Supporting Information

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SI Methods

Phenotype of *w*Mel After Direct Blood Feeding on Viremic Dengue Patients.

Summary of experimental design. This experiment was designed to compare the susceptibility of WT and *w*Mel-infected *Ae. aegypti* in a local HCMC background, against oral challenge with locally circulating DENVs of each serotype. Acutely infected dengue patients were enrolled through the HTD and were directly exposed to mosquitoes on either one or two occasions (depending on their day of illness at enrollment) within the first 96 h of their illness.

Ethics statement. The enrollment of patients for provision of viremic blood meals to WT and *w*Mel-infected *Ae. aegypti* was approved by the HTD EC and OxTREC (approval nos. HTD EC CS/NĐ/14/12 and OxTREC 45–14). Informed consent was obtained from all participants by trained study stuff.

Patient cohorts. Patients were enrolled through the two specialist dengue wards for adults within HTD wards C and D. Inclusion criteria for the study were as follows: (*i*) age ≥15 and ≤96 h of symptom onset, (*ii*) clinical suspicion of dengue and a positive dengue NS1 rapid test, and (*iii*) written informed consent. Patients were excluded from the study if (*i*) they were in intensive care, (*ii*) they had an intellectual disability and in the judgment of the attending clinician could not provide fully informed consent, (*iii*) they had a history of severe hypersensitivity reactions to mosquito bites or severe dermatological condition(s), and (*iv*) they were pregnant.

After completion of enrollment, patients were exposed to mosquitoes for either 1 or 2 d (based on day of illness at enrollment), with paired venous blood samples taken to quantify viremia and ascertain the infecting serotype. Demographic and clinical information were recorded into a case report form by clinical staff, with disease severity classified based on the WHO dengue classification guidelines from 2009.

Clinical adverse events. SAEs in this study were defined as clinically significant if a patient required a clinical intervention, prolonged stay in the hospital, or admission to the intensive care unit, when possibly, probably, or definitely related to the exposure of *Ae. aegypti* mosquitoes during the course of this study. No SAEs were reported.

Generation of mosquito lines and maintenance of mosquitoes. Mosquitoes used in these direct feeding experiments were all of HCMC genetic background. WT mosquitoes were colonized in the laboratory for three generations (as described in ref. 2) and then were maintained in subsequent generations with outcrossing of 10% field collected (F0) males every second generation to maintain genetic diversity. *w*Mel mosquitoes were produced with a backcrossing regime, starting with WT males from HCMC (F0) with *w*Mel-infected females from Cairns, Australia (1). After the initial F1 cross, we performed one generation of mating in a closed population, followed by five generations of backcrossing with males from the local HCMC population, to achieve >98% genetic homogeneity. As per the WT lines, colony maintenance beyond this point involved outcrossing with 10% field (F0) males from HCMC every second generation. The infection status of both WT and *w*Mel-infected HCMC lines was tested for every generation by RT-PCR (see *Diagnostics*), using 100 fourth instar larvae. In later generations, adult females were used for confirmation of *w*Mel infection.

All mosquitoes were maintained at 28 °C, 70–80% rH, and a 12:12 h light:dark cycle, with access to sucrose solution ad libitum. Populations were maintained in duplicate cages to preserve large population sizes each generation.

Direct human–mosquito blood feeding. A sample of ∼25 WT female *Ae. aegypti* and ∼25 *w*Mel female *Ae. aegypti* (1–4 d old and not previously blood-fed) were prepared for each patient exposure. WT and *w*Mel mosquitoes were placed in opposing sides of a purpose-designed mosquito chamber, with each of the chambers secured by a sheet of mesh. Mosquitoes were transported to the hospital wards, where they were held against the patient's forearm for the 5-min exposure. If the patients were enrolled with <72 h of illness, they were exposed to mosquitoes on two occasions, on subsequent days (unless they declined the second exposure). Those enrolled between 72 and 96 h of illness had only a single exposure, because lower viremias beyond this time are less efficient for infecting mosquitoes (2). In parallel to each direct feeding exposure, a sample of venous blood was taken to quantify virus concentration and determine the infecting serotype. Patient responses to mosquito feeding were monitored for adverse events. In some cases, residual blood from this sample was used for feeding mosquitoes for experiment II, described below.

