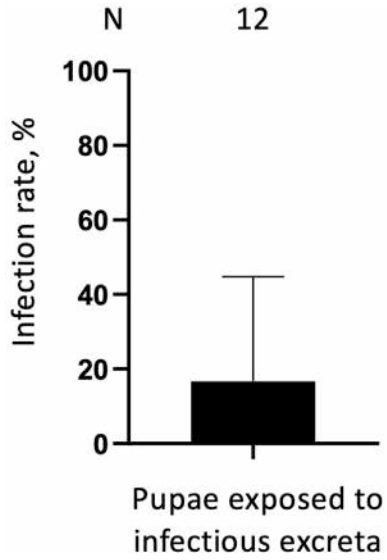

Figure 4. Susceptibility of pupae to infectious excreta

Bar shows infection rate +95% C.I. in adult mosquitoes exposed at the pupal stage to infectious excreta at a concentration of 4.6×10^3 PFU/mL. *N*, number of individual mosquitoes.

number of susceptible immature mosquitoes (S_L) and the resulting number of infected immature mosquitoes (I_L) based on excreted virions (W) from infected adult mosquitoes (I_A). The resulting basic reproduction number is $R^d_0 = \sqrt[3]{(((\tilde{N_L}) \sim \kappa\zeta\beta)/(\kappa+\mu)\nu\rho)}$ (see Table 1 for details of the parameters). This formula implies that the epidemiological potential of excreta-mediated infection increases with larval density ($\tilde{N_L}$), survival rate to emergence ($\kappa/(\kappa+\mu)$), excretion rate (ζ), infection rate (β), duration of the adult stage (ν^{-1}), and time before excreted virions lose their infectivity (ρ^{-1}). We also reasoned in density of immature mosquitoes and concentration of infectious particles delivered by excreta, and as a result our model is scale-free and applies to any size of mosquito population, or any spatial range. Moreover, our reproduction number represents solely the lower bound of the true basic reproduction number, as the model does not account for any other transmission route—namely horizontal, from vertebrate hosts to mosquitoes, and vertical, from female mosquitoes to eggs.[36]

Feeding the model with data from our study and the literature (Table 1), we inferred the distribution of R^d_0 as a function of larval density, ranging from 0 to 400 larva per L—a density range previously observed in the field.[41] Although extremely hard to assess in nature, the proportion of excreta falling into breeding sites is a key determinant of R^d_0. In the absence of data, we selected four boundaries at 0.01, 0.1, 1, 10, and 20% for the proportion of excreta falling into a breeding site to reflect all scenarios. The median basic reproduction number for excreta-mediated transmission rapidly increased from 0 to 25 larva/L and subsequently gradually increased until 400 larvae/L for all conditions (Figure 5B). The median basic reproduction number for 400 larvae/L was 0.03, 0.07, 0.15, 0.33, and 0.42 with 0.01, 0.1, 1, 10, and 20% excreta falling into breeding sites, respectively. A reproduction number lower than 1 indicates that excreta-mediated transmission does not amplify transmission. However, by computing reported variability between breeding sites, our model showed that the 90th percentile of the reproduction number reached 1 for 9 800, 980, 101, and 51 larvae/L for 0.1, 1, 10, and 20% excreta falling into breeding sites, respectively. Accordingly, when plotting the proportion of breeding sites suitable for excreta-mediated infection, we calculated that transmission could take place in some breeding sites (Figure 5C). Indeed, sustained excreta-mediated infection occurs in 0.06, 0.45, 2.7, 10, and 14% of breeding sites containing a low density of 100 larvae/L when 0.01, 0.1, 1, 10, and 20% excreta fall into breeding sites, respectively. Altogether, by combining detailed characterization of the parameters defining excreta-mediated infection of mosquitoes and comprehensive mathematical modeling, we revealed the possibility of transmission within mosquito populations through infectious excreta.

