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1 Dengue virus replicates and accumulates in *Aedes aegypti* salivary 2 glands

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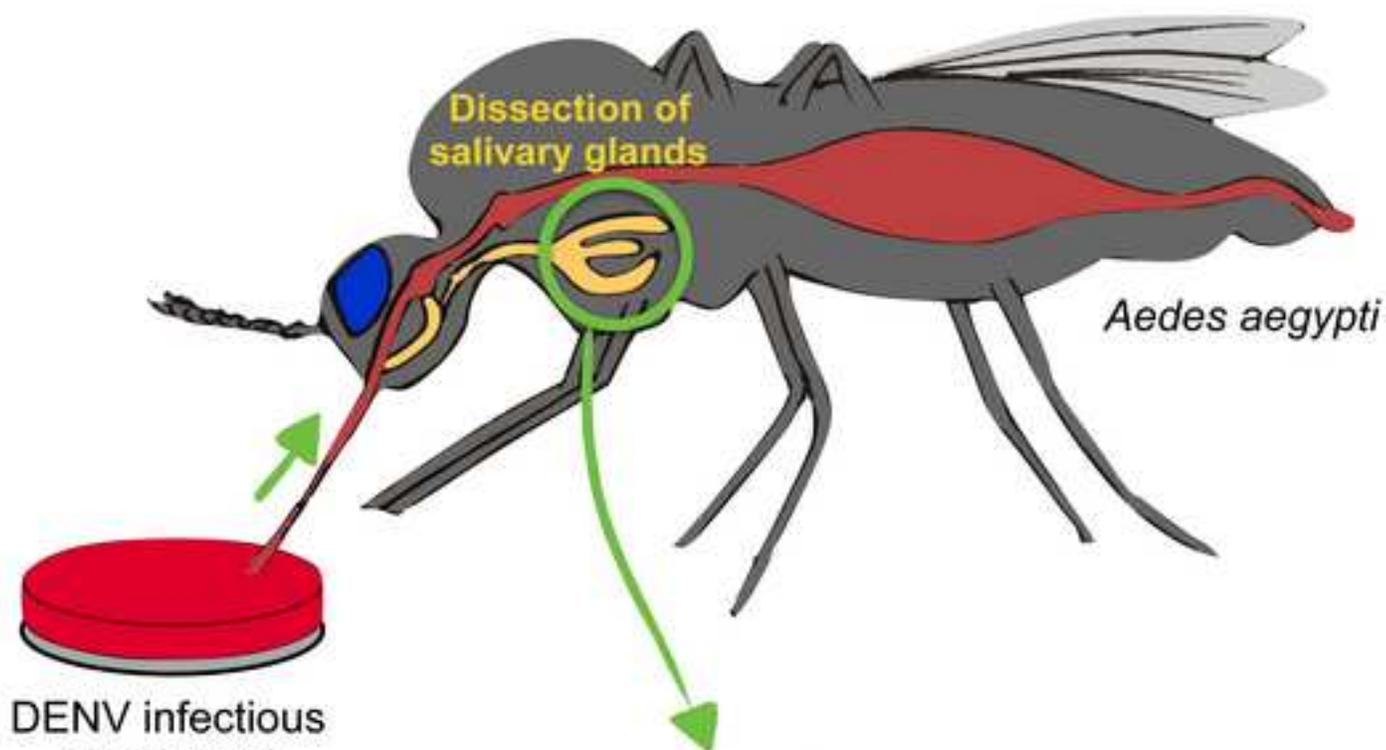
17 mosquito

18 salivary glands

19 virus replication

20 strand-specific RT-qPCR

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		Day post exposure			
		2	7	14	21
Strand-specific RT-qPCR	DENV (+) RNA		■	■	■
	DENV (-) RNA			■	■

□	■	■	■
No detection	3-4	4-5	5-6
(log ₁₀ RNA copies/sg)			

Highlights

- Strand-specific RT-qPCR allows accurate quantification of DENV (-) RNA in mosquito tissues
- Detection of DENV (-) RNA in salivary glands provides evidence of viral replication in this tissue
- Viral replication in salivary glands likely replenishes DENV genetic diversity prior to transmission

31 Abstract

32 Dengue virus (DENV) is an RNA virus transmitted among humans by mosquito vectors, mainly *Aedes*
33 *aegypti*. DENV transmission requires viral dissemination from the mosquito midgut to the salivary
34 glands. During this process the virus undergoes several population bottlenecks, which are stochastic
35 reductions in population size that restrict intra-host viral genetic diversity and limit the efficiency of
36 natural selection. Despite the implications for virus transmission and evolution, DENV replication in
37 salivary glands has not been directly demonstrated. Here, we used a strand-specific quantitative RT-
38 PCR assay to demonstrate that negative-strand DENV RNA is produced in *Ae. aegypti* salivary
39 glands, providing conclusive evidence that viral replication occurs in this tissue. Furthermore, we
40 showed that the concentration of DENV genomic RNA in salivary glands increases significantly over
41 time, indicating that active replication likely replenishes DENV genetic diversity prior to transmission.
42 These findings improve our understanding of the biological determinants of DENV fitness and
43 evolution.

