Transmission of Rift Valley fever virus from European-breed lambs to *Culex pipiens* mosquitoes

Rianka P. M. Vloet¹, Chantal B. F. Vogels², Constantianus J. M. Koenraadt², Gorben P. Pijlman³, Martin Eiden⁴, Jose L. Gonzales⁵, Lucien J. M. van Keulen¹, Paul J. Wichgers Schreur¹, Jeroen Kortekaas¹*¹

¹ Department of Virology, Wageningen Bioveterinary Research, Lelystad, the Netherlands, ² Laboratory of Entomology, Wageningen University, Wageningen, the Netherlands, ³ Laboratory of Virology, Wageningen University, Wageningen, the Netherlands, ⁴ Institute of Novel and Emerging Infectious Diseases, Friedrich-Loeffler-Institut, Greifswald—Insel Riems, Germany, ⁵ Department of Bacteriology and Epidemiology, Wageningen Bioveterinary Research, Lelystad, the Netherlands

* jeroen.kortekaas@wur.nl

Abstract

Background

Rift Valley fever virus (RVFV) is a mosquito-borne bunyavirus of the genus *Phlebovirus* that is highly pathogenic to ruminants and humans. The disease is currently confined to Africa and the Arabian Peninsula, but globalization and climate change may facilitate introductions of the virus into currently unaffected areas via infected animals or mosquitoes. The consequences of such an introduction will depend on environmental factors, the availability of susceptible ruminants and the capacity of local mosquitoes to transmit the virus. We have previously demonstrated that lambs native to the Netherlands are highly susceptible to RVFV and we here report the vector competence of *Culex (Cx.) pipiens* mosquitoes from the Netherlands. To subsequently investigate transmission of the virus under more natural conditions, mosquitoes were allowed to feed on RVFV-infected lambs during the viremic period. We found that RVFV is efficiently transmitted from lambs to mosquitoes, although transmission was restricted to peak viremia. Interestingly, in the mosquito-exposed skin samples, replication of RVFV was detected in previously unrecognized target cells.
Significance

We here report the vector competence of *Cx. pipiens* mosquitoes from the Netherlands for RVFV. Both laboratory-reared mosquitoes and well as those hatched from field-collected eggs were found to be competent vectors. Moreover, RVFV was transmitted efficiently from indigenous lambs to mosquitoes, although the duration of host infectivity was found to be shorter than previously assumed. Interestingly, analysis of mosquito-exposed skin samples revealed previously unidentified target cells of the virus. Our findings underscore the value of including natural target species in vector competence experiments.

Author summary

The consequences of first introductions of mosquito-borne viruses into previously unaffected areas depend on environmental factors, the availability of susceptible hosts and local vector populations. We have previously demonstrated that sheep breeds native to the Netherlands are highly susceptible to Rift Valley fever virus (RVFV), a mosquito-borne virus that causes severe outbreaks among domesticated ruminants and humans in Africa and the Arabian Peninsula. To gain further insight into the risk of a future RVFV introduction into the Netherlands, we have now investigated the vector competence of *Cx. pipiens*, the most abundant mosquito species in the country. Vector competence was first determined after artificial blood feeding and subsequently after feeding on viremic lambs. The results from artificial feeding experiments suggested that indigenous *Cx. pipiens* mosquitoes are competent vectors. The vector competence of *Cx. pipiens* was confirmed after feeding on viremic lambs. Transmission from lambs to mosquitoes was found to be very efficient, although largely confined to peak viremia. The localized inflammatory response resulting from mosquito bites was associated with enhanced virus replication in the skin.

Introduction

RVFV is a mosquito-borne zoonotic bunyavirus that predominantly affects domesticated and wild ruminants. Near simultaneous abortions of gestating sheep and high numbers of newborn lamb fatalities are characteristic features of RVF outbreaks. Human infections generally result in a self-resolving, acute and febrile illness, although a small percentage of infected individuals develop more severe complications including retinopathy, encephalitis or hemorrhagic fever, the latter with often fatal outcome. Since its discovery in the 1930s, the virus has spread across the African continent and invaded the Arabian Peninsula and several islands off the coast of Southeast Africa [1,2]. The worldwide distribution of mosquito species that are associated with transmission in endemic areas raises concerns that RVFV may follow in the footsteps of West Nile virus, chikungunya virus and Zika virus. RVFV has been isolated from over 30 species of mosquitoes (Diptera: *Culicidae*) belonging to 10 different genera [3]. Many of these mosquito species were found capable of transmitting the virus, at least under experimental conditions [2]. A landmark study by Linthicum and co-workers demonstrated that RVFV can be transmitted vertically to the eggs of the mosquito species *Aedes (Neomelaniconion) mcintoshi* Huang, a species that was misidentified and cited before 1985 as *Aedes lineatopennis* [4,5]. This mosquito is known as a “floodwater” mosquito, as females of this species deposit eggs near depressions that seasonally flood, known as “pans”
or “dambos” in endemic areas. The eggs need to dehydrate before they can hatch upon rehy-
dration. Eggs of such floodwater mosquitoes can survive long periods of drought, possibly con-
tributing to the persistence of RVFV during long interepidemic periods.

Upon hatching of infected mosquito eggs, the infected females may transmit the virus to
susceptible animals during blood feeding. The virus may circulate between floodwater mosqui-
toes and ruminants at low level in sylvatic cycles for many years without causing epizootics. In
those periods, human cases occur sporadically, as floodwater mosquitoes are generally zoo-
philic. After periods of above-average rainfall, mosquito populations can increase dramatically.
Various alternative species of mosquitoes may then contribute to transmission of the virus,
including anthropophilic mosquito species that may introduce the virus into the human popu-
lation. When such mosquito species become abundant, large outbreaks among humans may
follow [1,2].