DISCUSSION

While mosquito-vertebrate transmission (horizontal) remains the most prevalent route, repeated detection of multiple flaviviruses, including WNV, in non-blood feeding mosquito stages such as males, larvae, and pupae exposes the existence of alternative modes of transmission.[23–26,42–46] In our study, we demonstrate that transmission occurs when infected mosquitoes release excreta in breeding sites. We reported the presence of infectious WNV virions in mosquito excreta and quantified a potentially high concentration of infectious units per excreta. By defining the mosquito-related conditions for virion excretion, we observed that virion excretion occurs shortly after mosquito oral infection and remains constant for longer periods. We also found that the excreta viral load is independent of infection level, as previously observed.[28] These findings emphasize the infectiousness of excreta from infected mosquitoes. Furthermore, we reported the susceptibility of immature mosquitoes, especially pupae, to WNV infection, and demonstrated the capacity of infectious excreta to infect immature mosquitoes, uncovering an unreported mode of transmission. Finally, we modeled excreta-mediated transmission in the field and suggested its

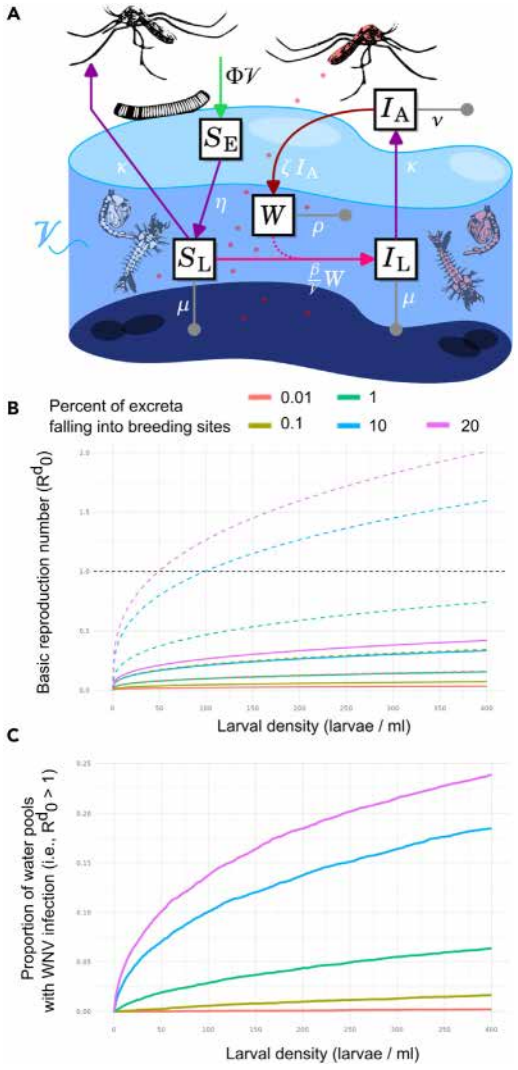

Figure 5. Mathematical modeling of excreta-mediated infection of mosquito aquatic stages

(A) Flow chart and mathematical formulation of the excreta-mediated flavivirus transmission model. V, breeding site volume. ΦV, egg laying rate. S_E, egg survival. η, egg hatching rate. S_L, immature mosquito susceptibility to infection. μm, immature mosquito mortality. βW/V, immature mosquito infection where W represents the WNV load in the breeding site (assumed well-mixed) and β the infection rate. I_L, infected immature mosquitoes. κ, adult emergence. I_A, infected adult mosquitoes. ν, adult mosquito mortality. ζ, rate of virion excretion into the breeding site. p, decay of excreted virions. Red mosquitoes indicate infection.

(B) Basic reproduction number, R^d_0, as a function of larval density and proportion of excreta falling into breeding sites. Solid curves indicate the median and dashed curve the 90th percentile.

(C) The proportion of breeding sites maintaining WNV infection ($R^d_0 > 1$) as a function of larval density and proportion of excreta falling into breeding sites. Color of solid and dashed curves indicates the different proportions of excreta falling into a breeding sites set at 0.01, 0.1, 1, 10, and 20%.

potential for maintaining WNV infection within mosquito reservoirs. Compared to horizontal and vertical transmissions, we propose to name the excreta-mediated transmission the "diagonal transmission".