44

45

46 1. Introduction

47 Dengue virus (DENV) is responsible for an estimated 390 million human infections each year,
48 mostly in tropical and sub-tropical regions (Bhatt et al., 2013). Over the last few decades, DENV has
49 become the most prevalent arthropod-borne virus (arbovirus) of humans (Guzman and Harris, 2015).
50 DENV is a positive-sense, single-stranded RNA virus that belongs to the genus *Flavivirus* in the family
51 *Flaviviridae*. It consists of four closely related serotypes (DENV-1 to -4) that are loosely antigenically
52 distinct (Katzelnick et al., 2015). Each DENV serotype can be subdivided into different genotypes
53 based on the divergence of viral gene sequences (Holmes and Twiddy, 2003). In the absence of
54 specific therapeutics, dengue prevention is limited to vector control, which has been largely
55 unsuccessful to date (Bowman et al., 2016). The current lack of antiviral therapeutics is partly due to
56 knowledge gaps in DENV biology (Diamond and Pierson, 2015).

57 The DENV infection cycle initiates with virus attachment to the target cell through interactions
58 between the viral envelope (E) protein and various cell surface receptors (Cruz-Oliveira et al., 2015).
59 Following receptor-mediated endocytosis, DENV genomic RNA is released into the cytoplasm and

60 translated into a polyprotein of ~3,400 amino acids that is further cleaved by viral and host proteases
1
2 61 into three structural (C, M and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and
3
4 62 NS5) proteins (Murray et al., 2008). DENV replication occurs with the formation of a membrane-bound
5
6 63 replication complex that incorporates viral genomic RNA, viral proteins and host factors (Welsch et al.,
7
8 64 2009). The DENV positive-strand (+) genomic RNA serves as a template to synthesize a negative-
9
10 65 strand (-) complementary RNA, which, in turn, is used for the production of multiple (+) RNA genomes
11
12 66 available for translation, initiation of new replication cycles or packaging into virions (Westaway et al.,
13
14 67 1999). DENV (-) RNA is considered a hallmark of viral replication in infected cells, thereby providing a
15
16 68 useful proxy to identify an active virus infection (Vaughan et al., 2002).

17
18 69 The primary DENV vector in nature is the mosquito *Aedes aegypti* (Lambrechts et al., 2010).
19
20 70 Following a blood meal on a viremic human, DENV initially establishes infection in the mosquito
21
22 71 midgut, before it disseminates systemically through the haemocoel (*i.e.*, the insect's body cavity) where
23
24 72 it can invade secondary tissues. Infection of the salivary glands and release of virions into the saliva is
25
26 73 the last step prior to virus transmission to the next human host. The time it takes between initial midgut
27
28 74 infection and subsequent transmission of DENV by *Ae. aegypti*, called the extrinsic incubation period,
29
30 75 ranges from 7 to 14 days at 25-30°C (Tjaden et al., 2013). Despite the critical role of mosquitoes in
31
32 76 DENV transmission and their potential as targets for control strategies, several aspects of DENV-
33
34 77 mosquito interactions are still poorly documented.

35
36 78 Several anatomical barriers impede DENV propagation in the mosquito, causing viral population
37
38 79 bottlenecks (Black et al., 2002). Such bottlenecks are stochastic drops in population size, which
39
40 80 combine with natural selection to shape the genetic composition of intra-host viral populations
41
42 81 (Lambrechts and Lequime, 2016; Lequime et al., 2016). Despite these population bottlenecks,
43
44 82 arboviruses typically maintain high levels of genetic diversity during transmission by mosquitoes
45
46 83 (Forrester et al., 2014). For example, while DENV populations undergo strong genetic drift due to a
47
48 84 tight population bottleneck during initial midgut mosquito infection, they subsequently display high
49
50 85 levels of genetic diversity in salivary glands (Lambrechts and Lequime, 2016; Lequime et al., 2016).
51
52 86 Replenishment of genetic diversity is thought to occur through *de novo* mutations during population
53
54 87 expansion, but the specific underlying mechanisms are unclear. In particular, it has long been
55
56 88 assumed that DENV replicates in *Ae. aegypti* salivary glands but, to the best of our knowledge, it has
57
58 89 never been unequivocally demonstrated.
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90 The common assumption that DENV replicates in salivary glands derives from the observation
91 during experimental infections that DENV genomic RNA and DENV antigens accumulate over time in
92 *Ae. aegypti* salivary glands (Anderson and Rico-Hesse, 2006; Luplertlop et al., 2011; Salazar et al.,
93 2007). However, viral RNA or antigen accumulation in salivary glands could simply result from a
94 continuous influx of virions from other tissues where the virus replicates. Direct evidence for viral
95 replication usually relies on detection of replicative intermediates such as (-) RNA or (+)/(-) dsRNA
96 duplex (Westaway et al., 1999). Because specific quantification of one RNA strand can be biased
97 during the reverse transcription reaction by false-priming events (*i.e.*, reverse transcription in the
98 absence of specific primer), tagged RT-PCR techniques have been developed to overcome this issue
99 (Bessaud et al., 2008; Horsington and Zhang, 2007; Kawakami et al., 2011; Peyrefitte et al., 2003;
100 Purcell et al., 2006). Here, we adapted a previously developed tag-based, strand-specific quantitative
101 RT-PCR (RT-qPCR) method to provide direct evidence that DENV replicates in the salivary glands of
102 *Ae. aegypti* based on detection and quantification of both (+) RNA and (-) RNA.

105 2. Material and Methods

107 2.1. Ethical statement

108 The Institut Pasteur animal facility has received accreditation from the French Ministry of
109 Agriculture to perform experiments on live animals in compliance with the French and European
110 regulations on care and protection of laboratory animals. Rabbit blood draws performed during the
111 course of this study were approved by the Institutional Animal Care and Use Committee at Institut
112 Pasteur under protocol number 2015-0032.