One of the largest “virgin soil” epidemics of RVF occurred in Egypt in 1977–78 [6]. This
outbreak followed the completion of the Aswan High Dam across the Nile river, which
resulted in new permanent fresh water breeding sites for mosquitoes. During this outbreak,
millions of animals and an estimated 200,000 humans became infected with the virus. Soon
after the Egyptian outbreak, two studies reported that almost all mosquitoes collected from the
affected areas of the Nile delta belonged to the *Cx. pipiens* complex [7,8]. These mosquitoes
were subsequently shown to transmit the virus to susceptible hamsters, thereby confirming
that *Cx. pipiens* is a competent vector of RVFV, at least in these regions [7,8]. Further studies
have demonstrated that *Cx. pipiens* mosquitoes from other areas, including the US and Europe,
are capable of transmitting RVFV, although significant differences in competence may exist
between vectors collected from different areas due to (epi)genetic and environmental factors
[9–16]. Consequently, to assess the risk of RVFV outbreaks in currently unaffected areas,
insight into the vector competence of local vector populations is crucial.

*Cx. pipiens* is the most abundant and widespread mosquito species in several European
countries, including the Netherlands [17,18]. To assess the risk of a future RVFV outbreak in
the Netherlands, our laboratory has previously evaluated the susceptibility of indigenous sheep
breeds [19–21]. We have now continued our risk-assessment with studies on the vector com-
petence of local *Cx. pipiens* mosquitoes. To facilitate our initial experiments, we made use of
the attenuated Clone 13 strain, which can be handled safely in biosafety level-2 (BSL-2) labora-
tories and was previously used successfully for RVFV vector competence studies [10,12]. After
initial experiments with Clone 13 and laboratory-reared mosquitoes, the vector competence of
indigenous *Cx. pipiens* mosquitoes was confirmed by experiments with wild-type RVFV and
mosquitoes hatched from field-collected eggs. Finally, the efficiency of transmission from vire-
mic lambs to mosquitoes was investigated. Our results show that *Cx. pipiens* mosquitoes of the
Netherlands are competent vectors of RVFV and that the virus is efficiently transmitted from
indigenous lambs to mosquitoes.

**Materials and methods**

**Virus and cells**

Culture media and supplements were obtained from Gibco unless indicated otherwise. C6/36 (*Aedes albopictus*) cells (ATCC CRL-1660) were cultured at 28˚C in absence of CO₂ in L15 medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Bodinco), 2% Tryptose Phosphate Broth (TPB), and 1% MEM nonessential amino acids solution (MEMneaa). Vero cells (ATCC CRL-1586) were cultured in DMEM GlutaMAX supplemented with 3% FBS, 1% Pen/Strep, and 1% Fungizone (DME**+) at 37˚C and 5% CO₂.
The Clone 13 virus was generously provided by Institut Pasteur. Clone 13 is a plaque-purified clone, derived from strain 74HB59, which was isolated from a human case in the Central African Republic. Clone 13 contains a 69% deletion in the gene encoding the non-structural NSs protein, which was shown to counteract host innate immune responses via several mechanisms and is thereby considered the major virulence determinant [22–27]. Indeed, viruses that lack NSs expression, such as Clone 13, are highly attenuated in mice [27], sheep [28], and cattle [29].

Wild-type recombinant strain 35/74 is derived from strain 35/74 that was isolated from the liver of a sheep that died during an RVFV outbreak in the Free State province of South Africa in 1974. The original virus was passaged in suckling mouse brain four times and subsequently four times in BHK cells. The full genome sequence of this virus was used to synthesize cDNA. Full genome sequences of this virus were deposited in GenBank under the accession numbers JF784386, JF784387, and JF784388. The virus was rescued using BSR-T7 cells and passaged once in BHK cells [30]. Recombinant 35/74 virus is highly virulent for sheep, as demonstrated in our previous studies [19–21].

Clone 13 and strain 35/74 were amplified in C6/36 cells, starting with a multiplicity of infection of 0.005. Culture medium was harvested after 4 days and cleared by slow-speed centrifugation. Virus titers were determined by end point dilution assay as 50% tissue culture infective dose per ml (TCID$_{50}$/ml). Briefly, Vero cells were seeded in 96 well plates at an approximate density of 20,000 cells/well in 100 μl DMEM. The next day, 10-fold dilutions of culture media containing virus were added to each well (50μl/well). After 4–5 days incubation, the wells were scored for cytopathic effect. Titers were determined as TCID$_{50}$/ml using the Spearman-Kärber algorithm [31,32].

For blood feeding experiments, blood was collected from cattle of Wageningen Bioveterinary Research (WBVR, Lelystad, the Netherlands). Erythrocytes were collected from freshly collected EDTA blood by slow-speed centrifugation (650 xg) and washed three times with PBS. Washed erythrocytes were subsequently resuspended in L15 complete medium (L15 + 10% FBS, 2% TPB, 1% MEMneaa) to a concentration that is four times higher than found in blood. To prepare a blood meal, one part of the erythrocyte suspension was mixed with two parts of culture medium containing virus.

**General procedures of artificial mosquito feeding, forced salivation and virus isolation**

Laboratory-reared *Cx. pipiens* mosquitoes were provided by the Laboratory of Entomology of Wageningen University. These mosquitoes were previously shown to be competent vectors of West Nile virus and Usutu virus [33,34]. The mosquitoes were reared from an above-ground collected pool of egg rafts originating from Brummen, the Netherlands. The colony was established in 2010 and maintained at 23˚C in BugDorm cages with a 16:8 h light-dark cycle and 60% humidity. The mosquitoes were provided with 6% glucose for general maintenance and with bovine (Carus, Wageningen, the Netherlands) or chicken (Kemperkip, Uden, the Netherlands) blood for egg production using a Hemotek PS5 feeder (Discovery Workshops). Egg rafts were allowed to hatch in tap water supplemented with Liquifry No. 1 (Interpet Ltd.). Larvae were fed with a mixture (1:1:1) of bovine liver powder, ground koi carp food, and ground rabbit food. In addition to this mosquito colony, we made use of *Cx. pipiens* mosquitoes hatched from eggs collected in Best, the Netherlands, and subsequently treated similarly as the colonized mosquitoes.