After feeding, mosquitoes were returned to the Oxford University Clinical Research Unit insectary (<250 m from the ward), cold anesthetized, and sorted. Only fully engorged mosquitoes were transferred to cups for maintenance, as per conditions described above.

Mosquito harvesting and survival. Each cohort of mosquitoes from a single blood feed were harvested either 10 and 14 d postinfection (dpi) or 12 and 16 dpi. The harvesting days for each cohort were preassigned, according to a randomly generated table with the allocations. At harvesting, blood-fed mosquitoes were cold anesthetized and had their wings and legs removed. Saliva was first collected by inserting the proboscis of these index mosquitoes into 6 μL of filtered saliva medium [a 1:1 solution 15% (vol/vol) sucrose in normal saline and heat-inactivated FCS] for 30 min. The expectorant was then transferred into a drawn microcapillary tube (tip diameter, <0.3 μm), and the expectorant was inoculated into the thorax of 5–6 susceptible (WT) *Ae. aegypti* (1–2 d old), with ∼1 μL per recipient mosquito. The abdomens of the index mosquitoes were then collected and stored individually at −80 °C. Inoculated, recipient mosquitoes were maintained for 5–7 d (as per conditions described above), with those surviving mosquitoes killed and stored at −80 °C. Together the abdomen tissues and inoculated mosquitoes were used as proxies for measuring infection and transmission of each mosquito that was exposed to the patient. All mosquitoes used in experiments were confirmed as being either *Wolbachia*-infected or -uninfected by PCR and analyzed accordingly.

Virus Susceptibility Phenotype of Field- and Laboratory-Reared Females.

Summary of experimental design. We assessed the effect of field- and laboratory-rearing conditions on the susceptibility of the resulting adult *w*Mel-infected and WT *Ae. aegypti* females to the viremic DENV-infected blood of hospitalized dengue patients via artificial membrane feeders. We did not perform direct human–mosquito feeding using F_0 mosquitoes for the small but persistent risk of vertical transmission of DENV (or other arboviruses) acquired by adult female mosquitoes in the field to their progeny and then to human participants.

Ethics statement. This study was performed under the umbrella of two equivalent studies, designed to allow the collection of blood from viremic dengue patients for the purpose of challenging mosquitoes to test susceptibility to DENV infection. Ethics approvals were granted for these studies by the HTD EC, OxTREC, and UoM HREC, as follows: study 1, HTD EC CS/NĐ/12/16,

OxTREC 30–12; study 2, HTD EC CS/NĐ/16/27, OxTREC 45–16, UoM HREC 1648095. Both prospective patient enrollment and the use of residual blood from research samples from other studies in which the patient fulfilled the current inclusion and exclusion criteria were utilized. All patients gave written informed consent for the prospective or alternative use of their specimens for such research purposes.

Patient cohorts. For the 50 patients whose blood was used for the indirect feeding experiments, each met the enrollment criteria of being NS1-positive, enrolled <96 h of illness, and >15 y of age. No patients were severely ill or pregnant. Patients were enrolled through the two specialist dengue wards at HTD (wards C and D).

Collection of mosquitoes. Mosquitoes used in experiments were all field-derived females (F_0). Collections were conducted in two field sites; these were (*i*) the WMP field site in central Vietnam, Tri Nguyen Village on Hon Mieu island, and (*ii*) the neighboring *Wolbachia*-free site, Nha Trang City.