114 2.2. Mosquitoes

115 All experiments were carried out with adult *Ae. aegypti* mosquitoes derived from a field population
116 originally sampled in 2013 in Thep Nakorn, Kamphaeng Phet Province, Thailand, where dengue is
117 endemic (Endy et al., 2002; Fansiri et al., 2013). Experiments took place within 6 generations of
118 laboratory colonization. The insectary conditions for mosquito maintenance were 28°C, 70% relative
119 humidity (RH) and a 12 h light: 12 h dark cycle. Eggs were hatched in dechlorinated tap water and

120 larvae were reared in 24×34×9 cm plastic trays with a standard diet of Tetramin fish food (Tetra).
121 Adults were maintained in 30×30×30 cm plastic cages (~500 individuals/cage) with permanent access
122 to 10% sucrose solution. Adult females were offered commercial rabbit blood (BCL) twice a week
123 through a membrane feeding system (Hemotek Ltd.) using pig intestine as the membrane. Eggs were
124 collected on blotting paper and stored for less than 4 months under insectary conditions.

2.3. Virus

127 A wild-type, low-passage DENV-1 isolate (KDH0030A) was obtained in 2010 from the serum of a
128 dengue patient attending Kamphaeng Phet Provincial Hospital, Thailand, as previously described
129 (Fansiri et al., 2013). Informed consent of the patient was not necessary because the virus isolated in
130 laboratory cell culture was no longer considered a human sample. Prior to its use in this study, the
131 virus isolate was passaged three times in C6/36 mosquito cells cultivated in Leibovitz's L-15 culture
132 medium (Gibco, ThermoFisher Scientific) supplemented with 10% Foetal Bovine Serum (FBS, Gibco,
133 ThermoFisher Scientific), 1% non-essential amino acids (Life Technologies) and 0.1% Penicillin-
134 Streptomycin (Gibco, ThermoFisher Scientific) at 28°C. The full-length consensus genome sequence
135 of the KDH0030A isolate is available from GenBank under accession number HG316482. Virus stock
136 was prepared as previously described (Fontaine et al., 2016). Infectious titer of the viral stock was
137 estimated at 8.5×10^7 focus-forming units (FFU) per mL using a standard focus-forming assay in
138 C6/36 cells as previously described (Fontaine et al., 2016).

2.4. Mosquito exposure to DENV

141 Oral mosquito exposure to DENV was conducted as previously described (Fontaine et al., 2016).
142 Briefly, eggs were hatched synchronously in a low-pressure chamber for 1 h. After hatching, first-instar
143 larvae were distributed into 24×34×9 cm plastic trays filled with 1.5 L of dechlorinated tap water at a
144 density of 200 ± 10 larvae per tray. Larvae were fed with 0.1 mg of Tetramin fish food (Tetra) per tray
145 every two days. Pupae were transferred to 30×30×30 cm plastic cages (~500 individuals/cage) to
146 emerge. Adults were maintained with 10% sucrose solution *ad libitum*. The day prior to oral virus
147 exposure, mosquitoes were deprived of sucrose and four- to 7-day-old females were transferred to a
148 biosafety level-3 insectary where they were maintained at 28°C, 70% RH and under a 12 h light: 12 h
149 dark cycle. On the day of the infectious blood meal, washed rabbit erythrocytes resuspended in

150 phosphate-buffered saline (PBS, ThermoFisher Scientific) were mixed 2:1 with pre-diluted DENV-1
151 viral stock and supplemented with 10 mM ATP (Sigma-Aldrich). The viral stock was pre-diluted in L-15
152 medium with 10% FBS, 0.1% Penicillin-Streptomycin and 1% sodium bicarbonate (Gibco,
153 ThermoFisher Scientific) to reach an infectious titer of 1×10^7 FFU/mL of blood meal. A control blood
154 meal was prepared identically except that the supernatant of mock-inoculated C6/36 cells replaced the
155 viral suspension. Mosquitoes were offered the infectious or mock blood meal for 30 min through an
156 artificial blood feeding system (Hemotek Ltd.) set at 37°C with a piece of desalted porcine intestine as
157 the membrane. A sample of the blood meal was saved and stored at -80°C for later titration. Following
158 the blood meal, fully engorged females were transferred to 1-pint cardboard boxes (20-30 females per
159 cup), with a minimum of two boxes per condition. Mosquitoes were incubated in an environmental
160 cabinet (Binder) at 28°C, 70% RH and under a 12 h light: 12 h dark cycle with permanent access to
161 10% sucrose.

2.5. Dissections and RNA isolation

162
163
164 On day 2, 7, 14 and 21 post DENV exposure, females were immobilized on ice and their midgut
165 and salivary glands were dissected under a magnifying glass on a clean glass slide, in a drop of 0.22
166 µm-filtered 1X PBS (Gibco, ThermoFisher Scientific). Midgut and salivary glands were rinsed twice in
167 a drop of 1X PBS prior to RNA isolation, in order to prevent contamination by viral RNA from adjacent
168 tissues or hemolymph. Special care was given to completely eliminate fat body surrounding salivary
169 glands. Dissection tools were decontaminated between each individual using Surfa'Safe (Anios) and
170 RNase AWAY (Life Technologies) to avoid cross contamination. Individual organs were transferred to
171 a tube containing 300 µL of RLT lysis buffer (Qiagen) and ~20 glass beads of 1 mm in diameter
172 (Biospec). Samples were homogenized for 30 sec at 6,000 rpm in a Precellys homogenizer
173 (ThermoFisher Scientific) and stored at -80°C. Total RNA was isolated using QIAamp viral kit (Qiagen)
174 following manufacturer's instructions and eluted in 35 µL of RNase-free water (Gibco, ThermoFisher
175 Scientific).

