"Chikungunya virus replication rate determines the capacity of crossing tissue barriers in mosquitoes"

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Abstract

Chikungunya virus (CHIKV) is a reemerging and rapidly spreading pathogen transmitted by mosquitoes. The emergence of new epidemic variants of the virus is associated with genetic evolutionary traits, including duplication of repeated RNA elements in the 3'UTR that seemingly favor transmission by mosquitoes. The transmission potential of a given variant results from a complex interplay between virus populations and anatomical tissue barriers in the mosquito. Here, we used the wild type CHIKV Caribbean strain and an engineered mutant harboring a deletion in the 3'UTR to dissect the interactions of virus variants with the anatomical barriers that impede transmission during the replication cycle of the virus in *Aedes* mosquitos. Compared to the 3'UTR mutant, we observed that the wild type virus had a shorter extrinsic incubation period after an infectious blood meal and was expectorated into mosquito saliva much more efficiently. We found that high viral titers in the midgut are not sufficient to escape the midgut escape barrier. Rather, viral replication kinetics play a crucial role in determining midgut escape and transmission ability of CHIKV. Finally, competition tests in mosquitoes co-infected with wild type and mutant viruses revealed that both viruses successfully colonized the midgut, but wild type viruses effectively displaced mutant viruses during systemic infection due to their greater efficiency of escaping from the midgut into secondary tissues. Overall, our results uncover a link between CHIKV replication kinetics and the effect of bottlenecks on population diversity, as slow replicating variants are less able to overcome the midgut escape barrier.

Importance

It is well established that selective pressures in mosquito vectors impose population bottlenecks for arboviruses. Here, we used a CHIKV Caribbean lineage mutant carrying

a deletion in the 3'UTR to study host-virus interactions *in vivo* in the epidemic mosquito vector, *Aedes aegypti*. We found that the mutant virus had a delayed replication rate in mosquitoes, which lengthened the extrinsic incubation period (EIP), and reduced fitness relative to the wild type virus. As a result, the mutant virus displayed a reduced capacity to cross anatomical barriers during the infection cycle in mosquitoes, thus reducing the virus transmission rate. Our findings show how selective pressures act on CHIKV non-coding regions to select variants with shorter EIPs that are preferentially transmitted by the mosquito vector.

Introduction

Chikungunya virus (CHIKV) is an arthropod-borne virus that after 60 years of exclusive circulation in Asia and Africa has recently spread into Europe and America producing about 1.7 million infections (1–5). CHIKV infection has thus emerged as a major public health concern since it may affect a large proportion of the population within an outbreak area (6). CHIKV infections are usually non-fatal and resolve over time, but they cause considerable pain, distress, and anxiety, as well as significant economic burden due to severe clinical manifestations (7–9) . There is no commercially available vaccine against CHIKV, and intervention efforts during outbreaks focus on preventing mosquito exposure and inhibiting local mosquito population growth (10, 11).

CHIKV cycles between mosquito and human hosts, and has evolved strategies that allow maintenance of efficient replication in these two disparate host environments. Research efforts have focused on the identification of viral genome sequences that determine the virus host range (12). CHIKV genome is a single stranded positive sense RNA of 11–12 kb that carries a 3'UTR containing 50-80 nucleotide-long sequence repetitions referred to as *direct repeats* (13, 14) that change in copy number among viral strains (15–17). Evidence shows that 3'UTR is subjected to conflicting selective pressures in mammalian and mosquito hosts, and that duplicated direct repeats are maintained in nature due to positive selection in the mosquito host (17). The Caribbean strains bear the longest 3'UTR among CHIKV lineages and display five copies of direct repeats. Previous work from our group showed that virus replication in mammalian cells results in the emergence of variants carrying large 3'UTR deletions that are cleared in mosquito (18). In addition, Chen *et al.* reported that for the Asian CHIKV strain, an intact 3'UTR provides

a selective advantage in mosquitoes over a virus with a shorter 3'UTR, as viruses with intact 3'UTR prevailed in the head of mosquitoes at 10 days after mixed infections (16). While in vitro studies demonstrate delayed replication rates of 3'UTR deletion mutants in C6/36 mosquito cells, a detailed investigation on the relevance of CHIKV replication kinetics in mosquitoes *in vivo* is still lacking. Moreover, consequences on transmission dynamics for viral variants with delayed growth have not yet been explored (19).

Transmission efficiency and the extrinsic incubation period (EIP) are two common indexes used to describe the interaction between viruses and their vectors. While the first one is related to the ability of the pathogen to be successfully transmitted to another susceptible host, the second one defines the interval of time for this infectious cycle to be completed (20, 21). Both parameters are highly dependent on four anatomical barriers or bottlenecks that viruses must cross within the mosquito in order to be transmitted (22–24). The first barrier is determined by the capacity of the virus to infect and replicate in midgut epithelial cells of the mosquito after blood-meal (midgut infection barrier). Once it has successfully established a midgut infection, escape from the midgut imposes a barrier for the virus to disseminate through the hemolymph to secondary organs and peripheral tissues, such as the fat body and trachea. The inability to disseminate at this step could result from defects in the release of virions from midgut epithelial cells (midgut escape barrier). The next anatomical barrier to infection occurs at the end of the dissemination process, when the virus has to reach the salivary glands (salivary gland infection barrier). Finally, in order to be successfully transmitted, viruses must replicate efficiently inside salivary glands to be released into the saliva, which is injected into a human host when the mosquito takes the next blood meal (salivary gland escape barrier). For CHIKV, the

98 salivary gland escape barrier has a very strong impact on virus transmission efficiency
99 (25–27).

100 In this work we addressed the relationship between CHIKV replication kinetics, and
101 its capacity to overcome successive physiological barriers and complete a replication
102 cycle in mosquitoes in order to be successfully transmitted. We gained insight into barriers
103 to arbovirus transmission using an engineered variant of the Caribbean strain of CHIKV
104 bearing the deletion of the first 500 nts of the 3'UTR as a tool. Our data show that delayed
105 growth kinetics in *Aedes* mosquitoes resulted in an extended EIP, which in turn
106 compromised transmission efficiency. We found that this effect on transmission is
107 associated with a severe bottleneck during escape from the midgut, and to a lesser extent
108 to impaired secretion into saliva. In addition, virus competition assays in mosquitoes
109 showing that a small amount of fast replicating viral variants were able to displace slow-
110 replicating viruses in disseminated tissues, provide novel insight into how mosquito
111 bottlenecks restrict arbovirus diversity.
112

113 ## Results

114 **Mosquito replication cycle of wild type and 3'UTR deletion mutant viruses**

115 To gain insight into the mosquito cycle of the Caribbean CHIKV strain in its epidemic
116 vector, we used *Aedes aegypti* mosquito infections to determine the EIP of wild type virus
117 and an engineered 3'UTR deletion mutant (hereafter referred to as ∆abb') that has been
118 previously described to show impaired growth rates in mosquito cells *in vitro* (18)(Fig 1A).
119 Laboratory colonies of *Aedes aegypti* mosquitoes were fed with an infectious blood-meal
120 containing 10^6 PFU/ml of wild type or ∆abb' mutant viruses. At 3, 6, 9 and 12 days post-