Late instar larvae and pupae were collected on a weekly basis in Nha Trang and Tri Nguyen and transported in an insulated box to HCMC by courier, taking <18 h. The delivery was received on a Friday morning. To ensure minimal effects of laboratory conditions, only those individuals that had emerged as adults by Sunday morning (~72 h after collection and removal from the field) were used in blood feeding, over the first 2–3 d of that week. No additional food was given to the larvae after entering the laboratory; the water in which they were collected was the water from which they emerged. In both Nha Trang City and Tri Nguyen, immature mosquitoes were collected from plastic drums (150–250 L), large jars (50–100 L), and tanks (1,000–2,000 L). Frequently plastic drums and jars were the most productive containers. Most containers were shaded for part of the day and exposed to sunlight for a number of hours.

Eggs that were obtained in the field were collected from ovitraps located close to the larval breeding sites. They were collected on a weekly basis and transported alongside the larvae to HCMC. As per our laboratory's standard, controlled rearing conditions, eggs were hatched by immersion and fed a combination of shrimp and pork liver daily. Water was changed daily, and larvae developed into pupae after an average of 7 d. Emergence of these laboratory-reared mosquitoes was synchronized with the delivery and emergence of field-reared material. All rearing and maintenance of adult mosquitoes were at a constant 28 °C, 80% humidity, and 12:12 h light:dark cycle.

Indirect mosquito blood feeding. Mosquitoes from each of the four groups (as yet still based on origin and rearing condition, as opposed to *Wolbachia* infection status and rearing condition) were maintained in separate cups and simultaneously offered the blood of NS1-positive dengue patients via artificial membrane feeders. Venous blood, collected into an EDTA tube, was drawn from patients in the hospital, and the blood was transported to the insectary, where it was offered to mosquitoes within 1 h of the blood draw. Only fully engorged females were maintained for incubation. An aliquot of the blood was taken for virus quantification and serotyping.

Mosquito harvesting. Individual mosquitoes were collected at days 10 and 14 after exposure to the virus. Upon harvesting, mosquitoes were tested for DENV infection in the abdomen and head/thorax. Quantitative and qualitative measures of infection in the abdomen and head/thorax samples were made. Saliva was also collected and inoculated into naïve mosquitoes as described in the direct human blood-feeding experiment to test for virus transmission. Recipient mosquitoes from a single index mosquito were pooled before testing for DENV by RT-PCR, with a positive result indicating that the index mosquito successfully transmitted infectious virus to one or more recipient mosquitoes in their saliva. All mosquitoes were confirmed for *Wolbachia* infection status in the triplex PCR as described above, testing for DENV and *Wolbachia* infection and the *Ae. aegypti* target. For analysis, *Wolbachia* infection status was based on PCR confirmation of the presence of *Wolbachia* in the mosquito abdomen, as opposed to the origin of mosquito collection.

Diagnostics.

Human diagnostics: Serology and molecular virology. For the direct human blood-feeding experiments, classification of primary and secondary dengue in patients was based on serology results (IgM and IgG antibody capture ELISAs; Panbio) on study day 5, with IgM/IgG ratios >1.8 interpreted as primary infection and <1.2 interpreted as secondary infection. IgM/IgG ratios <1.8 but >1.2 were called indeterminate. For both the direct and indirect feeding experiments, DENV plasma viremia levels were measured by a validated, quantitative serotype-specific RT-PCR assay that has been described previously (3). In the validation process, the RT-PCR assay was calibrated against infectious virus (grown in insect cells), and the ratio between genome copies per milliliter and plaque-forming units per milliliter was 214:1 for DENV-1, 73:1 for DENV-2, 436:1 for DENV-3, and 101:1 for DENV-4.

***Mosquito diagnostics: DENV,* Wolbachia + Ae. aegypti.** Mosquitoes exposed to blood meals in both studies were individually tested for DENV infection in a triplex-PCR, designed to amplify all four DENV serotypes, a *Wolbachia*-specific target, as well as an internal *Ae. aegypti* control gene (*RPS17*) (4). Mosquitoes were homogenized with a single bead in squash buffer (containing Tris base, EDTA, NaCl, and proteinase K), heated at 56 °C for 10 min and 98 °C for 15 min, before being cooled to 15 °C. Samples were centrifuged for 2 min to pellet the debris. A 2 μL aliquot of the clarified sample was added to the PCR. PCR-positivity for viral RNA was based on a Ct value of 35 or less for all targets.