Prior to artificial feeding experiments, mosquitoes were transported to Wageningen Bioveterinary Research (WBVR) and allowed to acclimatize for 3 days in an insect incubator
(KBWF 240, Binder) at 23°C at a humidity of 70% and a 16:8 h light:dark cycle. Feeding was performed in white buckets (1L) covered with mosquito netting, using a Hemotek PS5 feeder [33]. Of note, feeding with Clone 13 was performed inside the insect incubator, whereas feeding with virulent wild-type strain 35/74 was performed in a class-III biosafety cabinet (glovebox). After feeding on blood meals containing different amounts of virus as described for individual experiments in more detail below, fully engorged mosquitoes were collected with an automatic insect aspirator and maintained with sugar water (6% sucrose solution), provided in flasks with filter paper. After the required incubation periods, mosquitoes were sedated on a semi-permeable CO\textsubscript{2} pad connected to 100% CO\textsubscript{2}. Prior to the salivation assay, wings and legs were removed. Saliva was collected by forced salivation using 20 μl filter tips containing 7 μl of a 1:1 mixture of FBS and 50% sucrose (capillary tube method). After 1–1.5h, saliva samples were collected and incubated with Vero cell monolayers. After 3 h incubation at 37°C, the inocula were replaced by fresh medium. Cytopathic effect (CPE) was scored 3–5 days later.

Mosquito bodies were initially stored at -80°C. For analysis, bodies were thawed and homogenized in 500 μl DMEM+ with a pellet pestle (Sigma) and the homogenate was subsequently cleared by slow speed centrifugation. Part of the material (70μl) was used for virus isolation using the virus titration protocol described above. Another proportion of the material (200μl) was used for RVFV specific quantitative reverse-transcriptase PCR (qRT-PCR) as described previously [35].

The percentage of blood fed mosquitoes that contained virus after the incubation period as demonstrated by virus isolation from the mosquito body was defined as the infection rate. The transmission rate was defined as the percentage of blood fed mosquitoes that contained virus in their saliva after the incubation period.

**Influence of virus dose on infection and transmission rates**

In Experiment 1, laboratory-reared mosquitoes were fed with bovine erythrocyte suspensions, prepared as described above, spiked with 10\textsuperscript{5.3} (low dose), 10\textsuperscript{7.3} (medium dose) or 10\textsuperscript{9.3} (high dose) TCID\textsubscript{50}/ml of freshly prepared Clone 13 virus. Virus titers were confirmed retrospectively. Mosquitoes were subsequently cleared by slow speed centrifugation. Part of the material (70μl) was used for virus isolation using the virus titration protocol described above. Another proportion of the material (200μl) was used for RVFV specific quantitative reverse-transcriptase PCR (qRT-PCR) as described previously [35].

To investigate the vector competence of mosquitoes hatched from field-collected eggs, mosquitoes were fed with bovine erythrocyte suspensions containing a dose of 10\textsuperscript{9.3} TCID\textsubscript{50}/ml of Clone 13 (Experiment 2) or with blood meals containing 10\textsuperscript{8.0} TCID\textsubscript{50}/ml of Clone 13 or virulent strain 35/74 (Experiment 3). To enable the use of similar titers for the comparison of Clone 13 and strain 35/74, virus batches were prepared, titrated, set to equal titers and stored at -80°C until use. Mosquitoes were maintained for 14 days at 28°C, after which the infection and transmission rates were determined.

**Feeding of mosquitoes on viremic lambs**

To determine the infectious period of lambs for *Culex pipiens* mosquitoes (Experiment 4), two 12-week-old Dutch lambs (Texel/Swifter) were inoculated, under BSL-3 conditions, with 10\textsuperscript{5.0} TCID\textsubscript{50}/ml of the highly virulent RVFV strain 35/74 via intravenous route. EDTA blood samples, to be used for RVFV qRT-PCR and virus isolation, were collected every day after challenge. On days, 1, 2, 3, and 4 post inoculation, cardboard cups containing 50 female, laboratory-reared *Culex pipiens* mosquitoes were placed on the inner thigh of a hind leg of the sheep. A single feeding site was used on each animal. The wool was removed from this part of
the body using Veet hair removal cream, and the cup was fixed with elastic bandage. After 45 min the cups with mosquitoes were removed and brought to the BSL-3 laboratory. The mosquitoes were maintained for 7 days at 20°C and subsequently transferred to 28°C. Bodies and saliva samples were collected after 5–7 days at 28°C and analysed for the presence of virus by qRT-PCR and virus isolation as described above.

**Histopathology and immunohistochemistry**

After death or euthanasia of the two RVFV infected lambs, tissue samples obtained from the liver, spleen, adrenal gland and hepatic lymph node were collected for histopathological examination. In addition, the skin was sampled both from the site affected by the mosquito bites and from the unbiten skin of the opposite leg. Tissue samples were fixed for 48 h in 10% phosphate buffered formalin and processed routinely into paraffin blocks. Sections were cut on silane-coated glass slides and dried for at least 48 h in a 37°C incubator. Sections were stained routinely with haematoxylin and eosin (H&E) or immunostained for RVFV antigen. Briefly, endogenous peroxidase was blocked for 30 min in methanol/H₂O₂ followed by enzymatic digestion in 0.1% trypsin (Difco Laboratories) to retrieve relevant epitopes. As primary antibody, monoclonal antibody (mAb) 9 was used, which recognizes the nucleocapsid protein [36]. Mouse Envision peroxidase and DAB+ chromogen (Dakopatts, Denmark) were used as substrate, according to the manufacturer’s instructions.

**Ethics statement**

All animal experiments were conducted in accordance with the Dutch Law on Animal Experiments (Wet op de Dierproeven, ID number BWBR0003081) and were approved by the Animal Ethics Committee of Wageningen Bioveterinary Research (WBVR), in accordance with the regulations of EU directive 2010/63/EU and the Experiments on Animals Act, 1997. To minimize suffering of the animals from the RVFV infection, lambs were euthanized when they reached a predefined humane endpoint.

**Statistical analyses**

Statistical significance of the effect of the dose of ingested virus on virus titers in mosquito bodies after the incubation period was calculated using the Mann-Whitney test and differences between infection and transmission rates were calculated using Fisher’s exact test. The differences in virus titers in the bodies of mosquitoes after feeding on viremic lambs and the corresponding transmission rates were also calculated with the Mann-Whitney test and Fisher’s exact test, respectively. The threshold for significance was adjusted by applying the Bonferroni correction \( p < 0.05/n \), where \( n \) was the number of between group comparisons. These analyses were performed using GraphPad 6.