2.6. In vitro transcription of strand-specific viral RNA

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177
178 A 101 bp-fragment (position 1,102 to 1,203) of DENV-1 *E* gene was cloned into pCR II-TOPO Dual
179 Promoter vector (Invitrogen, Life Technologies) using one-shot *E. coli* TOP10 chemically competent

180 cells (ThermoFisher Scientific). Plasmid was recovered from a single transformant clone using Mini-
181 Prep kit (ThermoFisher Scientific) and sequenced by the Sanger method to determine the orientation
182 of the insert. Strand-specific DENV-1 RNA was synthesized in two separate *in vitro* transcription
183 reactions with the MEGAscript kit (Ambion, ThermoFisher Scientific) using 1.5 µg of linearized
184 recombinant plasmid and either T7 or Sp6 RNA polymerases. Contaminant DNA was removed using
185 TURBO DNA-free kit (Ambion, ThermoFisher Scientific) and synthetic DENV-1 (+) and (-) RNA were
186 purified on Qiagen RNeasy columns following manufacturer's instructions. RNA concentration was
187 measured with a Nanodrop spectrophotometer. Purified synthetic RNA was diluted to 1×10^{12}
188 copies/µL, aliquoted and stored at -80°C.

2.7. Strand-specific RT-qPCR of viral RNA

189
190 Specific DENV-1 *E* primers containing a 21-nt tag sequence at the 5' end absent from both DENV
191 and *Ae. aegypti* genomes (Table 1) were designed as previously described (Plaskon et al., 2009). To
192 specifically incorporate the tag sequence into cDNA generated from (+) or (-) DENV-1 RNA, only the
193 tag-D1EnvR or the tag-D1EnvF were added during RT, respectively. First-strand cDNA synthesis
194 reaction contained 0.5 µM of primers, 0.2 mM of dNTP mix, 10 µL of template RNA and RNase-free
195 water up to 14.5 µL. After incubation at 65°C for 15 min to denature dsRNA complexes, samples were
196 chilled on ice for 5 min. Each reaction consisted of 4 µL of 5X First-Strand buffer, 1 µL of 0.1 mM
197 Dithiothreitol, 40 units of RNase-OUT and 100 units of MML-V reverse transcriptase (ThermoFisher
198 Scientific) in a final volume of 20 µL. cDNA synthesis was conducted at 37°C for 50 min, then reaction
199 was inactivated at 70°C for 15 min. Unincorporated primers were digested with 20 units of
200 Exonuclease I (New England Biolabs) to avoid any primer competition during PCR. cDNA was
201 quantified using SYBR-green based quantitative PCR (qPCR). D1EnvR and D1EnvF qPCR primers
202 include a 12-bp helper sequence at their 5' end to increase fluorescence signal and facilitate cDNA
203 detection (Table 1). Strand-specific cDNA quantification was performed by using a tag-specific primer
204 (Table 1) mixed with either D1EnvF or D1EnvR primer for (+) or (-) cDNA, respectively. The qPCR mix
205 contained 800 nM of each primer, 10 µL of 2X SYBR-green I Master Mix (Roche), 2 µL of cDNA
206 template and PCR-grade water up to a final volume of 20 µL. After an initial denaturation at 95°C for 5
207 min, cDNA amplification consisted of 40 cycles at 95°C for 10 sec, 60°C for 20 sec and 72°C for 10
208 sec. Melting curve was obtained by heating at 95°C for 5 sec, 65°C for 60 sec and performing

210 continuous fluorescence acquisition up to 97°C with a ramp rate of 0.2°C/sec. Serial dilutions of (-)
 211 and (+) synthetic RNA transcripts from 1 x 10⁸ to 1 x 10¹ copies/μL were used as standards, in
 212 duplicate. Each RT-qPCR plate included a negative control RNA (from mosquitoes fed on an
 213 uninfected blood meal), an exonuclease control (only one qPCR primer added) as well as a no-
 214 template control.

Table 1. Primers used for strand-specific RT-qPCR

Oligonucleotide name	Reaction	Sequence (5'-3') ^{1,2}
<i>DENV (-) strand detection</i>		
tag-D1EnvF	RT	GGCCGTCATGGTGGCGAATAA TGCGCAAAGTGCATTGAAG
D1EnvR	qPCR	aataaatcataaGTTGCGTCTTGTCTTCCA
tag	qPCR	aataaatcataa GGCCGTCATGGTGGCGAATAA
<i>DENV (+) strand detection</i>		
tag-D1EnvR	RT	GGCCGTCATGGTGGCGAATAA GTTGCGTCTTGTCTTCCA
D1EnvF	qPCR	aataaatcataaTGCGCAAAGTGCATTGAAG
tag	qPCR	(see sequence above)