121 blood meal, we analyzed the presence of each virus in the body (as a proxy of infection
122 rate), in the head (as a proxy of dissemination rate to salivary glands) (28–30) and in the
123 saliva (as indicative of transmission rate) in individual mosquitoes (Fig 1B). For each
124 virus, the infection rate was estimated as the percentage of mosquitoes with infectious
125 viruses in their bodies (Fig 1C), measured by the development of cytopathic effect on
126 Vero cells inoculated with whole body extracts. At day 3, we observed that 100% of the
127 engorged mosquitoes were infected with the wild type virus, while only 50% of the
128 mosquitoes exposed to the mutant virus became infected. Eventually, infection with the
129 mutant virus progressed and the whole pool of mosquitoes was infected by day 12. This
130 result indicates that Δabb' mutant has no impediment in crossing the midgut infection
131 barrier. Therefore, differences in the infection rate at short times after blood feeding rather
132 reflect slower growth rate of the mutant compared to the wild type, resulting in longer
133 times to reach the threshold level to be detected by our method. Next, we determined the
134 dissemination rate, i.e. the ratio between the number of mosquito heads with detectable
135 virus and the number of infected mosquitoes (Fig 1D). Results showed 50%
136 dissemination rate for the wild type at day 3, and 100% by day 6. In contrast, the Δabb'
137 virus was detected in the heads of infected mosquitoes only after 6 days, and even at
138 later time points, it reached the head in no more than 50% of the individuals, pointing to
139 a defect at a stage between colonization of the midgut and arrival to salivary glands.
140 Finally, we measured the transmission rate, i.e. the ratio between the number of mosquito
141 salivas with detectable virus and the number of mosquitoes with disseminated infection
142 (Fig 1E). Transmission rate peaked to almost 40% for the wild type at day 6, and
143 decreased by day 9. In contrast, Δabb' CHIKV reached maximum transmission at day 12

144 with a rate of only 10%. For both dissemination and transmission rates, we used the
145 cytopathic effect assay to score infection as it is informative on the nature of the infectivity
146 of the virus in the disseminated tissues and importantly of the virus expectorated into
147 saliva, respectively. As noted, it may be possible that dissemination and transmission
148 rates are underestimated compared to molecular methods because of the limit of
149 detection of the assay. However, as opposed to the increase observed in the infection
150 rate of the mutant virus, dissemination rates did not increase over the course of the
151 experiment (compare days 6, 9, and 12 in Fig 1D), suggesting that the mutant virus likely
152 encounters a midgut escape barrier to infection. The results obtained for wild type
153 transmission rate are similar to previous reports and show that the salivary gland entry
154 and exit barriers impose the greatest limiting effect for transmission in nature (25, 26, 31).
155 Infection, dissemination and transmission rates of wild type and Δabb' viruses are
156 summarized in Table 1.

157 In order to determine whether the decreased dissemination rate of the mutant is
158 accompanied by lower viral titers in disseminated tissues, we measured the viral titer of
159 wild type and Δabb' viruses in mosquito heads at different times post-infection (Fig 1F).
160 Consistent with the estimates of dissemination rates, the wild type virus reached an
161 average titer of $2x10^3$ PFU/ml at day 3, while at this time point mutant viruses were not
162 detectable. However, as soon as infection disseminated at 6 days post infection, the
163 mutant virus reached viral titers comparable to the wild type. Therefore, the defect in
164 transmission is likely related to a growth delay rather than to a defect to reach high viral
165 titers.

166 To evaluate whether this phenomenon extends to other vector species of CHIKV,
167 the same experiment was performed infecting *Aedes albopictus* mosquitoes. Estimates
168 of infection and dissemination rates are presented in Figure 1G and H. The results
169 recapitulated our observations with *Ae. aegypti* mosquitoes, underscoring the role of viral
170 replication kinetics on viral dissemination and subsequent transmission, regardless of the
171 mosquito species.

172 Together, these data showed that, similar to replication in cell culture, the mutant
173 virus has a slow replication rate at the site of colonization (i.e. mosquito midguts) that
174 results in decreased ability to disseminate as well as to be secreted into the mosquito
175 saliva compared to the wild type virus. This defect is also reflected in a longer EIP, defined
176 as a quantitative trait of the mosquito population instead of a threshold time point at which
177 the first mosquito becomes infectious (29).

178

179 **Deficient dissemination of ∆abb' mutant virus is due to a defect to cross the midgut**
180 **escape barrier**

181 Delayed EIP of ∆abb' mutant virus could reflect either a problem of the virus to
182 leave midgut at the beginning of the infection, or a problem to spread through hemolymph
183 and reach secondary organs during dissemination. To differentiate between these two
184 possibilities, we assessed infection rates and viral titers of wild type or ∆abb' CHIKV in
185 the midgut and in the carcass (i.e. the rest of the body after removing the midgut) of
186 mosquitoes from day 2 to 8 after infectious blood-feeding (Fig 2A). Similar to EIP, both
187 viruses eventually reached almost 100% infection rate of midguts (day 2 vs. day 6 for wild
188 type and mutant viruses, respectively), indicating efficient colonization of the midgut (Fig

189 2B). Mean viral titers in midgut were significantly lower for the mutant at early time points,
190 and as of day 6 both viruses reached comparable titers (Fig 2C), indicating delayed
191 replication rates of ∆abb' compared to wild type. The rate of carcass infection was used
192 as a proxy for the ability to escape from the midgut and spread in the infected mosquito.
193 Results showed that the mutant virus was detected in carcasses later than the wild type,
194 and failed to infect the carcass in half of the individuals (Fig 2D), pointing to a defect to
195 escape from the midgut. Similar to midgut viral titers, carcass titers were significantly
196 lower for the mutant than for the wild type virus at earlier times after infection. Despite this
197 delayed replication kinetics, at day 8 both viruses reached comparable titers (Fig 2E).
198 Finally, we analyzed paired viral titers in midgut and carcass of each individual as of the
199 fourth day post infection. Viral titers in midgut were higher than 10^4 PFU/ml in 100% of
200 mosquitoes infected with the wild type virus, and in 96% of them, viral dissemination to
201 carcass was successful (Fig 2F). In the case of mosquitoes infected with the mutant virus,
202 although there was a slight drop in the number of individuals with midgut titers greater
203 than 10^4 UFP/ml (89% of the analyzed mosquitoes), the virus was able to cross the midgut
204 escape barrier in only 46% of these individuals (Fig 2G). A possible interpretation of this
205 result is that reaching a threshold value for viral titers in midgut is necessary but not
206 sufficient to guarantee a successful dissemination. In addition to a threshold titer, a
207 "window of opportunity" may define a timing effect that determines the ability to escape
208 the midgut barrier (32). To test this hypothesis, we repeated the experiment using five
209 times higher viral titers in the blood-meal to increase virus input in midgut cells (Fig 3).
210 We reasoned that increasing the viral titer in the input would allow the mutant to reach
211 threshold titers earlier in the mosquito cycle and it would favor escaping the midgut (33).