**Results**

**Influence of virus dose on infection and transmission rates**

To determine if *Cx. pipiens* mosquitoes native to the Netherlands are susceptible to RVFV infection and to study the effect of virus dose (Table 1, Experiment 1), mosquitoes were fed with bovine erythrocyte suspensions spiked with different doses of Clone 13. Virus titers in the bodies that were found positive after the incubation period are depicted in Fig 1A. The infection and transmission rates are depicted in Fig 1B. Mosquitoes from the low-dose group revealed an infection rate of 30% and a transmission rate of 8%, whereas those from the medium-dose group revealed an infection rate of 64% and a transmission rate of 14%. The
Table 1. Infection and dissemination rates in *Cx. pipiens* mosquitoes after oral exposure to Clone 13 or wild-type RVFV strain 35/74a.

| Exp. no: | Mosquito origin: | Feeding method: | Day of feeding on lambs: | Virus: | Titera: | Incubation temp (˚C)b: | Time (DPF)c: | N: | IR (%): | TR (%): |
|----------|------------------|-----------------|--------------------------|--------|---------|------------------------|-------------|-----|---------|---------|
| 1        | Laboratory reared| Hemotek         | N.A.                     | Clone 13 | 5.3     | 28                      | 14          | 40 | 30      | 8       |
|          |                  |                 |                          |         | 7.3     | 14                      | 44          | 64 | 14      |         |
|          |                  |                 |                          |         | 9.3     | 14                      | 34          | 74 | 24      |         |
| 2        | Field collected  | Hemotek         | N.A.                     | Clone 13 | 9.3     | 28                      | 14          | 40 | 60      | 18      |
| 3        | Field collected  | Hemotek         | N.A.                     | Clone 13 | 8.0     | 28                      | 14          | 45 | 24      | 2       |
|          |                  |                 |                          | 35/74   |         |                         | 14          | 20 | 60      | 25      |
| 4        | Laboratory reared| Lamb 1          | 35/74                    |         | 3.0     | 20˚C+28˚C                   | 7+7         | 14 | 0       | 0       |
|          |                  |                 |                          |         | 6.4     | 7+6                      | 23          | 91 | 30      |         |
|          |                  |                 |                          |         | 5.7     | 7+6                      | 11          | 18 | 0       |         |
|          |                  |                 |                          |         | 4.5     | 7+5                      | 11          | 18 | 0       |         |
|          |                  | Lamb 2          | 2.5                      |         |         |                         | 7+7         | 4  | 0       | 0       |
|          |                  |                 | 5.2                      |         |         |                         | 7+6         | 7  | 86      | 29      |
|          |                  |                 | 4.2                      |         |         |                         | 7+6         | 9  | 11      | 11      |

The most important variables in each experiment are shaded.

aTiters are depicted as $10^{\text{log TCID}_{50}/\text{ml}}$.
bMosquitoes were either maintained at one temperature or first at 20˚C and subsequently at 28˚C.
cWhen mosquitoes were incubated at two temperatures, the first number represents days incubated at 20˚C and the second number the days incubated at 28˚C.

DPF, days post feeding; N, Number of assayed mosquitoes; IR, infection rate; TR, transmission rate; N.A., Not Applicable.

https://doi.org/10.1371/journal.pntd.0006145.t001

**Fig 1. Influence of virus dose on infection and transmission rates.** Mosquitoes were allowed to feed on a suspension of bovine erythrocytes containing a low dose (LD, $10^{5.3}$ TCID$_{50}$/ml), medium dose (MD, $10^{7.3}$ TCID$_{50}$/ml) or high dose (HD, $10^{9.3}$ TCID$_{50}$/ml) of RVFV Clone 13 and were maintained for 21 days at 28˚C. (A) Symbols represent virus titers in the bodies of the mosquitoes that were found virus positive after the incubation period. Means with SDs (error bars) and the detection limit of the virus isolation assay (dashed line) are indicated. (B) Infection and transmission rates. Asterisks indicate statistically significant differences ($P<0.017$) as determined using the Mann-Whitney test (panel A) or Fisher’s exact test (panel B).

https://doi.org/10.1371/journal.pntd.0006145.g001
infection and transmission rates in the high dose group were 74% and 24%, respectively (Fig 1B and Table 1). These results indicate that Cx. pipiens mosquitoes from the Netherlands are competent to transmit RVFV and that infection rates increase with the dose of ingested virus. The effects of virus dose on transmission rates were not statistically significant, probably due to the low number of mosquitoes with infectious virus in their saliva.

Vector competence of Cx. pipiens hatched from field-collected eggs

To confirm the vector competence of Dutch Cx. pipiens mosquitoes for RVFV, mosquitoes hatched from field-collected eggs were fed with a blood meal containing $10^{9.3}$ TCID$_{50}$/ml of Clone 13. The virus titers in bodies and infection and transmission rates are depicted in Fig 2. Although the experiments were not performed at the same time, feeding with a dose of $10^{9.3}$ TCID$_{50}$/ml of Clone 13 in Experiment 1 resulted in an infection rate of 74% after incubation at 28˚C for 14 days, whereas feeding the same dose to mosquitoes hatched from field-collected eggs in the present experiment resulted in an infection rate of 60%. Transmission rates were also somewhat lower in the latter: 18% versus 24% (Table 1).

In Experiment 3, Cx. pipiens mosquitoes hatched from field-collected eggs were fed with a blood meal containing $10^{8.0}$ TCID$_{50}$/ml of Clone 13 or virulent strain 35/74. After incubation for 14 days at 28˚C, the titers in virus-positive bodies were found to be comparable between mosquitoes fed with Clone 13 and strain 35/74 (Fig 3A), whereas both infection and transmission rates were significantly higher in mosquitoes fed with strain 35/74 (Table 1 and Fig 3B). These results confirm that Cx. pipiens mosquitoes hatched from eggs collected in the Netherlands are competent vectors of wild-type RVFV.