¹The helper sequence is shown in lowercase

²The tag sequence is shown in bold font

2.8. Fluorescence microscopy of DENV-infected salivary glands

Dissected salivary glands still attached to the mosquito head were directly transferred to the fixative to avoid any damage, thereby keeping small fragments of fat body surrounding the tissue. Salivary glands were fixed for 24 h at 4°C in a 1.5-mL microtube containing 4% paraformaldehyde solution (Sigma-Aldrich) prepared in 1X PBS supplemented with 0.1% Tween-20 (Sigma-Aldrich). After three washes of 10 min each in 1X PBS + 0.1% Tween-20 (PBS-T), tissues were incubated for 1 h at 4°C in a blocking buffer consisting of 1X PBS-T supplemented with 5% bovine serum albumin, 5% FBS and 0.1% Triton-X 100 (Sigma-Aldrich). Tissues were incubated in 100 μL of anti-DENV complex primary antibody (MAB8705, Millipore) diluted 1:200 in blocking buffer, for 24 h at 4°C, followed by three washes in 1X PBS-T. After removing PBS-T, 100 μL of Alexa488-conjugated secondary antibody (Molecular probes, Invitrogen) diluted 1:1,000 in blocking buffer were added and tissues were incubated at 23°C for 2 h. After three washes in 1X PBS-T, heads were removed under a magnifying glass and salivary glands placed on a glass slide in 10 μL of ProLong Gold Antifade Mountant

233 (ThermoFisher Scientific) containing 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI). The
1 234 preparation was covered with a 22×22 mm coverslip and sealed with nail polish. Tissue observation
2 3 was conducted under an EVOS fluorescence microscope (ThermoFisher Scientific). Salivary glands
3 4 235 dissected from mosquitoes fed on an uninfected blood meal were used as a control.
4 5 236

7 237

10 238 *2.9. Statistical analyses*

11 239 The number of DENV RNA copies measured by strand-specific RT-qPCR was log₁₀-transformed
12 13 and analyzed as a function of time by analysis of variance (ANOVA). The (+) / (-) DENV RNA ratios
14 240 were analyzed by ANOVA as a function of time, tissue and their interaction. Time post virus exposure
15 16 241 was considered an ordinal variable. Normal distribution of the ANOVA residuals was assessed using
17 18 242 Shapiro-Wilk normality test. Pairwise differences of viral load or RNA ratios between time points were
19 20 243 determined by multiple t-tests with Holm correction for multiple testing. All statistical analyses were
21 22 244 performed in the R environment (<https://www.r-project.org/>, version 3.2.0).
23 24 245

26 246

27 247

30 248 **3. Results**

31 249

34 250 *3.1. Strand-specific quantification of DENV RNA*

35 251 We used a DNase-treated, synthetic DENV (+) RNA fragment of 101 bp in the *E* gene as a
36 37 252 template (concentration 1 × 10⁷ copies/μL) for reverse transcription (RT) in absence of primer to
38 39 253 measure the level of falsely-primed cDNA synthesis by the reverse transcriptase. The resulting cDNA
40 41 254 was quantified using qPCR with standard D1EnvF and D1EnvR primers (Table 1). In three replicate
42 43 255 reactions, primer-free reverse transcriptase activity resulted in an estimated mean of 2.4 × 10⁵
44 45 256 copies/μL of DENV RNA (Fig. 1A). Therefore, to avoid quantification of falsely-primed cDNA, we
46 47 257 performed separate RT reactions for DENV (-) or (+) RNA templates using either tag-D1EnvF or tag-
48 49 258 D1EnvR primers, respectively (Table 1). This allowed subsequent strand-specific quantification of
50 51 259 cDNA by qPCR using a tagged primer in combination with either D1EnvR or D1EnvF primers (Table
52 53 260 1). This resulted in a 146-bp amplicon consisting of 101 bp of the template sequence, 21 bp of the
54 55 261 strand-specific tag and 12 bp of helper sequences at both ends. Quantification of tagged cDNA
56 57 262 template with standard D1EnvF and D1EnvR primers was used as a positive control, resulting in the
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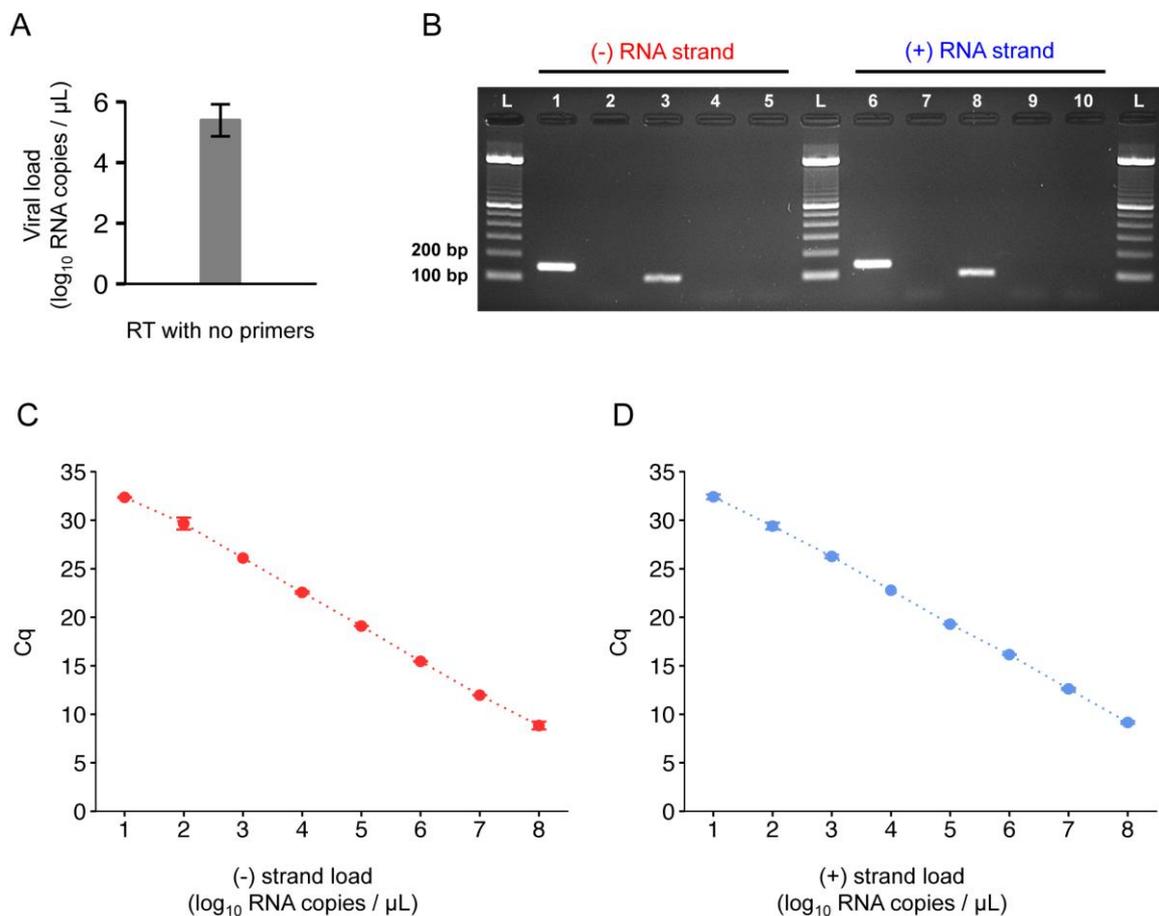
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263 expected 101-bp amplicon (Fig. 1B). We verified that the exonuclease treatment performed after the
 1 264 RT to eliminate unincorporated tagged primers effectively prevented non-strand-specific cDNA
 2 265 amplification by using only the D1EnvR or the D1EnvF primers during qPCR. As expected, no
 3 266 amplification was detected and no-template controls were also negative (Fig. 1B). We used ten-fold
 4 267 serial dilutions of DENV (+) and (-) RNA transcribed *in vitro* to evaluate the efficiency of our strand-
 5 268 specific RT-qPCR method. RT-qPCR efficiency was 99% and 97% for DENV (+) and (-) RNA,
 6 269 respectively, with a correlation fit (R^2) of 0.99 for both strands (Fig. 1C-D).