Statistical Analysis. Statistical analysis was performed in R, version 3.2.4 Revised (R Foundation for Statistical Computing).

Direct human blood-feeding experiment. The probability of successful human–mosquito transmission in abdomen and similarly in saliva was modeled based on a marginal logistic regression model, which used a working exchangeable correlation structure with mosquito strain (*w*Mel vs. WT) as the main covariate. Adjusted marginal logistic regressions were subsequently used to analyze the additional effects of virus serotype, and plasma viremia and day of harvesting on the DENV infection status. Robust (sandwich) SEs were employed to account for potential within-subject correlation due to the paired design of exposing patients simultaneously to *Ae. aegypti* ± *w*Mel and multiple exposures for the same patient and overdispersion. We performed these regressions on PCR results for DENV infection obtained from testing abdomen tissue and mosquitoes inoculated with collected saliva. Scatterplots, stratified by strain, illustrate the proportion of saliva-infected mosquitoes plotted against $\log_{10}$ plasma viremia (RNA copies per milliliter), with the overlaid smooth curves based on logistic regression for each serotype.

By employing a marginal logistic regression model with dpi and strain as the main covariates and interaction terms between dpi and strains, the predicted EIP_{50} of each strain, being the duration for 50% of mosquitoes to complete the EIP, which corresponds to the odds of infected saliva being equal to one, was derived from the marginal logistic regression equation with known variable strain and unknown variable dpi. The mean difference between the two EIP_{50}s of the two strains was computed for each serotype. CIs and corresponding *P* values for the mean difference used SE estimated based on the "delta method" (5, 6).

Field- and laboratory-reared mosquito comparisons. Probabilities for DENV infection in the abdomen, head/thorax, and saliva (proxies for midgut infection, dissemination, and transmission) were calculated, assessing the effect of immature rearing conditions within each of the WT and *w*Mel mosquito groups. We used a marginal logistic regression model, with a working exchangeable correlation structure with immature rearing conditions (laboratory

vs. field) as the main covariate. The model was later adjusted for the effect of additional factors on DENV infection: virus serotype, strain (each of the four mosquito groups, with PCR-confirmed *Wolbachia* infection status), plasma viremia, and day of harvesting. Robust (sandwich) SEs were employed to account for potential within-subject correlation due to the paired design of exposing patients simultaneously to the four mosquito groups and overdispersion. Sensitivity analysis for pairwise interactions between harvesting day, serotype, and strain were similarly performed. Scatterplots and the overlaid corresponding smooth curve of the proportion of infected mosquitoes stratified by strain with a separated panel for different serotype vs. rearing were similarly performed. To quantify and visualize effect sizes, we also calculated the percent reduction in saliva infection for each WT–*w*Mel paired cohort among field- and laboratory-reared mosquitoes and presented this as a boxplot.

1. Walker T, et al. (2011) The wMel Wolbachia strain blocks dengue and invades caged Aedes aegypti populations. *Nature* 476:450–453.
2. Nguyet MN, et al. (2013) Host and viral features of human dengue cases shape the population of infected and infectious Aedes aegypti mosquitoes. *Proc Natl Acad Sci USA* 110:9072–9077.
3. Hue KDT, et al. (2011) Validation of an internally controlled one-step real-time multiplex RT-PCR assay for the detection and quantitation of dengue virus RNA in plasma. *J Virol Methods* 177:168–173.
4. Joubert DA, et al. (2016) Establishment of a Wolbachia superinfection in Aedes aegypti mosquitoes as a potential approach for future resistance management. *PLoS Pathog* 12:e1005434.
5. Jackson CH (2011) Multi-state models for panel data: The msm package for R. *J Stat Softw* 38:1–29.
6. Oehlert GW (1992) A note on the delta method. *Am Stat* 46:27–29.