Transmission of RVFV from viremic lambs to Cx. pipiens mosquitoes

To determine if Dutch Cx. pipiens mosquitoes become infected after feeding on viremic lambs, two lambs were inoculated intravenously with $10^5$ TCID$_{50}$/ml RVFV strain 35/74. Every
following day, groups of 50 female mosquitoes were allowed to obtain a blood meal. The procedure is visualized in Fig 4A–4C.

Both lambs developed fever (Fig 4D) and high viremia on the second day post infection (DPI 2) as determined by qRT-PCR and virus isolation (Fig 4E). One lamb succumbed to the infection on DPI 3, whereas the other was euthanized on DPI 4 after reaching a humane endpoint. This lamb was euthanized by exsanguination, after being anesthetized with 50 mg/kg sodium pentobarbital (EuthasolH, ASTfarma BV, The Netherlands) applied via the intravenous route. Post-mortem analysis revealed massive hepatic necrosis in both lambs, which is characteristic of a fatal outcome of RVFV infection.

Some engorged mosquitoes were found dead upon arrival in the BSL-3 laboratory. To maintain adequate group sizes, we decided to maintain the mosquitoes at 20˚C instead of 28˚C during the first 7 days. To stimulate virus dissemination, the mosquitoes were subsequently placed at 28˚C. Although it was our intention to maintain the mosquitoes for 7 days at 28˚C, we decided to sample some of the groups earlier to maintain adequate sample sizes (Table 1).

Surprisingly, the infection rates were very high (86–91%) in the groups of mosquitoes fed on DPI 2, whereas relatively low infection rates (11–18%) were detected in the groups of mosquitoes fed on DPI 3 (Fig 5A and 5B). This was particularly surprising as viral RNA levels in the blood were equally high, or even higher on DPI 3 (Fig 4E). However, virus isolation demonstrated that infectious virus titers in the blood were higher on DPI 2 than on DPI 3 (Fig 4E). Apparently, non-infectious virus or viral RNA rapidly accumulated in the blood of the lambs between DPI 2 and 3, which can be visualized by the ratios of viral RNA levels and infectious virus titers (Fig 4F). Importantly, the lower infection rates (Fig 5A and 5B) correlated well with transmission rates, being 29–30% in mosquitoes that fed on DPI 2 and 0–11% in mosquitoes fed on DPI 3 (Fig 5C and 5D). These results suggest that efficient transmission from viremic lambs to mosquitoes is largely limited to peak viremia.
Increased virus replication at mosquito feeding sites

Samples from unexposed skin (Fig 6A) and mosquito-exposed skin (Fig 6B) were examined by H&E staining and immunohistochemistry (IHC). H&E staining revealed extensive haemorrhages in the superficial and deep dermis at the site of mosquito bites (Fig 6B). Blood vessels were severely dilated centrally filled with erythrocytes while neutrophils and thrombocytes showed margination at the periphery of the blood vessels (Fig 6C). In the dermis, an increased influx of both neutrophils and macrophages was noticed compared to the unbitten skin of the opposite leg (Fig 6D). The epidermis showed hydropic degeneration of keratinocytes, acantholysis and cleft formation. Exocytosis of neutrophils was observed with crust formation on the epidermis (Fig 6B).
Immunostaining was performed with mAb 9 specific for the N protein (Fig 7). Analysis of mosquito-exposed skin revealed heavy staining of RVFV antigen in endothelial cells of dermal blood vessels, smooth muscle cells, lipocytes, keratinocytes and fibroblasts (Fig 7). Strikingly, RVFV antigen was associated with the margination of thrombocytes observed in the blood vessels (Fig 7C). RVFV antigen was detected in the cytoplasm of infiltrating macrophages but not in neutrophils (Fig 7A and 7C). In the epidermis, localized areas of positively stained keratinocytes were observed located in the stratum basale, stratum spinosum and stratum granulosum (Fig 7G). Importantly, no RVFV antigen was observed in skin samples obtained from the other leg of the same animal or in control sections of the skin of an uninfected sheep (Fig 7A).

Discussion

To gain insight into the consequences of a potential future introduction of RVFV into the Netherlands, our laboratory previously investigated the susceptibility of indigenous sheep breeds. These studies demonstrated that local sheep breeds are highly susceptible to RVFV, resulting in mortality rates varying from 20% to 70% [19–21]. Here, we show that Cx. pipiens mosquitoes, the most abundant and widespread mosquito species in the Netherlands and elsewhere in Europe, are competent vectors of RVFV, as was previously demonstrated for Cx. pipiens mosquitoes from other areas [8–10,13,37–39]. Interestingly, results obtained from a direct comparison between Clone 13 and wild-type RVFV are in line with earlier indications that NSs contributes to replication in mosquitoes [13,14,40].
To evaluate whether the mosquitoes would be competent vectors under more natural conditions, *Cx. pipiens* mosquitoes were allowed to feed on viremic lambs during different stages of viremia. This resulted in high infection rates of 86–91% and transmission rates of 29–30%. As expected, most mosquitoes were infected after feeding during peak viremia, occurring on day 2 after infection of the lambs. It was, however, surprising to find that almost no transmission took place from viremic lambs to mosquitoes during the following days. Although viral RNA levels were comparable the day after peak viremia, virus isolation demonstrated that the levels of infectious virus declined between days 2 and 3 and further declined the following day. This finding was correlated with a rapid rise of the RNA:TCID$_{50}$ ratio (Fig 4F) and may be explained by the rapid accumulation of defective particles or release of viral RNA from dying cells. Importantly, this finding makes clear that experiments in which only viral RNA levels are measured should be interpreted with caution. Even if we disregard viral RNA levels and only take infectious virus into account, an interesting observation can be made. Feeding on day 2 on a lamb with viremia of $10^{5.2}$ TCID$_{50}$/ml (lamb 2) resulted in an infection rate of 86%, whereas feeding on day 3 on the other lamb with comparably high viremia ($10^{5.7}$ TCID$_{50}$/ml) resulted in an infection rate of only 18%. Although based on limited data, this observation suggests that some factor in the blood interfered with infection of the mosquitoes on day 3. It is relevant to note that the survival rates of the mosquitoes that had fed on day 3 on both lambs were strikingly lower than those of mosquitoes that had fed on day 2. Specifically, whereas 79% and 78% of the mosquitoes that had fed on day 2 on lamb 1 and 2, respectively, survived...
Transmission of Rift Valley fever virus from lambs to *Culex pipiens* mosquitoes
until the moment of analysis, only 41% and 50% of the mosquitoes that had fed on day 3 on lambs 1 and 2, respectively, survived until the moment of analysis. The cause of these declines in survival rates is unclear, but may be correlated with the rapidly declining transmission rates.