272 **Fig. 1. Strand-specific quantification of DENV RNA.** (A) Falsely-primed DENV cDNA detected in
 273 qPCR following RT in the absence of primer. The error bar represents the standard deviation of the
 274 mean ($n=3$). (B) Tagged primers allow strand-specific RT-qPCR amplification of DENV RNA. The
 275 picture shows amplification products separated by electrophoresis on a 2% agarose gel stained with
 276
 277

278 ethidium bromide. Amplification of a 146-bp tagged cDNA fragment using (-) or (+) strand-specific
1 279 primers is shown in lane 1 and lane 6, respectively. Absence of falsely-primed cDNA detection
2 3
4 280 following (-) or (+) strand-specific qPCR amplification of a cDNA template produced in the absence of
5
6 281 RT primers is shown in lane 2 and lane 7, respectively. Detection of a 101-bp cDNA fragment
7
8 282 following non-strand-specific qPCR amplification of (-) or (+) tagged cDNA template is shown in lane 3
9
10 283 and lane 8, respectively. Controls for RT primer digestion by Exonuclease I show no amplification of (-
11
12 284) and (+) tagged cDNA following qPCR with only the strand-specific primer not complementary to the
13
14 285 tag sequence in lane 4 and lane 9, respectively. No-template controls for tagged cDNA amplification
15
16 286 using (-) and (+) strand-specific primers are shown in lane 5 and lane 10, respectively. Product size
17
18 287 was estimated using a 100-bp size ladder (L). (C, D) RT-qPCR allows efficient and accurate
19
20 288 quantification of both DENV RNA strands. Standards curves used for DENV (-) RNA (C) and DENV
21
22 289 (+) RNA (D) quantification are shown. Error bars represent the standard deviation of the means ($n=2-3$
23
24 290 per template dilution level).