212 Figure 3A shows that both viruses infected midguts at similar rates. In contrast to
213 infections with low input, infections with higher dose disseminated into the carcass as of
214 day 2 for both viruses and differences in dissemination rates disappeared at day 8 (Fig
215 3C). Analysis of paired midgut and carcass viral titers further confirmed the effect of the
216 input on the ability of the mutant virus to disseminate; we found that ∆abb' CHIKV
217 achieved successful dissemination in 70% of mosquitos with midgut titers higher than 10^4
218 PFU/ml (Fig 3F). Thus, it appears that the delay to reach this threshold titer negatively
219 impacted on viral dissemination of the mutant, likely due to an impairment to overcome
220 the midgut escape barrier. In summary, these results indicate that the initial dose and
221 viral replication kinetics have a strong effect on the ability of CHIKV to escape the midgut.
222
223 **Deficiency in viral replication capacity also occurs in secondary tissues during**
224 **dissemination.**
225 With the aim of assessing if slow replication kinetics of the ∆abb' virus impacts
226 barriers other than the midgut escape barrier during the mosquito replication cycle, we
227 infected mosquitoes through the intrathoracic route to bypass the first two barriers that
228 occur during an infectious blood feeding (i.e. the midgut infection and escape barriers)
229 (Fig 4A). Mosquitoes were intrathoracically injected with 2500 PFU of wild type or Δabb'
230 mutant virus so that initial viral titers in the mosquito hemolymph were the same for both
231 viruses. Next, infection and transmission rates as well as viral titers in the body of infected
232 mosquitoes were measured every two days. Mosquito infection rates, estimated as the
233 presence of viruses in the body at different times post-injection, was 100% for both viruses
234 at all tested time points (Fig 4B). Virus titration in the bodies showed ~10-fold higher viral

titers for the wild type than for the mutant at days 2 and 4, and as of day 6 both viruses showed the same titers (Fig 4C). In turn, the overall trend of transmission rate, estimated as the presence of viruses in the saliva, was slightly lower in the mutant than in the wild type (Fig 4D). These data indicate that mutant virus growth rate is also affected in secondary tissues, impacting on its ability to cross the salivary glands barriers and thus contributing to a deficient transmission of the virus.

Wild type CHIKV displays a fitness advantage to escape from the mosquito midgut.

To directly address the impact of CHIKV growth rate on fitness we performed competition experiments between wild type and Δabb' viruses. *Ae. aegypti* mosquitoes were fed with an infectious blood-meal containing 10^6 PFU/ml of a mixture of wild type and Δabb' viruses in a 1:10 ratio in order to give a quantitative advantage to the virus with the impaired phenotype (Fig 5A). At different times post blood-meal, total RNA was purified from individual mosquitoes and subjected to reverse transcription reactions with an oligo(dT) primer. The pool of viral cDNAs was used to amplify viral 3'UTRs, which yielded fragments of different lengths for the wild type and mutant viruses. The gel in Figure 5B shows the amplification product of wild type and mutant viruses in a 1:10 ratio in the input used for the blood-meal (amplification products of the wild type and the Δabb' 3'UTRs were used as a reference). The relative abundance of viruses with full-length or Δabb' 3'UTR was assessed by agarose gel electrophoresis analysis of the RT-PCR products amplified from individual mosquitoes at 2, 5, and 9 days after feeding (Fig 5C). The gels show the fragments amplified from 12 individual mosquitoes at each time point. For each lane, we scored the ratio of intensities of the bands corresponding to wild type

and mutant 3'UTR and plotted the average ratio for each time point (Fig 5D). The 1:10 ratio in the input was quickly reversed to 1:1 ratio at the earliest time point evaluated. This rapid displacement of Δabb' by wild type virus in vivo indicates a fitness advantage of the wild type virus during mosquito infections.

We next assessed whether the fitness advantage of the wild type reflected the observed differences in the ability of wild type and mutant viruses to cross the midgut escape barrier. To this end, *Ae. aegypti* mosquitoes were fed with a blood meal containing a mixture of both viruses at a 1:1 ratio (Fig 5B). Midgut and carcass were dissected at different time points, total RNA was extracted, and the presence of virus was evaluated by RT-PCR (Fig 5E). Representative agarose gels of midgut and carcass from day 4 post infection illustrate the differential mobility of wild type and Δabb' 3'UTR amplification products (Fig 5F). When analyzing the presence of viruses as a function of time, we observed that both viruses were detected in all mosquito midguts even at 8 days post infection (Fig 5G top). Based on previous reports, we reasoned that incoming viruses likely formed independent foci of infection within the midgut and thus, coexisted independently of their growth rates (24, 26, 34, 35). Wild type virus was readily detected as of 2 days post infection in the carcasses, while the mutant virus was only detected after 8 days, indicating that the wild type had a higher dissemination rate than the mutant virus at all times post-infection (Fig 5G bottom). Altogether, our experiments demonstrate that wild type CHIKV has a fitness advantage over the ∆abb' CHIKV due to a faster replication rate that enhances its ability to escape the midgut.

Discussion

The infection kinetics of arboviruses in their mosquito vectors have long been recognized as a powerful determinant of transmission and epidemiology (29). Viral genetic variations influence growth kinetics and their interaction with mosquito barriers, which together contribute to the overall phenotype of virus transmission (23, 24, 36, 37). For instance, comparisons between dengue serotypes and even between strains from single serotypes, showed differences in EIP, that are most likely due to differences in viral replication kinetics in mosquitoes (29, 38). For CHIKV, the emergence of new viral lineages has been linked to large variations in the 3'UTR, which enhances replication in mosquito cells in vitro (15, 16, 18, 39, 40). Using an engineered 3'UTR deletion mutant of the Caribbean lineage of CHIKV we characterized the interaction of this mutant with mosquito barriers *in vivo*. We found that the replication rate of the 3'UTR mutant is compromised in *Aedes* mosquitoes, and based on our results we propose a model (Fig 6) where viral replication rate is intimately linked to viral capacity to overcome barriers within mosquitoes. Viruses with fast replication rates efficiently infect mosquitoes, disseminate to secondary tissues and reach the mosquito saliva, resulting in a short EIP that assures transmission. In contrast, viruses with slow replication rates experience hurdles to overcome the barriers imposed by the mosquitoes, resulting in a longer EIP and lower transmission.