The infectious threshold for RVFV transmission to mosquitoes was previously proposed to be $10^{4.5}$ plaque-forming units/ml, corresponding to $10^{4.6}$ TCID$_{50}$/ml [41]. Based on the levels and duration of viremia in ruminants, transmission of RVFV from these animals to mosquitoes was predicted to occur within a time period of 4 days [41]. Although this theoretical assumption was plausible, our data suggests that a much narrower window of opportunity exists for the virus to infect mosquitoes. However, it is also likely that infectious thresholds differ among mosquito species or even biotypes of *Culex pipiens*, as, for example, transmission rates for West Nile virus differed greatly among biotypes of *Cx. pipiens* from The Netherlands [34]. Moreover, the vector competence of *Ae. vexans* for RVFV was found to vary greatly depending on the area from which the mosquitoes were collected [11]. Clearly, more research is needed to determine the duration of infectivity of different hosts for specific mosquito species, preferably taking into account environmental and (epi) genetic factors.

Another unexpected observation was that one lamb suddenly succumbed to the infection and that the other lamb had to be euthanized when a humane end point was reached. This high disease burden warranted a thorough post mortem examination of the two lambs. Gross examination revealed severe liver necrosis, which is usually observed in lambs that succumb to RVFV infection, but no other aberrant pathological findings. Examination of the mosquito feeding sites on the inner thighs of the lambs, however, yielded more remarkable results. Apart from extensive haemorrhages in the dermis and an influx of neutrophils and macrophages, IHC staining revealed extensive replication of RVFV in keratinocytes, the endothelium of dermal blood vessels, smooth muscle cells, fat cells and fibroblasts. Macrophages in the dermis were also strongly positive for RVFV antigen, which may have resulted from phagocytosis of virus particles or from replication in these cells. Importantly, similar samples obtained from the other leg of the same animal revealed no signs of RVFV infection, suggesting that this enhanced, localized replication was mediated by the inflammatory response resulting from the mosquito bites. Another striking observation was the margination of RVFV antigen in the blood vessels together with thrombocytes. This may be explained by an interaction of the virus with blood platelets that responded to vascular damage or inflammation. Since the marginalized thrombocyte aggregates did not appear to contain fibrin filaments, some component in the mosquito saliva may have interfered with the normal coagulation cascade.

It is relevant to note that the host inflammatory response to mosquito bites was previously shown to enhance the severity of Semliki Forest virus and Bunyamwera virus infection [42]. Furthermore, saliva from *Aedes* mosquitoes was previously shown to modulate RVFV pathogenicity for mice. Interestingly, this was not observed when using salivary gland extracts from *Cx. pipiens* [43]. The latter may be a first clue that the influence of mosquito saliva on arbovirus infection varies among virus-host-mosquito combinations.

In conclusion, a future introduction of RVFV into the Netherlands or elsewhere in Europe could result in significant spreading of the virus as Europe, and particularly the Netherlands, is
home to high densities of target animals and mosquitoes that have now been shown susceptible to the virus. However, before a proper risk-assessment can be made, additional research is needed on the vector competence of other relevant mosquito species, particularly those of the genus *Aedes*, which can also be abundant certain times of the year and in specific areas [44]. In addition, research on the influence of mosquito bites on the outcome of RVFV infections is warranted not only to gain more fundamental insight into RVF pathogenesis and epidemiology, but also to address the ability of vaccines to protect animals from natural exposure to the virus.

**Acknowledgments**

We thank the animal care takers of WBVR, Bregtje Smid and Jet Kant for technical support and Rob Moormann for useful discussions. We thank the rearing staff of the Laboratory of Entomology (Wageningen University, the Netherlands) for providing the *Cx. pipiens* mosquitoes used in our experiments. We thank Jan Lundström, Jenny Hesson (Uppsala Universitet, Sweden), Marian Dik, Adolfo Ibáñez-Justicia, Ernst Jan Scholte, Wietse Den Hartog and Arjan Stroo (Centre for Monitoring Vectors, Wageningen, the Netherlands) and Ilse Scheperskeuter for useful discussions and their assistance in the pilot experiments preceding the present study. We thank Martin Groschup of the Friedrich-Loeffler-Institut for providing mAb 9.

**Author Contributions**

**Conceptualization:** Chantal B. F. Vogels, Constantianus J. M. Koenraadt, Gorben P. Pijlman, Paul J. Wichgers Schreur, Jeroen Kortekaas.

**Formal analysis:** Paul J. Wichgers Schreur, Jeroen Kortekaas.

**Funding acquisition:** Jeroen Kortekaas.

**Investigation:** Rianka P. M. Vloet, Lucien J. M. van Keulen, Paul J. Wichgers Schreur, Jeroen Kortekaas.

**Methodology:** Chantal B. F. Vogels, Constantianus J. M. Koenraadt, Lucien J. M. van Keulen, Paul J. Wichgers Schreur, Jeroen Kortekaas.

**Project administration:** Jeroen Kortekaas.

**Resources:** Chantal B. F. Vogels, Martin Eiden, Jeroen Kortekaas.

**Supervision:** Jeroen Kortekaas.

**Validation:** Jose L. Gonzales.

**Writing – original draft:** Rianka P. M. Vloet, Jeroen Kortekaas.

**Writing – review & editing:** Chantal B. F. Vogels, Constantianus J. M. Koenraadt, Gorben P. Pijlman, Paul J. Wichgers Schreur, Jeroen Kortekaas.