Excreta-mediated transmission depends on several parameters. First, infectious virions have to be shed through excretion. The Malpighian tubules are the main excretory organs and accumulate wastes as primary urine, which is then transferred to the hindgut for excretion.[47] Flavivirus infection of the Malpighian tubules[28] can result in virion accumulation in urine and subsequent excretion. Infections of Malpighian tubules by WNV in *Culex* mosquitoes and by Zika virus in *Aedes* mosquitoes were previously reported.[48,49] Interestingly, while WNV antigens in Malpighian tubules are detected as early as 3 days, their infection rate seems to peak at 7 days post infection and then slowly decreases until 14 days post infection.[48] This pattern is coherent with the peak of excreta infection rate we observed at 8 days post infection and the subsequent decrease at 10 and 12 days post infection. Alternatively, following initial infection of the midgut, virions can be secreted into the gut lumen and channeled to the hindgut for excretion. Other authors detected a very low WNV inoculum in excreta from *Cx. annulirostris*,

Table 1. List of parameters involved in the computation of the WNV diagonal reproduction number

Parameter	notation	dimension (unit)	value	source
Larval stage duration	τ_L	duration (d)	$\tau_L \sim$ Gamma(1176, 143)	Eastwood et al.[37]
Pupal stage duration	τ_P	duration (d)	$\tau_P \sim$ Gamma(5.99, 5.21)	Eastwood et al.[37]
Emergence rate	κ	probability per unit time (d^{-1})	$\kappa = 1/(\tau_L + \tau_P)$	
Pre-imaginal survival	q_P	probability	$q_P \sim$ Unif(0.77, 0.96)	Ciota et al.[38]
Pre-imaginal mortality rate	μ	probability per unit time (d^{-1})	$\mu\ (1/q_P - 1) \cdot \kappa$	
Adult lifespan	τ_A	duration (d)	$\tau_A \sim$ Gamma(61.9, 1.88)	Moser et al.[39]
Adult mortality rate	ν	probability per unit time (d^{-1})	$\nu := 1/\tau_A$	
Excretion flow	ξ	number of excreta produced per mosquito per unit time (d^{-1})	$\xi := 62.5$	Pilotte et al.[40]
Breeding-site excretion proportion	χ	daily proportion of excreta falling in a breeding site	$\chi \in \{0.2, 0.5\}$	Estimation
Excretion viral load	υ	viral load per single excreta (PFU)	$\upsilon \sim$ LogNormal(2.62, 2.92)	Data shown in Figure 1C
Viral excretion rate	ζ	viral load per mosquito per unit time ($PFU.d^{-1}$)	$\zeta = \xi \cdot \chi \cdot \upsilon$	
Viral decay rate	ρ	probability per unit time (d^{-1})	$\rho \sim$ Gamma(1.56, 0.0206)	Data shown in Figure 3A
Infection rate	β	probability per viral concentration per unit time ($PFU^{-1}.mL.d^{-1}$)	$\beta \sim$ Gamma(0.447, 58.5)	Data shown in Figure 3C
Larval density	$\tilde{N}_L$	number per unit volume (mL^{-1})	$N_L \in [0,400]$	Amara Korba et al.[41]
Volumic demographic inflow	Φ	number of surviving eggs laid per unit volume per unit time ($mL^{-1}.d^{-1}$)	$\Phi = (\kappa + \mu) \cdot \tilde{N}_L$	Result from demographic equilibrium.

D, day(s). 1, dimensionless. Gamma distributions are parametrized by their shape (first argument) and their rate (second argument). Log Normal distributions are parametrized by the mean and the standard deviation of log-scale counterpart variable.

suspecting degradation by proteases.[29] Based on our observed sensitivity to time for excreted WNV, we posit that the previously observed low infectivity resulted from the bi-daily excreta collection.