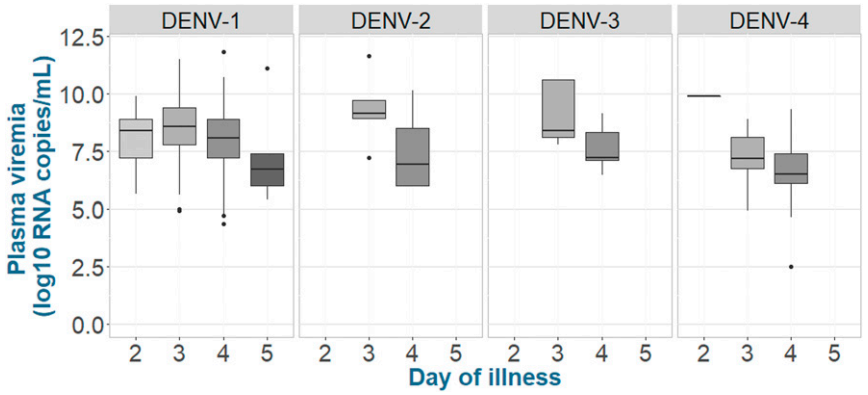

Fig. S1. Boxplots of plasma viremia levels measured for 197 PCR-positive blood meals, associated with independent direct feeding events. Plasma viremia is stratified by serotype and the day of illness in which the human–mosquito feeding took place. Fourteen samples out of the total 211 human–mosquito exposures performed were excluded: 13 feeding events (from 12 patients) had undetectable levels of plasma viremia and are thus excluded because they could not be assigned a serotype. For one feeding event, all mosquitoes died before they were harvested for testing.