**References**

1. Bird BH, Ksiazek TG, Nichol ST, Maclachlan NJ (2009) Rift Valley fever virus. J Am Vet Med Assoc 234: 883–893. https://doi.org/10.2460/javma.234.7.883 PMID: 19335238

2. Pepin M, Bouloy M, Bird BH, Kemp A, Paweska J (2010) Rift Valley fever virus (*Bunyaviridae: Phlebovirus*): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention. Vet Res 41: 61. https://doi.org/10.1051/vetres/2010033 PMID: 21188836

3. Meegan JM, Bailey CL (1989) Rift Valley fever. Boca Raton, FL, USA: CRC Press Inc.
4. Linthicum KJ, Davies FG, Kairo A, Bailey CL (1985) Rift Valley fever virus (family Bunyaviridae, genus Phlebovirus). Isolations from Diptera collected during an inter-epizootic period in Kenya. J Hyg (Lond) 95: 197–209.

5. Clements AN (2012) Transmission of Rift Valley fever virus. The biology of mosquitoes. pp. 298–321.

6. Meegan JM (1979) The Rift Valley fever epizootic in Egypt 1977–78. 1. Description of the epizootic and virological studies. Trans R Soc Trop Med Hyg 73: 618–623.

7. Hoogstraal H, Meegan JM, Khalil GM, Adham FK (1979) The Rift Valley fever epizootic in Egypt 1977–78. 2. Ecological and entomological studies. Trans R Soc Trop Med Hyg 73: 624–629. PMID: 44038

8. Meegan JM, Khalil GM, Hoogstraal H, Adham FK (1980) Experimental transmission and field isolation studies implicating Culex pipiens as a vector of Rift Valley fever virus in Egypt. Am J Trop Med Hyg 29: 1405–1410. PMID: 7446827

9. Turell MJ, Dohm DJ, Fonseca DM (2014) Comparison of the potential for different genetic forms in the Culex pipiens Complex in North America to transmit Rift Valley fever virus. J Am Mosq Control Assoc 30: 253–259. https://doi.org/10.2987/14-6441R.1 PMID: 25843130

10. Arnaoudi F, Krida G, Bouattour A, Rhim A, Daaboub J, et al. (2012) Culex pipiens, an experimental efficient vector of West Nile and Rift Valley fever viruses in the Maghreb region. PLoS One 7: e36757. https://doi.org/10.1371/journal.pone.0036757 PMID: 22693557

11. Turell MJ, Wilson WC, Bennett KE (2010) Potential for North American mosquitoes (Diptera: Culicidae) to transmit Rift Valley fever virus. J Med Entomol 47: 884–889. PMID: 20939385

12. Moutaille S, Bouloy M, Failloux AB (2007) Short report: efficient oral infection of Culex pipiens quinquefasciatus by Rift Valley fever virus using a cotton stick support. Am J Trop Med Hyg 76: 827–829. PMID: 17488999

13. Moutaille S, Krida G, Madec Y, Bouloy M, Failloux AB (2010) Replication of Clone 13, a naturally attenuated avirulent isolate of Rift Valley fever virus, in Aedes and Culex mosquitoes. Vector Borne Zoonotic Dis 10: 681–688. https://doi.org/10.1089/vbz.2009.0246 PMID: 20854021

14. Moutaille S, Krida G, Schaffner F, Vazeille M, Failloux AB (2008) Potential vectors of Rift Valley fever virus in the Mediterranean region. Vector Borne Zoonotic Dis 8: 749–753. https://doi.org/10.1089/vbz.2008.0009 PMID: 18620510

15. Turell MJ, Linthicum KJ, Patrician LA, Davies FG, Kairo A, et al. (2008) Vector competence of selected African mosquito (Diptera: Culicidae) species for Rift Valley fever virus. J Med Entomol 45: 102–108. PMID: 18283949

16. Turell MJ, Linthicum KJ, Patrician LA, Davies FG, Kairo A, et al. (2008) Vector competence of selected African mosquitoes (Diptera: Culicidae) species for Rift Valley fever virus for transmission. J Med Entomol 45: 102–108. PMID: 18283949

17. Meegan JM, Khalil GM, Hoogstraal H, Adham FK (1979) The Rift Valley fever epizootic in Egypt 1977–78. 1. Description of the epizootic and virological studies. Trans R Soc Trop Med Hyg 73: 618–623.

18. Turell MJ, Linthicum KJ, Patrician LA, Davies FG, Kairo A, et al. (2008) Vector competence of selected African mosquito (Diptera: Culicidae) species for Rift Valley fever virus. J Med Entomol 45: 102–108. PMID: 18283949

19. Turell MJ, Linthicum KJ, Patrician LA, Davies FG, Kairo A, et al. (2008) Vector competence of selected African mosquito (Diptera: Culicidae) species for Rift Valley fever virus. J Med Entomol 45: 102–108. PMID: 18283949

20. Turell MJ, Linthicum KJ, Patrician LA, Davies FG, Kairo A, et al. (2008) Vector competence of selected African mosquito (Diptera: Culicidae) species for Rift Valley fever virus. J Med Entomol 45: 102–108. PMID: 18283949

21. Turell MJ, Linthicum KJ, Patrician LA, Davies FG, Kairo A, et al. (2008) Vector competence of selected African mosquito (Diptera: Culicidae) species for Rift Valley fever virus. J Med Entomol 45: 102–108. PMID: 18283949

22. Billecocq A, Spiegel M, Vialat P, Kohl A, Weber F, et al. (2004) NSs protein of Rift Valley fever virus blocks interferon production by inhibiting host gene transcription. J Virol 78: 9798–9806. https://doi.org/10.1128/JVI.78.18.9798-9806.2004 PMID: 15331713

23. Habjan M, Pichlmair A, Elliott RM, Overby AK, Glatte T, et al. (2009) NSs protein of Rift Valley fever virus induces the specific degradation of the double-stranded RNA-dependent protein kinase. J Virol 83: 4365–4375. https://doi.org/10.1128/JVI.02148-08 PMID: 1921744a