Important bottlenecks have been reported for arboviruses such as West Nile virus, Western Equine Encephalitis virus, Sindbis virus and CHIKV during infection of their natural vectors (26, 33, 34, 41–43). These bottlenecks have been found at the midgut level or/and at the salivary gland level. By assessing viral infection rates in midgut and carcass we found that, although there were no differences in the infectivity rate of both

304 viral variants, the mutant virus had impaired ability to leave the midgut, suggesting a
305 strong midgut escape barrier effect. The outcome is a proportion of the mosquito
306 population exhibiting dissemination and the rest exhibiting no dissemination. This
307 scenario of mosquito subpopulation structure has already been reported for DENV (38).
308 In turn, a dose-dependent effect has also been associated to escape from the midgut and
309 occurred only when low doses of virus had been ingested (24). In agreement, in this work
310 we found that increasing blood meal viral titers reduced the midgut escape barrier effect.
311 Once midgut infection has been established, in order to disseminate, virus must
312 cross the basal lamina surrounding the midgut epithelium. It has been shown that after a
313 blood meal both an alteration of the expression of specific enzymes in the mosquito
314 midgut as well as a mechanical distention occur (32, 44–46). Several works have
315 proposed that this results in transient degradation and increased permissibility of the
316 basal lamina promoting a “window of opportunity” of 48 hs during which large quantities
317 of CHIKV are allowed to disseminate (32, 44). In this sense, viruses with longer mosquito
318 replication cycles such as DENV or ZIKV may not benefit as much from early transient
319 degradation of the basal lamina following a blood meal (23). Interestingly, a recent work
320 has demonstrated that acquisition of a second non-infectious blood meal significantly
321 shortens the EIP of all these viruses in infected *Aedes* by triggering a mechanical
322 distention in the basal lamina and thus enhancing virus dissemination from the mosquito
323 midgut (46). Our results suggest that CHIKV may need to reach threshold viral titers within
324 midgut cells that are necessary but not sufficient to cross the midgut escape barrier and
325 spread into secondary tissues. We speculate that the ∆abb’ CHIKV mutant may miss that
326 window of opportunity because it does not reach threshold titers required to disseminate

in early times after infection. Whether administration of a second bloodmeal with the mutant virus has a positive effect on dissemination as a consequence of the mechanical distention in the basal lamina remains to be tested. Altogether, our data indicate that the slow replication rate of the 3'UTR mutant has a strong effect on the ability of CHIKV to escape the midgut at the onset of the infection.

It is well established that selective pressures in the mosquito vector impose important population bottlenecks to arboviruses (23, 36, 37, 47). Given that viral infection cycle in mosquitoes moves in a stepwise fashion, selective pressures in an initial tissue might have effects on the viral kinetics in downstream tissues (38, 48). CHIKV replication in mammalian cells was previously shown to generate virus variants with shorter 3'UTR including large deletions of direct repeat elements similar to the engineered mutation evaluated here (18). Furthermore, viruses with shorter 3'UTRs seemingly display a replicative advantage in mammalian cells. Similar to previous work (16), by using virus competitions in mosquitoes co-infected with wild type and mutant viruses, we observed a displacement of the mutant virus by wild type virus. In addition, we found that this fitness advantage is due to an increased capacity to escape from the midgut to secondary tissues, which results in a shift in the composition of the viral population. Interestingly, both viruses were simultaneously detected in the midguts of most of the mosquitoes even at 8 days post infection. This suggests that co-infecting viruses formed independent foci of infection within the midgut, allowing both viruses to coexist independently of their replication rates (24, 26, 34, 35). These results widen the notion of how intra-host diversity plays a role in transmission, with variants with a fitness advantage spreading faster, and eventually displacing those with lower fitness (38, 49). Epidemiological consequences

might also be possible, like the 2008 large outbreak of dengue in Australia that was attributed to the very short EIP of the DENV3 strain in the mosquito (50). In nature, a significant proportion of mosquitoes are expected to die before they are capable of transmitting virus and in this scenario a virus variant with shorter EIP would confer an evolutionary advantage by increasing their probability of transmission (5, 29, 51).

Taken together, our results show that a precisely timed replication rate is required for CHIKV to reach necessary threshold titers to exit the midgut during the onset of the infection cycle, indicating that the viral replication rate is a determining factor in the ability to cross anatomical barriers and complete a successful replication cycle in mosquitoes. Understanding the factors that affect viral trajectories between mosquito infection and viral transmission will help to predict viral epidemic potential and design strategies to disrupt viral transmission cycle.

Materials and Methods

Cells and viruses

Mammalian BHK and Vero cells were grown at 37˚C in DMEM medium supplemented with 10% Foetal Bovine Serum (Gibco) and 1% penicillin/streptomycin (Gibco). Mosquito C6/36 (*Aedes albopictus*, ATCC, CRL-1660) cells were grown at 28˚C in Leibovitz L-15 medium supplemented with 10% FBS, 1% nonessential amino acids (Gibco), 2% tryptose phosphate broth (Sigma) and 1% penicillin-streptomycin. For RNA transfections, cell lines were grown to 60–70% confluence and transfected in 24-well plates using Lipofectamine 3000 (Invitrogen) following manufacturer's instructions. Caribbean wild type and Δabb' infectious clones were obtained as described in (18). Viral stocks were obtained by

373 transfection of 500 ng of in vitro transcribed viral RNA and harvested from cell culture
374 supernatant at different times post-transfection. Viruses were quantified by plaque
375 assays. To this end, 10^5 Vero cells per well were seeded in 24-well plates and allowed to
376 attach overnight. Viral stocks were serially diluted and 0.1 ml was added to the cells and
377 incubated for 1 h. Then, 1 ml of overlay (1X DMEM medium, 2% fetal bovine serum, 1%
378 of pen-strep and 0.8% agarose) was added to each well. Cells were fixed 3 days post-
379 infection with 4% paraformaldehyde and stained with crystal violet.
380
381 **Mosquitoes rearing**
382 Laboratory colonies of *Ae. aegypti* mosquitoes (17th generation, collected originally in
383 Kamphaeng Phet Province, Thailand) and *Ae. albopictus* (19th generation, collected
384 originally in Phu Hoa, Binh Duong Province, Vietnam) were used. The insectary
385 conditions for mosquito maintenance were 28˚C, 70% relative humidity, and a 12-h light
386 and 12-h dark cycle. Adults were maintained with permanent access to 10% sucrose
387 solution. Adult females were offered commercial rabbit blood (BCL) twice a week through
388 a membrane feeding system (Hemotek Ltd.).
389
390 **Experimental infections of mosquitoes**.
391 (i) Infectious blood meals. Infection assays were performed with 7- to 10-day-old females
392 starved 24 h prior to infection in a biosafety level 3 (BSL-3) laboratory. Mosquitoes were
393 offered the infectious blood meal for 30 min through a membrane feeding system
394 (Hemotek Ltd) set at 37˚C with a piece of desalted pig intestine as the membrane. The
395 blood meal was composed of washed human erythrocytes resuspended in phosphate-

buffered saline mixed 2:1 with pre-diluted viral stock and supplemented with 10 mM ATP (Sigma-Aldrich). The viral stock was prediluted in Leibovitz L-15 medium with 0,1% sodium bicarbonate (Gibco) to reach an infectious titer ranging from $1x10^6$ to $1x10^7$ focus forming units and back titrated to ensure similar presented doses (the exact titer of each infectious blood meals is noticed in each experiment). Following the blood meal, fully engorged females were selected and incubated for at 28˚C, 70% relative humidity and under a 12 h light: 12 h dark cycle with permanent access to 10% sucrose. At different times post-infection mosquitoes were cold-anesthetized for salivation and dissection. For saliva collection, wings and legs were removed from each individual, and its proboscis was inserted into a 20-µl tip containing 10 ul of FBS for 30 min at room temperature. Saliva-containing FBS was expelled in 90 µl of Leibovitz L-15 medium (Gibco) for amplification and titration. Following the collection of saliva, mosquitoes were dissected and body parts were homogenized in microtubes containing steel beads (5mm diameter) and 300 µl of DMEM supplemented with 2% FBS using a TissueLyser II (QIAGEN) at 30 shakes/second for 2 minutes. Homogenates were clarified by centrifugation and stored at 80°C until further processing. Viral titers in individual samples were determined by plaque assay. For detection of 3'UTR RNA from whole mosquitoes or mosquito parts, RNA Trizol-extracted from homogenates was used for reverse transcription using oligo reverse 5'-TTTTTTTTTTTTTTTTTTTGAAATAT-3', complementary to the poly(A) tail plus the last 7 nucleotides of CHIKV genomes. PCRs were then carried out (DreamTaq - Thermo Fisher]) using the same oligo reverse and oligo forward 5'-CTAATCGTGGTGCTATGC-3'. The length of viral 3'UTRs was estimated by resolving