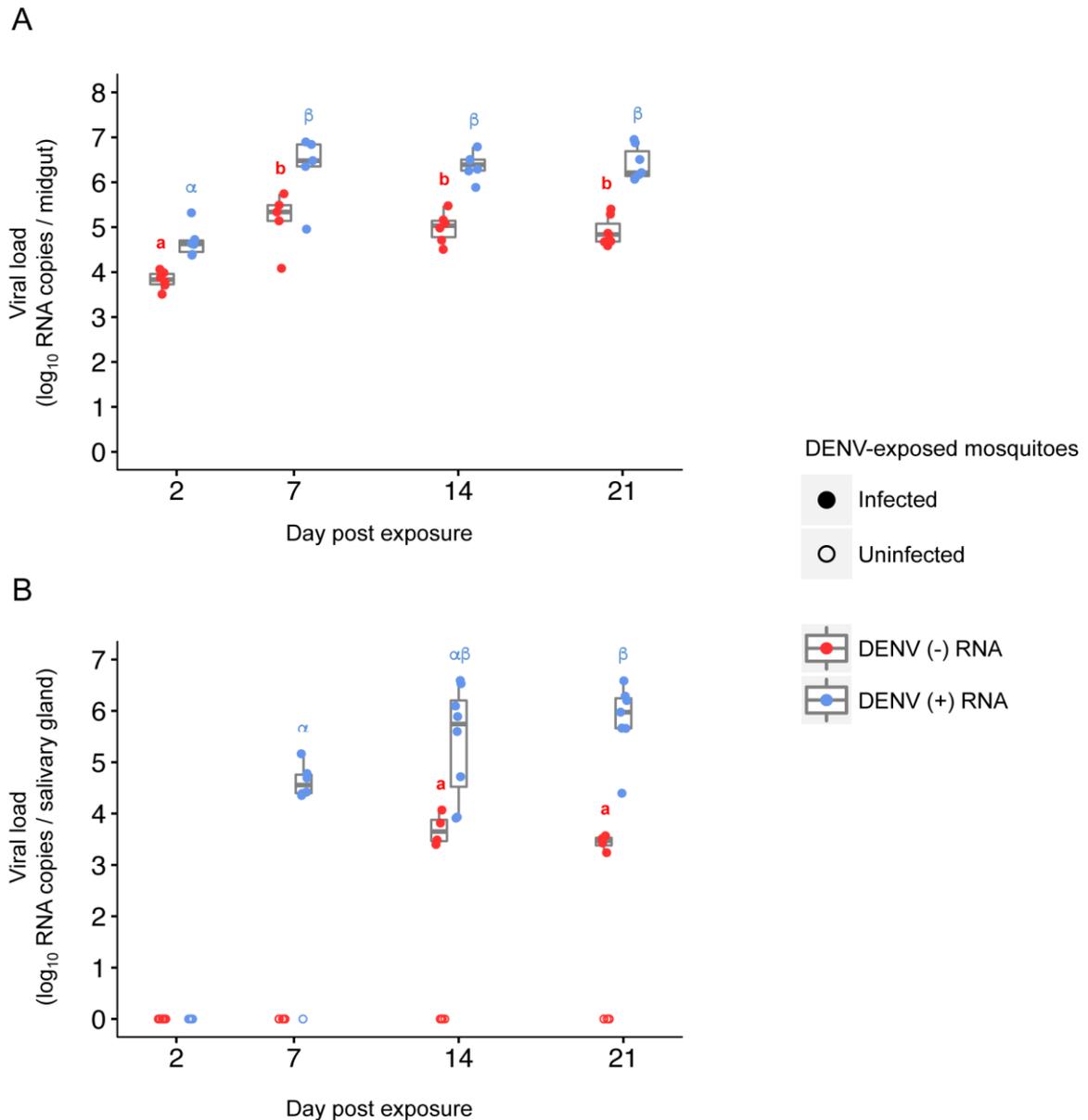
28 292 3.2. DENV replicates and accumulates in *Ae. aegypti* salivary glands

30 293 Our strand-specific RT-qPCR assay allowed quantification of both DENV (+) and (-) RNA loads
31
32 294 isolated from the midgut and salivary glands of individual mosquitoes previously exposed to an
33
34 295 infectious blood meal (Fig. 2A). All the midguts tested ($n=24$) were infected, with both DENV (-) and
35
36 296 (+) RNA detected in all the samples regardless of the day post exposure (Fig. 2A). The midgut viral
37
38 297 load varied significantly according to the time post exposure for DENV (-) and (+) RNA (ANOVA: $P <$
39
40 298 0.0001). There was a significant increase (multiple t-test: $P < 0.05$) in both DENV (+) and (-) RNA
41
42 299 loads between day 2 ($n=5$) and day 7 ($n=5$) post exposure. The viral load did not significantly vary
43
44 300 between day 7 ($n=6$), day 14 ($n=6$) and day 21 ($n=7$) post exposure (Fig. 2A).

46 301 All mosquitoes tested were negative for DENV RNA in salivary glands on day 2 post exposure
47
48 302 ($n=9$) (Fig. 2B). On day 7 post exposure, only DENV (+) RNA was detected in salivary glands in 6 out
49
50 303 of 7 individuals (Fig. 2B). All the mosquitoes tested on day 14 ($n=8$) and on day 21 ($n=7$) were positive
51
52 304 for DENV (+) RNA in salivary glands (Fig. 2B). DENV (-) RNA, however, was only detected in salivary
53
54 305 glands in 4 out of 8 individuals on day 14 post exposure, and in 4 out of 7 individuals on day 21 post
55
56 306 exposure (Fig. 2B). The analysis of viral load as a function of time post exposure indicated that DENV
57
58 307 (-) RNA load did not significantly vary between day 14 and day 21 post exposure (ANOVA: $P = 0.18$).

308 However, DENV (+) RNA load increased significantly over time (ANOVA: $P = 0.01$), indicating that
 1
 2 309 genomic viral RNA accumulates in salivary glands. DENV (+) RNA load was significantly lower
 3
 4 310 (multiple t-test: $P < 0.05$) on day 7 than on day 21 post exposure (Fig. 2B). Together, these results
 5
 6 311 indicate that DENV actively replicates and accumulates in mosquito salivary glands. Accordingly, we
 7
 8 312 observed accumulation of viral proteins by immunofluorescence assay in salivary glands (Fig. S1).