Second, mosquitoes have to deposit excreta in breeding sites. Our mathematical modeling demonstrates that the rate of excretion in breeding sites is a determining factor. Excretion in mosquitoes occurs continuously but more frequently when the insect imbibes liquids, given the osmoregulation function of excretion.[47,50] Accordingly, mosquitoes exhibit an excretion peak shortly after blood feeding.[51] Additionally, excreta could be released during egg laying as the hindgut is compressed during the process. There are thus multiple events that could increase the probability of excreta falling into breeding sites. In our model, we selected several estimates (i.e., from 0.01 to 20%) of excreta proportion falling into breeding sites to account for the unknown, and almost impossible to measure, variability in the field. Overall, the reproduction rate resulting from excreta-mediated transmission was lower than one, even for the highest proportion, indicating that excreta-mediated transmission does not amplify viruses at the mosquito population level. However, water pools vary in their biophysical and biochemical properties and, based on this computed variability, excreta-mediated WNV infection was sustained in a fraction of the breeding sites, suggesting local maintenance of the virus.

Third, there must be immature mosquitoes in the breeding sites. Mosquito selection of breeding sites with specific characteristics[52,53] and attraction to breeding sites containing conspecific eggs[54,55] due to egg aggregation pheromone[56,57] should favor this condition. Fourth, viruses have to be stable in breeding water. WNV half-life in cell culture media is 17 h.[58] Viral stability is expected to fluctuate depending on breeding site biophysical conditions, such as pH, oxygen level, temperature, and organic matter concentration. A prior study observed that Zika viruses, another mosquito-borne flavivirus, remain infectious in sewage for as long as 192 h.[59] Lastly, immature mosquitoes have to be susceptible to infection. Although both larvae and pupae were susceptible to WNV infection, we observed higher susceptibility in pupae. Of note, similar titers in rearing water obtained from infectious excreta or virus stock derived from cell culture resulted in different infection rates for pupae. This discrepancy could indicate differential infectivity or stability between the two inoculum sources. Infection of immature mosquitoes was previously observed for Zika and dengue viruses,[59,60] and the differential susceptibility between larvae and pupae was also previously reported.[59] Infection of immature mosquitoes may occur when viruses come in contact with midgut epithelial cells. However, the larvae midgut has a protective peritrophic membrane that is absent in pupal stage,[35,61] potentially explaining the differential susceptibility between the two immature stages. Alternatively, changes in the cuticle during the pupal stage might favor virus penetration.[62] Overall, our study demonstrates that each of the conditions required for excreta-mediated transmission is fulfilled.

Excreta-mediated transmission potentially occurs in all arbovirus-mosquito systems because all the required conditions are conserved in the different arbovirus-mosquito systems. Multiple flaviviruses such as dengue, Usutu, Murray Valley viruses,[28,63,64] and alphaviruses such as Ross River virus[29] shed virions in excreta from *Aedes* and *Culex* mosquitoes, although excreta infectivity has not been tested. WNV[65] and Zika virus[59] survive in water from potential breeding sites, while all four serotypes of dengue virus remain infectious in cell media.[58] Finally,

immature mosquitoes from *Aedes* and *Culex* are susceptible to Zika,[59] dengue,[60] and Rift Valley Fever viruses.[66] Importantly, conservation of excreta-mediated transmission across different arbovirus systems implies the potential for excreta-mediated transmission to act as a transmission bridge for viruses between different mosquito vectors. Indeed, breeding sites usually contain several different mosquito species.[67,68] A shift in mosquito vectors to more anthropophilic species could promote the emergence of zoonotic arboviruses.

Understanding arbovirus transmission routes is critical to deploy efficient vector control strategies. Our results showing the possibility of an excreta-mediated (diagonal) mode of transmission further emphasize the importance of water management.