418 the product in 1% agarose gels. Intensity of the bands was measured with ImageJ
419 software.
420 (ii) Intrathoracic inoculations of mosquitoes. 7- to 10-day-old Female mosquitoes were
421 cold-anesthetized and injected with a transfection mix of CellFectin II reagent (Thermo
422 Fisher) with 50nl of Liebovitz's L-15 medium containing $2{,}5x10^3$ PFU of virus. The
423 injection was performed intrathoracically using a nanoinjector (Nanoject III, Drummond
424 Scientific) and a glass capillary needle. At 2, 4, 6 and 8 days post-injection, mosquitoes
425 were cold-anesthetized and dissected.
426
427 **Virus titration and quantification.**
428 The presence of infectious virus particles in mosquito bodies, midguts, carcass and heads
429 extracts were determined by plaque assay in homogenate samples following mosquito
430 dissection. Briefly, 100 µl of sample homogenates were serially diluted in cell culture
431 media and used to infect Vero cells in 24-well plates as described for virus titration.
432 Mosquito salivas were amplified in C6/36 cells for 5 days and viral presence in amplified
433 supernatants was assessed by cytopathic effect in Vero cells. The data were analyzed
434 quantitatively for most of the samples (PFU/ml) and qualitatively for saliva samples and
435 some body and head samples (i.e., presence or absence of infectious virus in
436 heads/bodies). Infection Rate (IR) was calculated as the proportion of mosquitos infected
437 among all tested females. Dissemination Rate (DR) was defined as the proportion of
438 females with infected head tissues among those that were infected (i.e., in which the virus
439 successfully disseminated from the midgut). Dissemination efficiency (DE) was calculated
440 as the proportion of females with infected head tissues among all tested females.

Transmission rate (TR) was defined as the proportion of females with infectious saliva among those that developed a disseminated infection. Transmission efficiency (TE) was calculated as the overall proportion of females that had infectious saliva (i.e., among all tested females with or without a disseminated infection).

Human blood and ethics statement

Human blood used to feed mosquitoes was obtained from healthy volunteer donors. Healthy donor recruitment was organized by the local investigator assessment using medical history, laboratory results and clinical examinations. Biological samples were supplied through participation of healthy volunteers at the ICAReB biobanking platform (BB-0033-00062/ICAReB platform/Institut Pasteur, Paris/BBMRI AO203/[BIORESOURCE]) of the Institut Pasteur to the CoSImmGen and Diagmicoll protocols which have been approved by the French Ethical Committee (CPP) Ile-de-France I. The Diagmicoll protocol was declared to the French Research Ministry under the reference: DC 2008–68 COL 1.

Statistics.

All statistical analyses were performed in GraphPad Prism 6. Significant differences between virus infection, dissemination and transmission rates were determined by Fisher's exact test. For viral titers, where the data did not follow a Gaussian distribution, a Mann-Whitney U test was used to replace the t test. Statistical significance is represented as follows, * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

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635 **Figure 1. Extrinsic incubation period of wild type and ∆abb' mutant CHIKV viruses**
636 **in *Aedes* mosquitoes. (**A) Schematic representation of the genomes of wild type (WT)
637 and ∆abb' mutant viruses. The ∆abb' bears a deletion of the first 500 nucleotides of the
638 3'UTR. (B) Extrinsic incubation period of WT and ∆abb' CHIKV. Mosquitoes were blood-
639 fed with 10^6 PFU/ml of WT or ∆abb' mutant viruses and the presence of virus was
640 analyzed in the body (as a proxy of infection rate), in the head (as a proxy of dissemination
641 rate to salivary glands) and in the saliva (as indicative of transmission rate) at different
642 times post-infection. (C, D and E) Bar graphs showing infection, dissemination, and
643 transmission rates of WT and ∆abb' viruses in infected *Aedes aegypti* mosquitoes. (C)
644 Infection rate was calculated as the percentage of infected mosquito bodies at each time
645 point. (D) Dissemination rate was scored as the number of infected mosquito heads over
646 the number of infected bodies. (E) Transmission rate was measured as the ratio between
647 the number of mosquito saliva with detectable virus and the number of mosquitoes in
648 which dissemination was successful. Bars for infection, dissemination and transmission
649 rates represent cumulative data of two independent experiments (n = 48). Data were
650 analyzed by Fisher's exact test. (F) Dot plot showing mean viral titers and SD of WT and
651 ∆abb' viruses in the heads of infected mosquitoes. Infectious virus titers were measured
652 in the heads of mosquitos displaying positive CPE at each time point by plaque assay in
653 Vero cells. Data represent the titer of individual mosquitoes. Statistics were performed by
654 Mann–Whitney U test. (G and H) Infection and dissemination rates in *Aedes albopictus*
655 mosquitoes. Bar graphs for (H) infection and (G) dissemination rates (n = 24). Data were
656 analyzed by Fisher's exact test.

Figure 2. Δabb' mutant CHIKV is impaired to escape the midgut. (A) Midgut escape barrier assay. Mosquitoes were blood-fed with 10^6 PFU/ml of wild type (WT) or Δabb' mutant CHIKV and dissected from days 2 to 8 to separate midguts and carcasses. Infection rates and viral titers were measured in each sample. (B) Bar graph showing midgut infection rates. Data represent the percentage of infected mosquito midguts at each time point. (C) Dot plot showing mean viral titers and SD of WT and Δabb' viruses in midguts of infected mosquitoes. Virus titers in midgut extracts scored positive by CPE assay were measured by plaque assay. Data represent titers of individual midguts. (D) Bar graph showing carcass infection rates. Data represent the percentage of infected carcass at different times post blood-feeding and reflect virus dissemination efficiencies. (E) Dot plot showing mean viral titers and SD of WT and Δabb' viruses in carcasses of infected mosquitoes. Virus titers in carcass extracts were measured by plaque assay. Data represent titers of individual carcasses. (F and G) Scatter plot of viral titers in midgut vs. carcass for individual mosquitoes from the fourth to the eighth day post infection. The dotted line indicates the threshold titer needed to leave the midgut was set at 10^4 PFU/ml. The percentage of mosquitoes above this threshold with disseminated infection was measured for (F) wild type and (G) mutant viruses. Statistics on infection rates were performed by Fisher's exact test on cumulative data (n = 24) of two independent experiments. Statistics on viral titers were performed by Mann–Whitney U test.