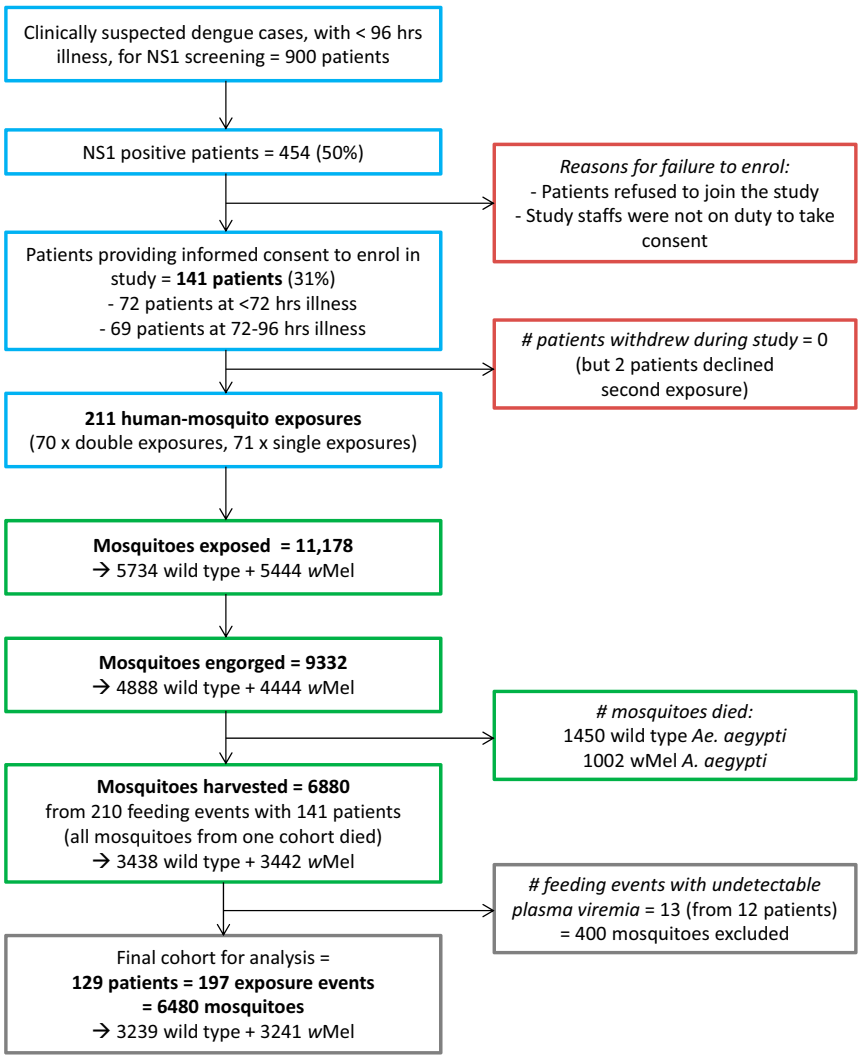

Fig. S2. Flowchart of patient enrollment, mosquito exposures, and processing for the direct human–mosquito blood-feeding experiment.

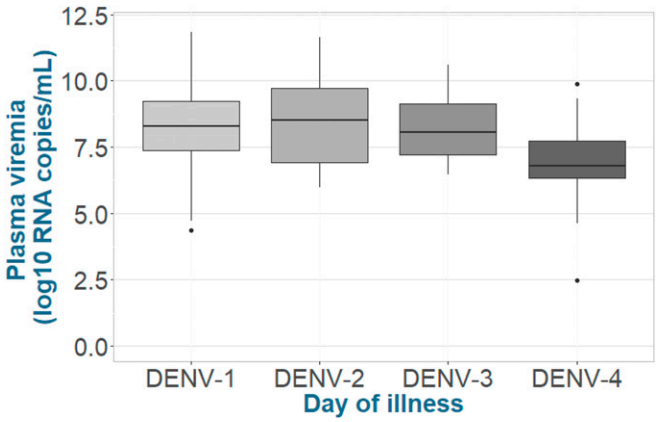

Fig. S3. Boxplot of plasma viremia for the 55 patients [with a median of 4 d of illness (interquartile range = 3–4 d) at the time of participation] enrolled in the indirect feeding study comparing field- and laboratory-rearing conditions.