Limitations to the study

There are several limitations to our discovery of excreta-mediated transmission. First, the weak stability of the collected excreted viruses limits the accurate quantification of infectious units. Although we collected excreta at short intervals (i.e., 1 h), our analysis revealed a swift degradation of viruses, which suggests an actually higher concentration of infectious units per excreta. Second, our findings are difficult to directly translate to the field for several reasons. One can hardly imagine following mosquitoes to determine where they excrete and there is a dearth of knowledge on this aspect of mosquito behavior. Nonetheless, mathematical modeling provides some evidence that excreta-mediated transmission can occur in the field. Third, we did not evaluate whether mosquitoes infected during the aquatic stages with excreta could transmit to a vertebrate host. There is evidence that mosquitoes infected during the aquatic stages with Zika viruses transmit to a mouse model.[59] However, our study restricted its conclusion to the discovery of excreta-mediated transmission within mosquito populations.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Julien Pompon (Julien.pompon@ird.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data: All data reported in this paper will be shared by the lead contact upon request.
- Code: This paper does not report original code.
- All other requests: Any additional information required to reanalyze the data reported will be shared by the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization: R.H., Q.N., and J.P.; methodology: R.H., Q.N., and I.S.-P.; software: M.T.S.; formal analysis: R.H., Q.N., M.T.S., and J.P.; investigation: R.H., Q.N., and M.S.; resources: C.G., A.B., S.W., and D.M.; writing – original draft: R.H., M.T.S., and J.P.; writing – review and editing: all; visualization: R.H., Q.N., M.T.S., and J.P.; supervision: R.H. and J.P.; project administration: R.H. and J.P.; funding acquisition: R.H. and J.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work the authors used ChatGPT 4.0 in order to correct grammar and spelling. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Infectious clone WNV-IS98; GenBank: KR107956.1	Pasteur Institute – Paris. Bahuon et al.[69]	N/A
Critical commercial assays		
T7 RiboMAX Express Large Scale RNA Production System kit	PROMEGA	P1320
Experimental models: Cell lines		
Mosquito: C6/36 cells	ATCC	CRL-1660
Monkey: Vero cells	ATCC	CCL-81
Experimental models: Organisms/strains		
Culex quinquefasciatus, strain SLAB	Georghiou et al.[70]	SLAB
Oligonucleotides		
Primer: WNV gRNA, forward ATTCGGGAGGAGACGTGGTA	This paper	N/A
Primer: WNV gRNA, reverse CAGCCGCCAACATCAACAAA	This paper	N/A
Primer: T7-WNV forward, TAATACGACTCA CTATAGGGATTCGGGAGGAGACGTGGTA	This paper	N/A
Software and algorithms		
R version 4.4.1 (2024-06-14)	R Core Team (2024). R: A Language and Environment for Statistical Computing_. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.	
Prism 8.0.2 (GraphPad)	www.graphpad.com	

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cells, viruses, and mosquitoes

C6/36 cells (ATCC CRL-1660) derived from *Aedes albopictus* and Vero cells (ATCC-CCL-81) derived from green monkey (*Chlorocebus sabaeus*) kidney were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, France) supplemented with 10% fetal bovine serum (FBS) (Eurobio, France) and 1% penicillin-streptomycin (Gibco, France). The insect cells medium was also supplemented with 1% non-essential amino acid (Gibco, France). Mosquito cells were grown at 28°C and mammalian cells at 37°C, both in 5% CO_2.

A WNV infectious clone derived from IS98, a highly virulent strain isolated from a white stork, Ciconia ciconia (IC-WNV-IS98; GenBank accession number: KR107956.1), was received from Dr. Philippe Desprès,[69] propagated in C6/36 cells and titered with Vero cells before storage at −70°C. The same virus stock was used throughout the study.

The *Culex quinquefasciatus* strain SLAB originating from California[70] was bred in the Montpellier Vectopole Sud facility. After egg hatching, larvae were maintained in plastic trays (Gilac, France) with distilled water and fed a mixture of pelleted rabbit food (Hamiform, France) and fish TetraMin flake (Tetra, France). L1 larvae were also initially given yeast solution. Pupae were transferred to a new tank and placed in a net cage (29 × 18 × 22 cm) (Custom manufacturing) with water and sugar solution (10%) for emerging adults. Mosquitoes were maintained at 26°C–28°C, 70–80% humidity with a 12h:12h photoperiod. Mosquitoes were regularly offered rabbit blood meal to produce eggs and maintain the colony.