677 **Figure 3. Increasing the infectious dose decreases the midgut escape barrier effect.**
678 Mosquitoes were blood-fed with $5x10^6$ PFU/ml wild type (WT) or ∆abb' mutant CHIKV
679 and dissected from days 2 to 8 to separate midguts and carcasses. Infection rates and
680 viral titers were measured in each sample. (A) Bar graph showing midgut infection rates.
681 (B) Dot plot showing mean viral titers and SD of WT and ∆abb' viruses in midguts of
682 infected mosquitoes. (C) Bar graph showing carcass infection rates. (D) Dot plot showing
683 mean viral titers and SD of WT and ∆abb' viruses in carcasses of infected mosquitoes. (E
684 and F) Scatter plot of viral titers in midgut vs. carcass for (E) wild type and (F) mutant
685 viruses. Statistics on infection rates were performed by Fisher's exact test on cumulative
686 data (n = 24) of two independent experiments. Statistics on viral titers were performed by
687 Mann–Whitney U test.
688

Figure 4. Salivary glands impose a tight barrier to CHIKV transmission (A) Intrathoracic injections of *Ae. aegypti* mosquitoes with wild type (WT) and Δabb' CHIKV. In order to bypass the midgut barrier, *Ae. aegypti* mosquitoes were intrathoracically injected with 2500 PFU of WT or mutant virus. (B) Bar graph showing infection rates in bodies after intrathoracic injection of viruses. Infection rate was calculated as the percentage of mosquitoes with viral presence in the body at different times post-injection. (C) Dot plot showing mean viral titers and SD in the bodies of intrathoracically injected mosquitoes. For the viral titers, statistics were performed by Mann–Whitney U test. (D) Bar graph showing transmission rates after intrathoracic injection of viruses. Transmission rate was calculated as the percentage of mosquitoes with viral presence in the saliva at different times post-injection.

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Figure 5. Wild type CHIKV has a fitness advantage over ∆abb' CHIKV to cross the midgut escape barrier. (A) Experimental setup of wild type (WT) vs. ∆abb' competitions in *Aedes aegypti* mosquitoes. Mosquitoes were offered an infectious blood meal containing a mixture of WT and ∆abb' viruses in a 1:10 ratio (10^6 PFU/ml). Total RNA was purified from individual mosquitoes at different time points post infection and the presence of WT and ∆abb' 3'UTRs was assessed. (B) RT-PCR product of the RNA extracted from the infectious blood meal containing wild type and ∆abb' virus in 1:1 and 1:10 ratio was resolved alongside fragments corresponding to wild type and ∆abb' 3'UTRs for reference. (C) Agarose gel electrophoresis of 3'UTR amplification products from individual mosquitoes. The presence of WT and ∆abb' viruses was assessed by RT-PCR and agarose gel electrophoresis on 12 individual mosquitoes at three different times after blood meal. (D) Bar graph showing the ratio of wt:∆abb' 3'UTR in the input and in mosquito individuals during the time course of the experiment. Bars represent the average of the ratio of intensities for the bands corresponding to the products of amplification of WT and ∆abb' 3'UTR in individual mosquitoes at each time point. (E) Competition assays to assess the ability of WT and ∆abb' CHIKV to cross the midgut escape barrier. Infectious blood feeding of *Ae. aegypti* mosquitoes was performed with blood containing a mixture of both viruses at 1:1 ratio (10^6 PFU/ml). At different times post-infection, midgut and carcass were dissected, total RNA was extracted, and the presence of virus was evaluated by RT-PCR as described above. (F) Representative agarose gels showing the products of amplification from midgut (top) and carcass (bottom) samples of 12 individual mosquitoes at 4 days post infection. (G) Bar graph showing the presence of WT and ∆abb' viruses in midgut as a function of time (top). Bars

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represent the percentage of midguts where WT and Δabb' viruses were detected. Bar graph showing the presence of WT and/or Δabb' viruses in carcasses as a function of time (bottom). Bars represent the percentage of carcasses where WT and/or Δabb' viruses were detected.

729 **Figure 6. Model for the effect of viral growth rate on the ability to cross barriers**
730 **during the infectious cycle in mosquitoes.** Infection rate in *Aedes* mosquitoes (midgut
731 infection barrier) is almost 100%, regardless of virus growth rate. Within midgut cells, wild
732 type (WT) CHIKV replicates and reaches the necessary threshold (> 10,000 PFU) to cross
733 the midgut escape barrier and spread into secondary tissues. A slow growing virus
734 accomplishes leaving midgut at later times and it spreads to secondary tissues in only
735 50% of individuals. WT disseminated viruses colonize the salivary glands and are
736 successfully secreted into the saliva in 40% of individuals. Secretion into saliva of mutant
737 viruses is only achieved in 10% of mosquitoes with disseminated infection. The outcome
738 is a longer EIP and lower transmission efficiency of mutant (5%) vs. WT CHIKV (35%).
739 After peaking (between 4 and 8 dpi for WT and between 9 and 12 dpi for ∆abb'),
740 transmission efficiency drops to undetectable levels.

741 **Table 1. Infection, dissemination and transmission rates (%) estimated at different**

742 **days after exposure of *Ae. aegypti* to CHIKV wild type or Δabb'.**

Days Post-Infection	CHIKV wild type			CHIKV Δabb'		
	IR n (%)	**DR n (%)**	**TR n (%)**	**IR n (%)**	**DR n (%)**	**TR n (%)**
3	48 (100)	28 (58)	0 (0)	48 (55)	2 (6)	0 (0)
6	48 (100)	45 (93)	17 (38)	48 (84)	18 (45)	1 (7)
9	48 (100)	46 (96)	13 (29)	48 (91)	25 (58)	1 (4)
12	48 (98)	45 (96)	3 (7)	48 (100)	26 (54)	2 (10)

743

744 Abbreviations: IR infection rate, DR dissemination rate, TR transmission rate, n number

745 of mosquitoes analyzed.