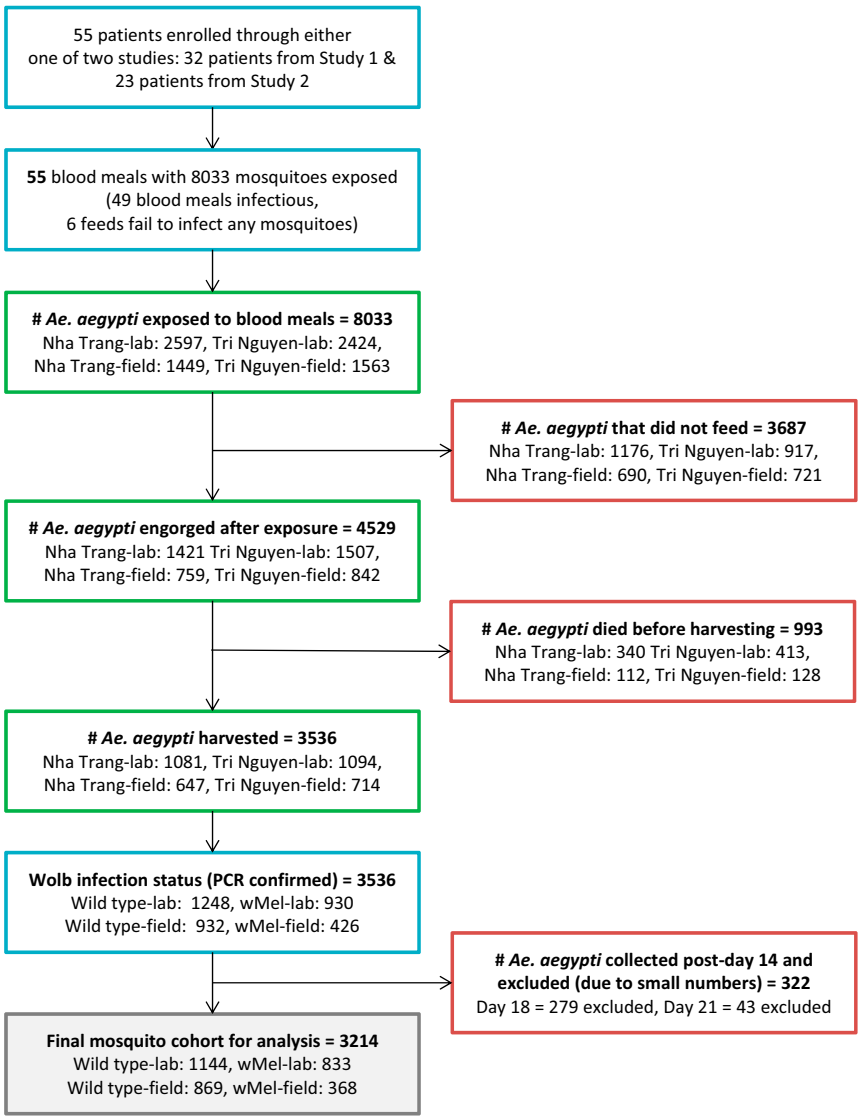

Fig. S4. Flowchart of patient enrollment, mosquito exposures, and processing for the indirect human–mosquito blood-feeding experiment, investigating laboratory- and field-rearing differences in mosquito susceptibility.

Table S1. Baseline patient data for those enrolled in the direct human–mosquito feeding study

	Undefined	DENV-1	DENV-2	DENV-3	DENV-4	Overall
Covariate	*N* = 12	*N* = 73*	*N* = 7	*N* = 6	*N* = 43	*N* = 141*
Gender, male	2 (16.6)	28 (37.4)	4 (57.1)	5 (83.3)	24 (55.8)	63 (44.7)
Age, years	23 (19–27)	24.5 (21–32)	22 (21–25)	31.5 (30–34)	26 (20–32)	25 (21–32)
Plasma viremia, $\log_{10}$ RNA copies per milliliter	<LOD	8.2 (7.7–9.2)	8.5 (7.2–9.4)	7.8 (7.5–8.2)	6.7 (6.2–7.5)	7.8 (6.5–8.7)
Infection history, serology						
Primary	1 (8.3)	20 (27.4)	0 (0)	0 (0)	1 (2.3)	22 (15.6)
Secondary	9 (75)	53 (72.6)	7 (100)	6 (100)	42 (97.7)	117 (83)
Indeterminate	2 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.4)
Classification, clinical						
Dengue	5 (41.7)	40 (54.8)	3 (42.9)	4 (66.7)	22 (51.2)	74 (52.5)
Dengue with warning signs	6 (50)	31 (42.5)	4 (57.1)	2 (33.3)	21 (48.8)	64 (45.4)
Severe dengue	1 (8.3)	1 (1.4)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.4)

Serology results were based on IgM and G antibody capture ELISAs (PanBio). For age and viral load, values are presented as median (interquartile range). All remaining values are presented as number (percent). LOD, limit of detection. LOD for DENV-1, 5 copies per reaction; DENV-2, 1 copy per reaction; DENV-3, 5 copies per reaction; DENV-4, 10 copies per reaction.
*Data from one patient were missing.

Table S2. Overview of the number of mosquitoes exposed and collected for testing DENV infection throughout the duration of the experiment

	WT		*w*Mel		Total	
Experimental stage	No.	%	No.	%	No.	%
Mosquitoes exposed	5,734		5,444		11,178	
Mosquitoes harvested (total)	3,438	60.6	3,442	62.5	6,880	61.5
Day 10	980	28.5	921	26.8	1,901	55.9
Day 12	905	26.3	912	26.5	1,817	53.4
Day 14	804	23.4	833	24.2	1,637	48.1
Day 16	749	21.8	776	22.5	1,525	44.8

Wolbachia infection status was confirmed by PCR.