METHOD DETAILS

Oral infection

Adult mosquitoes aged 3 to 5-day-old were sedated at +4°C in the fridge, sorted at a density of 50 females and 5 males per box and starved for 24h. Mosquitoes were then transferred to the BSL3 insectary to acclimatize at 28°C with 80% humidity for 3 h. The Hemotek membrane feeding system (Hemotek Ltd, United Kingdom) was used for oral infection using chicken skin and an infection mixture consisting of 1,500 µL PBS-washed-rabbit blood (IRD animal facility, accreditation number H3417221), 150 µL FBS, 150 µL of 5 mM ATP (Sigma-Aldrich, France),

700 μL Roswell Parc Memorial Institute medium (RPMI) (Gibco, France) and WNV stock to obtain either 10^5 or 10^7 pfu/mL of blood. Of note, blood meal titer may not exactly correspond to the calculated titer based on the virus stock titer. Mosquitoes were allowed to feed on the blood mixture maintained at 37°C for 1h15. Fully engorged mosquitoes were then sorted and maintained in an appropriate container with *ad libitum* access to water and sugar solution (10%).

Collection of excreta

To avoid detecting viruses secreted during feeding on the WNV-blood meal, mosquitoes were transferred into new containers 2–3 days post exposure (DPE), when the blood was digested. Mosquitoes were then offered sucrose solution. Pooled or single excreta were used to assess different aspects of excreta-mediated transmission and were collected using different types of containers. Methods were inspired from previous studies.[28,29,40]

For collection of pooled excreta, female mosquitoes were grouped in 250 mL jars (Nalgene, France) at a density of 25 mosquitoes/jar at 6 DPE. Mosquitoes were offered sugar solutions (10%) containing a blue food colorant (Vahiné, France). Excreta were then collected over intervals of 1h–1h30 by adding 500 μL of DMEM containing 1% antibiotic-antimycotic (Gibco, France) and collecting the media containing the diluted excreta. Before adding media, the number of excreta was visually counted as blue dots.

For collection of single excreta, female mosquitoes were maintained in round-bottomed 14 mL polypropylene Falcon tubes (Fisher Scientific, France) that were capped with a 3D-printed cap to allow mosquito feeding on a sugar solution (10%) and safe mosquito transfer from one tube to another to collect excreta without sedating mosquitoes (Figure S1). Excreta were collected in 500 μL of DMEM containing 1% antibiotic-antimycotic (Gibco, France) on the 4^{th}, 6^{th}, 8^{th}, 10^{th} and 12^{th} DPE. On the twelfth day, mosquitoes were collected and analyzed. During excreta collection, mosquitoes were maintained in a climatic chamber at 28°C, 80% humidity and a 12h:12h photoperiod.

Infection of cells with excreta

The collection media containing pooled excreta was filtered through 0.22 μm filter (Milex-GV, Fisher Scientific, France) and 150 μL of the filtrate was combined with 350 μL of DMEM and used to inoculate T25 flasks containing 8.5×10^5 Vero cells for 1h15 at 37°C. After removing the inoculum and washing cells with DMEM to remove completely the inoculum, cells were incubated for 6 days at 37°C with 5% CO_2. Supernatant was collected, filtered (filter exclusion size 0.45 μm, Fisher Scientific, France) to clear bacteria, used for RNA extraction before RT-PCR detection and used for plaque assay.

RNA extraction

Single adult mosquitoes previously exposed to blood meal were homogenized with a plastic pestle in a 1.5 mL Eppendorf tube containing 500 μL of TRI Reagent (Euromedex, France) before RNA extraction according to manufacturer's instructions. Single larval and pupal mosquitoes previously exposed to inoculum were similarly homogenized in 500 μL of TRI Reagent before RNA extraction according to manufacturer's instructions. RNA from 150 μL of excreta solution was extracted by adding 600 μL of RAV1 lysis buffer using the NucleoSpin virus RNA kit (Macherey-Nagel, France).