746

747

Figure 1

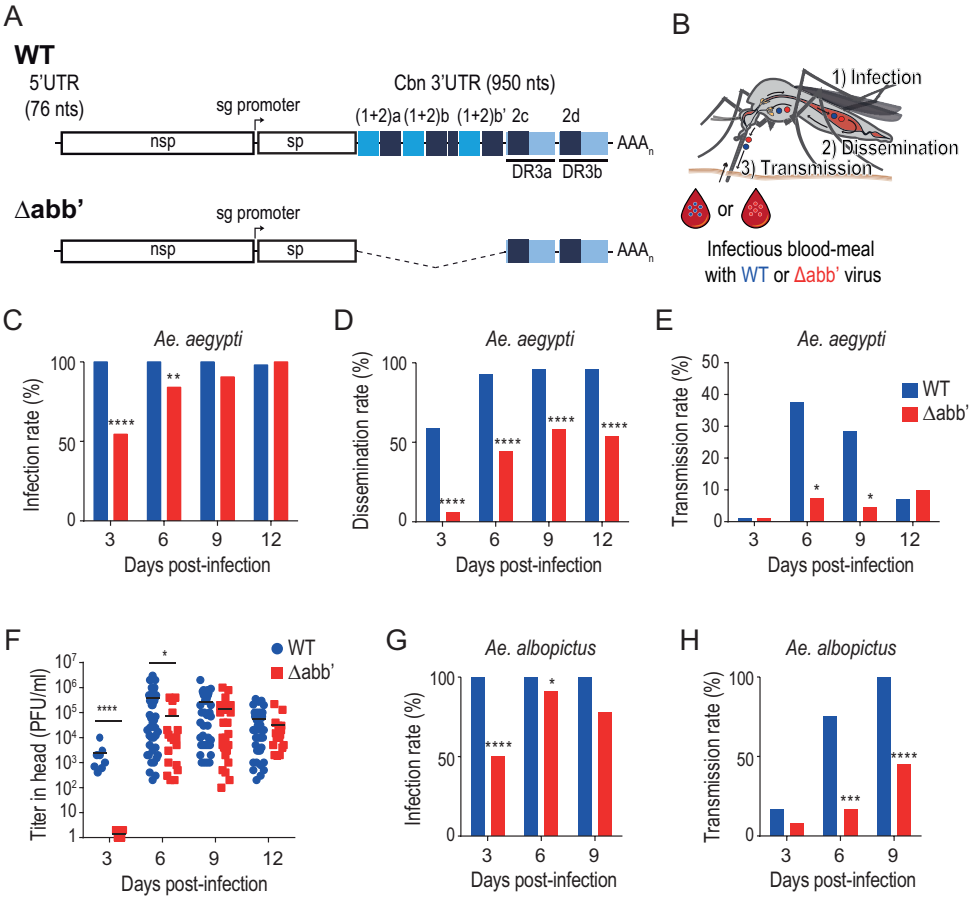
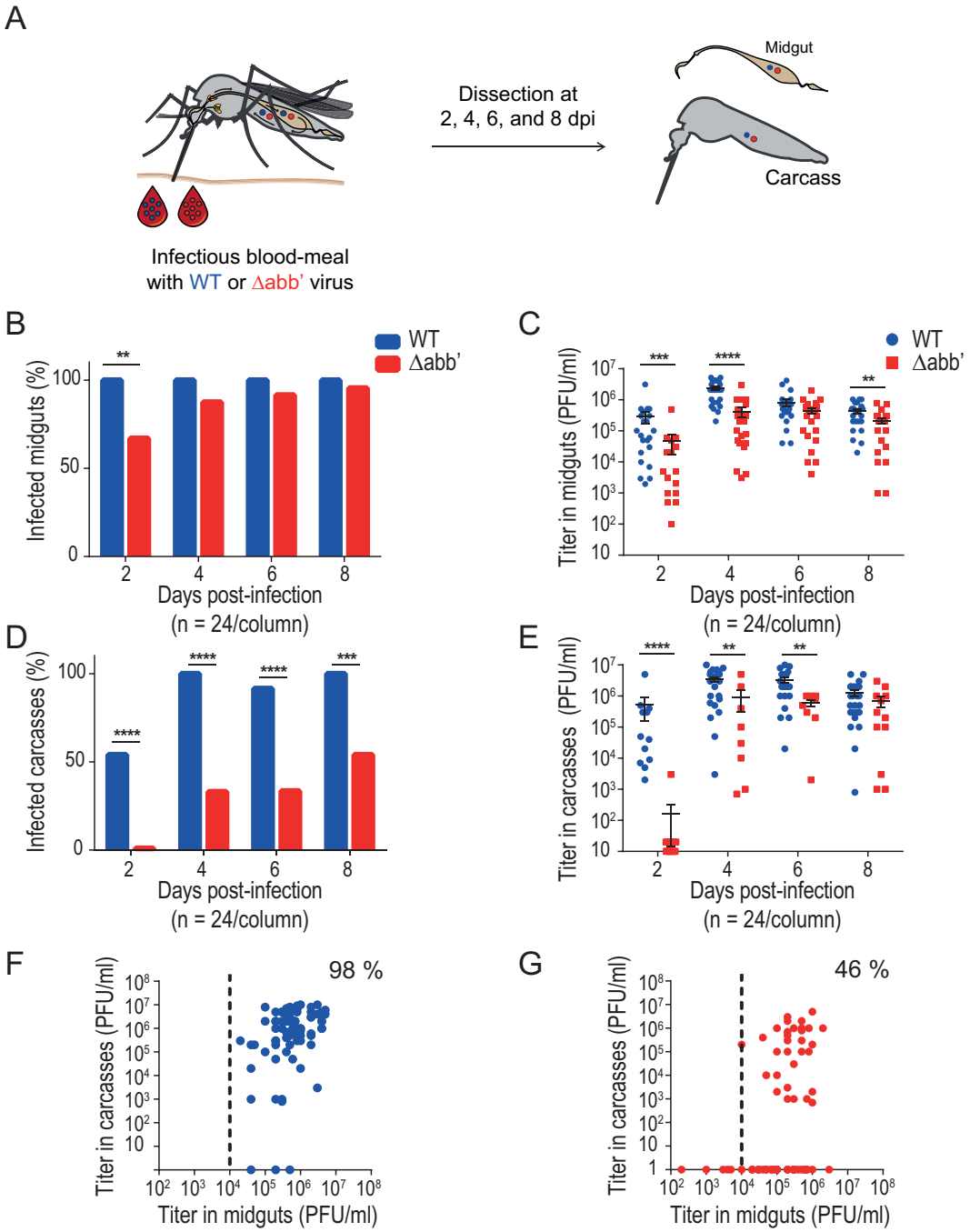