24. Ikegami T, Narayanan K, Won S, Kamitani W, Peters CJ, et al. (2009) Dual functions of Rift Valley fever virus NSs protein: inhibition of host mRNA transcription and post-transcriptional downregulation of protein kinase PKR. Ann N Y Acad Sci 1171 Suppl 1: E75–85.
25. Ikegami T, Narayanan K, Won S, Kamitani W, Peters CJ, et al. (2009) Rift Valley fever virus NSs protein promotes post-transcriptional downregulation of protein kinase PKR and inhibits eIF2alpha phosphorylation. PLoS Pathog 5: e1000287. https://doi.org/10.1371/journal.ppat.1000287 PMID: 19197350

26. Kalveram B, Lihoradova O, Ikegami T (2011) NSs protein of Rift Valley fever virus promotes posttranslational downregulation of the TFIH subunit p62. J Virol 85: 6234–6243. https://doi.org/10.1128/JVI.02255-10 PMID: 21543505

27. Muller R, Saluzzo JF, Lopez N, Dreier T, Turell M, et al. (1995) Characterization of Clone 13, a naturally attenuated avirulent isolate of Rift Valley fever virus, which is altered in the small segment. Am J Trop Med Hyg 53: 405–411. PMID: 7485695

28. Dungu B, Louw I, Lubisi A, Hunter P, von Teichman BF, et al. (2010) Evaluation of the efficacy and safety of the Rift Valley fever Clone 13 vaccine in sheep. Vaccine 28: 4581–4587. https://doi.org/10.1016/j.vaccine.2010.04.085 PMID: 20470972

29. von Teichman B, Engelbrecht A, Zulu G, Dungu B, Pardini A, et al. (2011) Safety and efficacy of Rift Valley fever Smithburn and Clone 13 vaccines in calves. Vaccine 29: 5771–5777. https://doi.org/10.1016/j.vaccine.2011.05.055 PMID: 21664400

30. Kortekaas J, Oreshkova N, Cobos-Jimenez V, Vloet RP, Potgieter CA, et al. (2011) Creation of a nonattenuated avirulent isolate of Rift Valley fever virus, which is altered in the small segment. Am J Trop Med Hyg 53: 405–411. PMID: 21957302

31. Spearman C (1908) The method of "right and wrong cases" ("constant stimuli") without Gauss’s formulae. Br J Psych 2: 227–242.

32. Kärber G (1931) Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Arch Exp Path Pharmac 162: 480–483.

33. Fros JJ, Geertsema C, Vogels CB, Roosjen PP, Failloux AB, et al. (2015) West Nile Virus: High transmission rate in North-Western European mosquitoes indicates its epidemic potential and warrants increased surveillance. PLoS Negl Trop Dis 9: e0003956. https://doi.org/10.1371/journal.pntd.0003956 PMID: 26225555

34. Vogels CB, Fros JJ, Goertz GP, Pijlman GP, Koenraad CJ (2016) Vector competence of northern European Culex pipiens biotypes and hybrids for West Nile virus is differentially affected by temperature. Parasit Vectors 9: 393. https://doi.org/10.1186/s13071-016-1677-4 PMID: 27388451

35. Kortekaas J, Antonis AF, Kant J, Vloet RP, Vogel A, et al. (2012) Efficacy of three candidate Rift Valley fever vaccines in sheep. Vaccine 30: 3423–3429. https://doi.org/10.1016/j.vaccine.2012.03.027 PMID: 22449427

36. Jackel S, Eiden M, Dauber M, Balkema-Buschmann A, Brun A, et al. (2014) Generation and application of monoclonal antibodies against Rift Valley fever virus nucleocapsid protein NP and glycoproteins Gn and Gc. Arch Virol 159: 535–546. https://doi.org/10.1007/s00705-013-1867-4 PMID: 24100475

37. Turell MJ, Rossi CA, Bailey CL (1984) Replication and dissemination of Rift Valley fever viruses lacking the NSs and/or NSm genes in mosquitoes: potential role for NSm in mosquito infection. PLoS Negl Trop Dis 6: e1639. https://doi.org/10.1371/journal.pntd.0001639 PMID: 22563517

38. Brubaker JF, Turell MJ (1998) Effect of environmental temperature on the susceptibility of Culex pipiens (Diptera: Culicidae) to Rift Valley fever virus. J Med Entomol 35: 918–921. PMID: 9835680

39. Turell MJ, Gargan TP, 2nd, Bailey CL (1984) Replication and dissemination of Rift Valley fever virus in Culex pipiens. Am J Trop Med Hyg 33: 176–181. PMID: 6696176

40. Crabtree MB, Kent Crockett RJ, Bird BH, Nichol ST, Erickson BR, et al. (2012) Infection and transmission of Rift Valley fever viruses lacking the NSs and/or NSm genes in mosquitoes: potential role for NSm in mosquito infection. PLoS Negl Trop Dis 6: e1639. https://doi.org/10.1371/journal.pntd.0001639 PMID: 22563517

41. Golnar AJ, Turell MJ, LaBeaud AD, Kading RC, Hamer GL (2014) Predicting the mosquito species and vertebrate species involved in the theoretical transmission of Rift Valley fever virus in the United States. PLoS Negl Trop Dis 8: e3163. https://doi.org/10.1371/journal.pntd.0003163 PMID: 25211133

42. Pingen M, Bryden SR, Pondivele E, Schnettler E, Kohl A, et al. (2016) Host inflammatory response to mosquito bites enhances the severity of arbovirus infection. Immunity 44: 1455–1469. https://doi.org/10.1016/j.immuni.2016.06.002 PMID: 27332734

43. Le Coupanc A, Babin D, Fiette L, Jouvion G, Ave P, et al. (2013) Aedes mosquito saliva modulates Rift Valley fever virus pathogenicity. PLoS Negl Trop Dis 7: e2237. https://doi.org/10.1371/journal.pntd.0002237 PMID: 23785528

44. Ibanez-Justicia A, Stroo A, Dik M, Beeuwkes J, Scholte EJ (2015) National mosquito (Diptera: Culicidae) survey in the Netherlands 2010–2013. J Med Entomol 52: 185–198. https://doi.org/10.1093/jme/tju058 PMID: 26336303