Table S3. Adjusted marginal logistic regressions of the abdomen infection and transmission of virus in saliva in the direct human–mosquito feeding study, which enrolled 141 NS1-positive dengue patients

	Abdomen			Saliva		
Covariate	OR	95% CI	*P*	OR	95% CI	*P*
Log10 viremia, +1	2.815	(2.141, 3.701)	**<0.001**	1.536	(1.364, 1.729)	**<0.001**
Dpi, +1	1.044	(1.007, 1.082)	**0.021**	1.136	(1.095, 1.179)	**<0.001**
DENV-1, reference						
DENV-2	9.579	(2.812, 32.63)	**<0.001**	0.679	(0.391, 1.182)	0.171
DENV-3	0.508	(0.155, 1.666)	0.264	0.684	(0.370, 1.265)	0.226
DENV-4	0.339	(0.172, 0.669)	**0.002**	0.412	(0.283, 0.601)	**<0.001**
WT, reference						
*w*Mel-infected	0.361	(0.275, 0.473)	**<0.001**	0.376	(0.313, 0.453)	**<0.001**

Significant effects are highlighted in boldface type.

Table S4. Point estimates of the mean percentage of infection among WT and *w*Mel-infected mosquitoes, tested for infection in the abdomen, head/thorax, and saliva, under field- or laboratory-rearing conditions, along with the number of mosquitoes tested in each group

Strain	Abdomen, %	Head/thorax, %	Saliva, %	Sample size, *n*
Laboratory-reared WT	67.7	43.2	30.8	1,144
Field-reared WT	76.3	63.6	49.7	869
Laboratory-reared *w*Mel	58.0	25.2	12.1	833
Field-reared *w*Mel	52.7	31.3	9.5	368

Table S5. Baseline analysis comparing the effect of field- and laboratory-rearing conditions among WT females and *w*Mel-infected females after feeding indirectly on patient-derived blood meals

		Abdomen			Head/thorax			Saliva		
Group	Covariate	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
WT mosquitoes	Laboratory-reared (reference)									
	Field-reared	1.122	(0.999, 1.26)	0.051	1.601	(1.234, 2.076)	**<0.001**	1.678	(1.331, 2.115)	**<0.001**
*w*Mel-infected mosquitoes	Laboratory-reared (reference)									
	Field-reared	0.777	(0.621, 0.97)	**0.026**	0.868	(0.537, 1.403)	0.564	0.676	(0.387, 1.18)	0.168

Data show that the odds of developing a saliva DENV infection in WT field-reared females is underestimated by rearing equivalent mosquitoes in the laboratory. Significant effects are highlighted in boldface type.

Table S6. Adjusted marginal logistic regression testing the effect of additional covariates on the susceptibility of field- and laboratory-reared WT and *w*Mel-infected mosquitoes to DENV infection in the abdomen, head/thorax, and saliva

	Abdomen			Head/thorax			Saliva		
Covariate	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
Log_{10} viremia, +1	2.223	1.587–3.113	**<0.001**	1.436	1.128–1.829	**0.003**	1.529	1.239–1.886	**<0.001**
Dpi, +1	1.022	0.988–1.056	0.202	1.093	1.031–1.159	**0.003**	1.151	1.084–1.223	**<0.001**
Laboratory WT, reference									
Field WT	1.179	1.019–1.363	**0.027**	1.736	1.306–2.308	**<0.001**	1.896	1.459–2.463	**<0.001**
Laboratory *w*Mel	0.589	0.444–0.781	**<0.001**	0.393	0.296–0.522	**<0.001**	0.28	0.191–0.41	**<0.001**
Field *w*Mel	0.375	0.257–0.547	**<0.001**	0.367	0.23–0.586	**<0.001**	0.178	0.094–0.339	**<0.001**
DENV-1, reference									
DENV-2	1.538	0.499–4.743	0.454	0.559	0.204–1.526	0.256	0.564	0.208–1.524	0.259
DENV-4	0.31	0.119–0.809	**0.017**	0.252	0.095–0.666	**0.006**	0.311	0.142–0.683	**0.004**

Significant effects are highlighted in boldface type.