Figure 2



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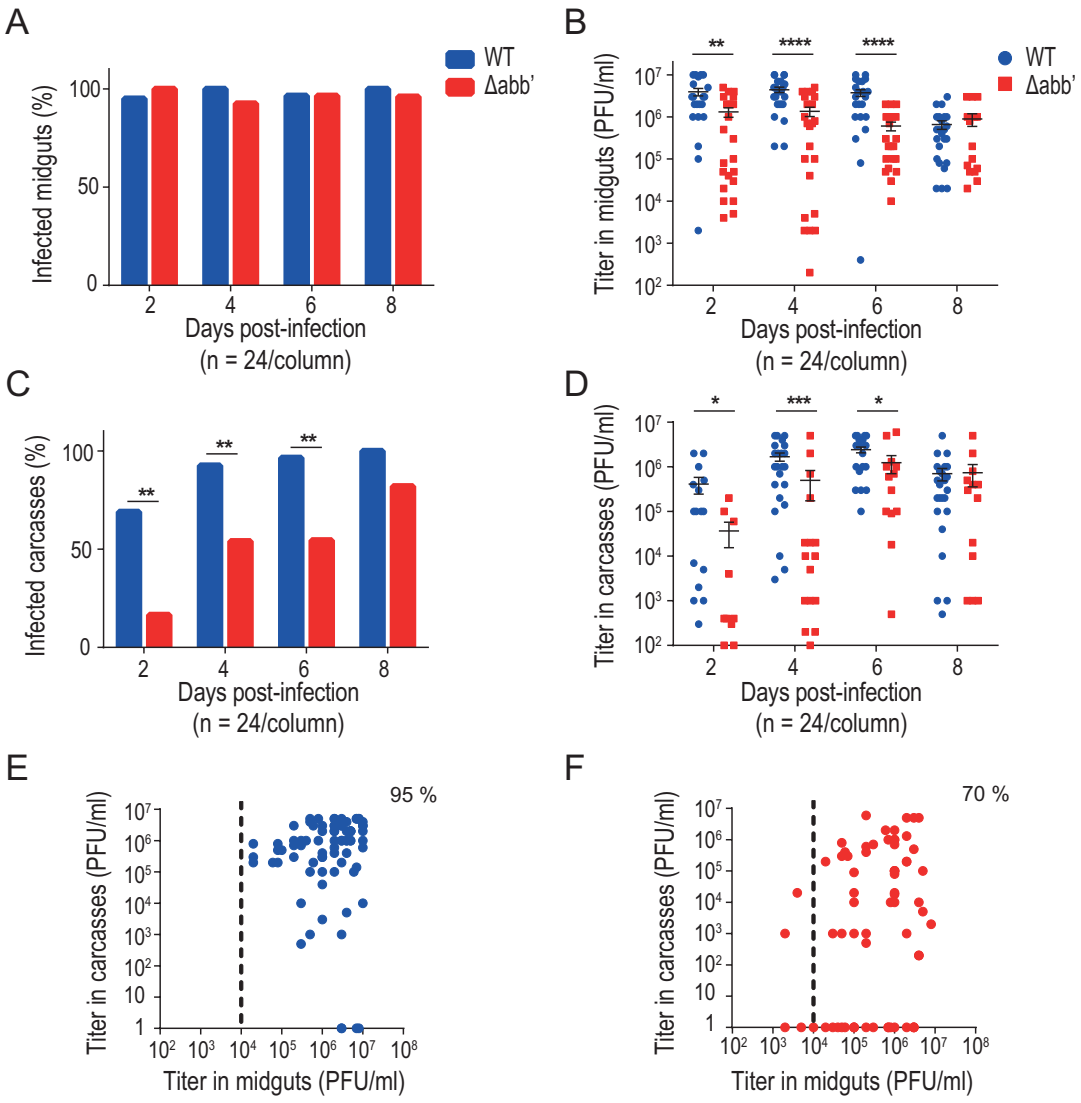

Figure 4

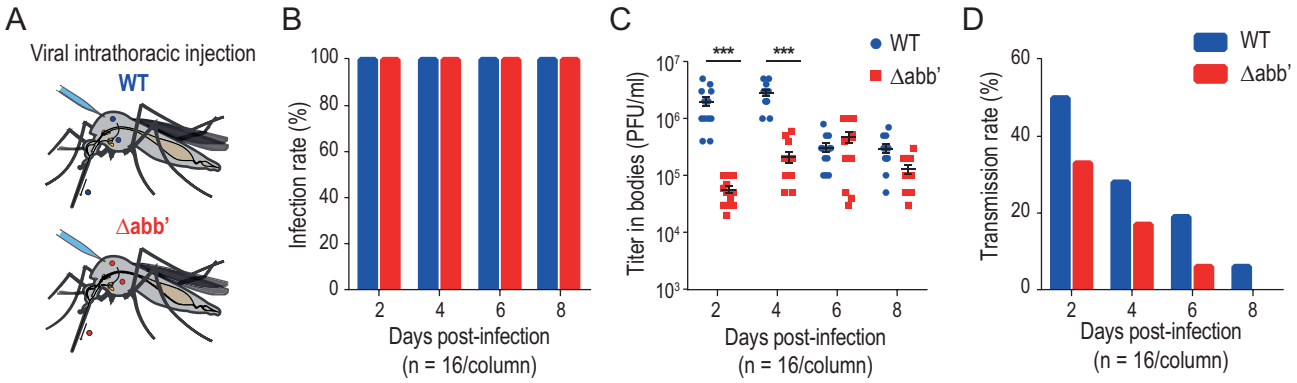

Figure 5

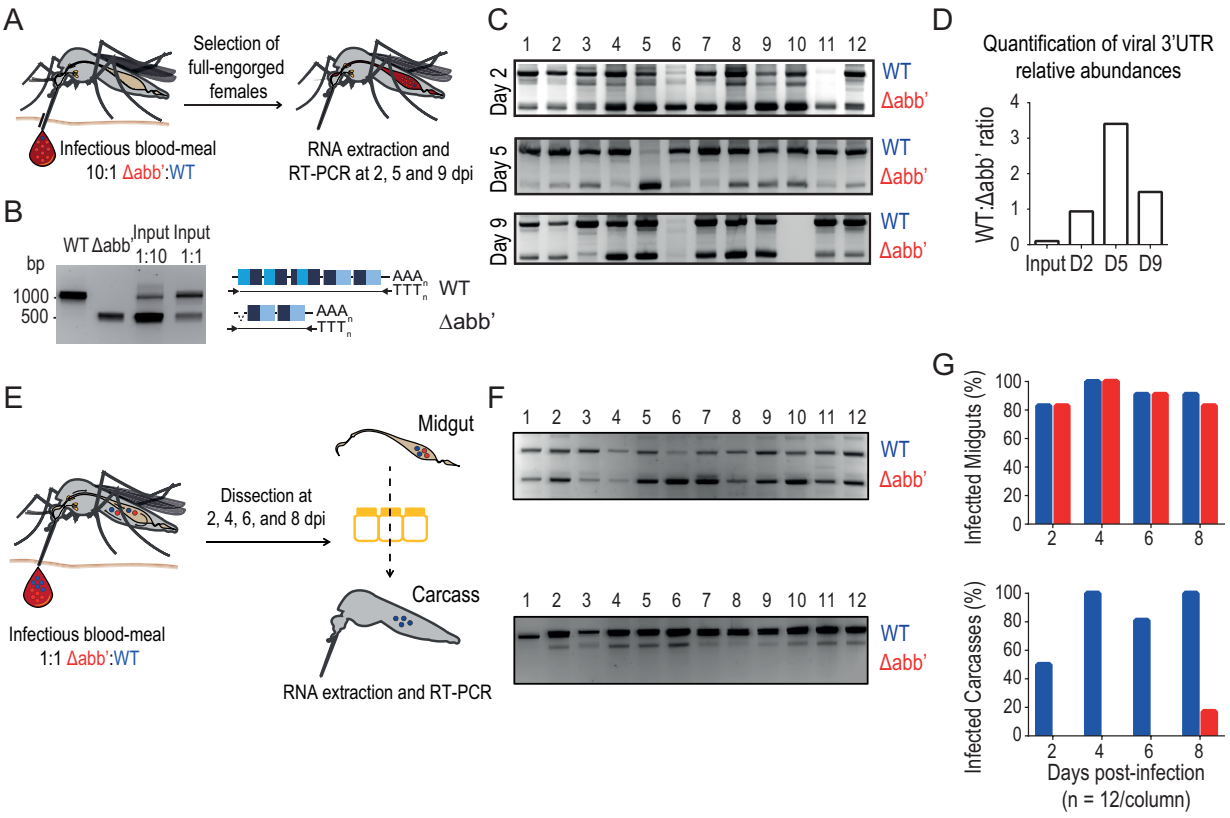

Figure 6