WNV gRNA detection by RT-PCR

RT-PCR was performed using AccessQuick RT-PCR System (Promega, France) in a total reaction volume of 25 μL with 5 μL of RNA extracts and 400 nM of forward primer (5′-ATTCGGGAGGAGACGTGGTA-3′) and reverse primer (5′-CAGCCGCCAACATCAACAAA-3′) to amplify a 129 base pairs (bp) region in the WNV envelope region. Reactions were conducted at 42°C for 45 min, 95°C for 2 min followed by 45 cycles of 20 s at 95°C, 20 s at 58°C and 20 s at 72°C and a 2 min-final step at 72°C. PCR products were visualized on 2% agarose gel.

WNV gRNA quantification by RT-qPCR

One-step RT-qPCR was conducted using the GoTaq 1-Step RT-qPCR System kit (Promega, France) in a total reaction volume of 20 μL containing 2 μL of RNA extracts and 300 nM of the same forward and reverse primers as for gRNA detection. Amplification was conducted on an AriaMax Real-Time PCR system (Agilent, France) and consisted of an initial RT step at 42°C for 20 min, 95°C for 10 min, followed by 45 cycles of 10 s at 95°C, 15 s at 60°C and 20 s at 72°C, and a final melting curve analysis. Viral RNA was absolutely quantified by establishing a standard equation using serial dilutions of known amounts of the *in vitro* transcribed qPCR RNA target. The amplicon target was amplified from WNV cDNA using the qPCR primers with the forward primer flanked by the T7 sequence (5′-TAATACGACTCACTATAGGGATTCGGGAGGA GACGTGGTA-3′) and transcribed using the T7 RiboMAX Express Large Scale RNA Production System kit (Promega, France). RNA was purified by ethanol precipitation, quantified by a NanoDrop spectrophotometer (FisherScientific, France) and converted to the concentration of molecular copies by using the following formula: number of Viral RNA copy/μL = [(g/μL of RNA)/(transcript length in bp x 340)] x 6.02×10^{23}. Methods were inspired from previous studies.[71,72]

WNV titration

Triplicates of 1.8×10^5 Vero cells in 24-well plates were infected with 10-fold serial dilutions of 250 μL of excreta solution or cell supernatant at 37°C for 1h15. After washing, cells were overlaid with DMEM containing 2% carboxymethylcellulose (CMC, Sigma-Aldrich, France), 2% FBS and 1% of antibiotic-antimycotic (Gibco, France). Cells were incubated at 37°C with 5% CO_2 for 7 days. The overlay medium was then

aspirated, and cells were incubated for 30 min at room temperature with 3.7% formaldehyde diluted in PBS, washed twice with PBS, and incubated with crystal violet solution (3.7% formaldehyde and 0.1% crystal violet in 20% ethanol) for 1h. After two washes, plaques were counted and used to calculate PFU/ml with the following formula:

$$PFU/ml = \frac{number\ of\ plaques}{dilution\ factor * 0,25}$$

WNV stability

5×10^4 PFU/mL of WNV was incubated in water supplemented with larval food at 28°C. 200 μL of liquid was collected after 0 min, 30 min, 1h, and 2h for viral titration.

Infection of mosquito aquatic stages with virus stock

Fifteen L1 *Cx. quinquefasciatus* larvae were incubated for 1h in one Petri dish (Nunclon, FisherScientific, France) containing 2 mL of food-supplemented water and different concentrations of WNV stock. Larvae were then transferred to plastic tubes (Nalgen, France) capped with cotton and containing 3 mL of distilled water with larval nutrient solution and incubated at 28°C, 80% humidity. On day 5 post exposure, L4 larvae were collected, rinsed twice in distilled water and collected for RNA extraction.

Twenty-five pupae were incubated for 1h in a similar Petri dish containing 2 mL of food-supplemented water (to homogenize conditions with larvae) and different concentrations of WNV stock. After exposure, pupae were transferred inside a rearing cage and kept at 28°C, 80% humidity with a sugar solution (10%). RNA extraction was performed on adult mosquitoes collected three days after emergence.

Infection of mosquito aquatic stages with infectious excreta

Seven and eight pupae were separately incubated with 300 μL of pooled excreta solution in one well of a 48-well flat-bottom plate (Falcon, Fisher Scientific, France). Pupae were placed in a climatic chamber with rearing conditions. Adults were collected in 500 μL of TRI Reagent for RNA extraction three days after emergence.

Mathematical modeling of stercoraceous transmission

A mathematical model governed by an autonomous non-linear dynamical system of five ordinary differential equations (ODE) (see below) was analyzed through the next-generation theorem[73] to derive a closed-formed expression of the basic reproduction number for transmission through mosquito excreta, R^d_0 (see below). Such compartmental models based on ODEs constitute the reference approach to quantify dynamics of both host and pathogen species, i.e., in ecology and epidemiology. Specifically, their mathematical formulation allows the greatest analytical tractability, hence providing closed-form solutions of key metrics, such as the reproduction number, as a function of the model parameters. Consequently, closed-form solutions can be simplified by biologically realistic approximations and dimension reduction.

$$\frac{dS_E}{dt} = \Phi\,\nu - \eta\,S_E \qquad \text{(Equation 1)}$$

$$\frac{dS_L}{dt} = \eta\,S_E - \left(\frac{\beta}{\nu}\,W + \kappa + \mu\right) S_L \qquad \text{(Equation 2)}$$

$$\frac{dI_L}{dt} = \frac{\beta}{\nu}\,W\,S_L - (\kappa + \mu)\,I_L \qquad \text{(Equation 3)}$$

$$\frac{dI_A}{dt} = \kappa\,I_L - \nu\,I_A \qquad \text{(Equation 4)}$$

$$\frac{dW}{dt} = \zeta\,I_A - \rho\,W \qquad \text{(Equation 5)}$$

$$\text{and } R_0^d = \sqrt[3]{\frac{\tilde{N}_L \kappa \zeta \beta}{(\kappa+\mu)\nu\rho}} \qquad \text{(Equation 6)}$$

Where S_E stands for surviving eggs; S_L immature mosquito susceptibility; I_L infected immature mosquitoes; I_A infected adult mosquitoes; and W for the viral load in breeding site in PFU. The other parameters are defined in Table 1.

The distribution of the R^d_0 was calculated using the Monte-Carlo method by computing its value across a large number (10,000) of parameter sets, independently drawn (both within and between sets) from distributions fitted from data either found in the literature or generated by the current study (Table 1). Note that the volumic demographic inflow Φ is linked to the (volumic) larval density N_L, defined as the value of $(S_L + I_L)/V$ (i.e., the total number of larvae and pupae in the breeding site, whether susceptible or infected, per unit volume) and evaluated at the demographic equilibrium (i.e., by canceling out the ODE 1–3).

Modeling assumptions included the well-mixed nature of the breeding site water volume, the exponential distribution of the time-to-events (conditionally to the knowledge of their expectations), the negligibility of the WNV infection impact on both immature and mature stage survival, the non-susceptibility of the eggs and the density-dependence of mosquito demography restricted by breeding site volume [in line with empirical studies suggesting fitness reduction in overcrowded habitat[74]].

All calculations and visualisations of the modeling part were performed on R,[75] using the package fitdistrplus[76] for distribution fitting.

QUANTIFICATION AND STATISTICAL ANALYSIS

Differences in infection rate were tested with Chi-square. One-way repeated-measures ANOVA was used to test the effect of DPE on infection intensity. Statistical analyses were conducted with Prism v8.0 (GraphPad). Significance levels (*p* values) are indicated in legends of each figure, showing *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.