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 10 313



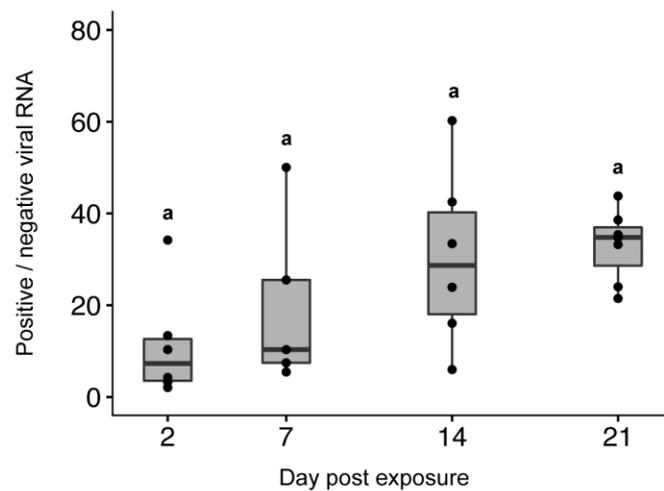
51
 52 314

53
 54 315 **Fig. 2. Strand-specific DENV RNA loads in midguts and salivary glands of *Ae. aegypti*.** Boxplots
 55
 56 316 show the amount of DENV (+) RNA (blue circles) or DENV (-) RNA (red circles) quantified by strand-
 57
 58 317 specific RT-qPCR in individual midguts (**A**) or salivary glands (**B**). Solid circles represent DENV RNA
 59
 60 318 positive samples and open circles represent individuals with no detectable viral RNA. Latin letters (a-

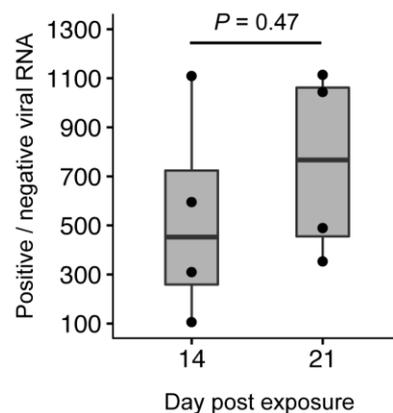
319 b) and Greek letters (α - β) above the graph indicate statistically significant differences between time
320 points (multiple t-tests: $P < 0.05$) in (-) RNA loads and (+) RNA loads, respectively. Viral loads are not
321 significantly different between time points sharing a letter.

322
323 In individual mosquito tissues in which both strands of DENV RNA were detected, the (+) / (-) ratio did
324 not vary according to time post exposure neither in the midgut nor in the salivary glands (Fig. 3). In the
325 midgut, the mean (+) / (-) RNA ratio was 30 on day 14 and 33 on day 21 (Fig. 3A). In the salivary
326 glands, it was 530 on day 14 and 750 on day 21 (Fig. 3B). Whereas the time post exposure had no
327 effect (ANOVA: $P = 0.19$), the mean (+) / (-) RNA ratio was significantly higher in the salivary glands
328 than in the midgut (ANOVA: $P < 0.0001$).

A



B



329

330 **Fig. 3.** Ratio of positive to negative DENV RNA strands in *Ae. aegypti* midgut and salivary glands.
1
2 331 Boxplots represent the ratio of (+) / (-) RNA strands in the midgut (A) and in the salivary glands (B) of
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4 332 individual mosquitoes at different time points post exposure. The same letter above the boxplots of
5
6 333 panel A indicate the lack of statistically significant differences (multiple t-tests: $P > 0.05$) in the mean
7
8 334 viral load between time points. The P -value above panel B was obtained by t-test.

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13 14 337 **4. Discussion**

15
16 338 We used a strand-specific RT-qPCR method to detect and quantify genomic and replicative RNA
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18 339 forms of a low-passage DENV-1 isolate in the midgut and in the salivary glands of individual *Ae.*
19
20 340 *aegypti* mosquitoes from a field-derived population. An earlier study on DENV replication in *Ae.*
21
22 341 *aegypti* focused on the midgut and did not use a strand-specific method (Richardson et al., 2006). This
23
24 342 study likely quantified falsely-primed DENV cDNA and therefore overestimated viral replication. We
25
26 343 detected DENV replicative RNA in the midgut of *Ae. aegypti* using a strand-specific method and
27
28 344 confirmed that this tissue is an active DENV replication site. This is consistent with the detection in
29
30 345 mosquito cell culture and in the mosquito midgut of virus-derived small interfering RNAs produced by
31
32 346 the antiviral RNA interference pathway that align to both (+) and (-) strands of the DENV genome
33
34 347 (Hess et al., 2011; Sánchez-Vargas et al., 2009; Schirtzinger et al., 2015). In agreement with previous
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36 348 studies, the amount of genomic DENV RNA that we detected in the midgut increased until day 7 post
37
38 349 exposure but remained stable thereafter (Amuzu et al., 2015; Choy et al., 2015; Salazar et al., 2007;
39
40 350 Sánchez-Vargas et al., 2009). DENV (-) RNA was detected in the midgut as soon as two days post
41
42 351 exposure. Replicative RNA load remained stable from day 7 to day 21 post exposure indicating that
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44 352 DENV persistently replicates in the midgut. In contrast with the RNA load, DENV infectious titer in the
45
46 353 midgut decreases during the late stage of infection (Salazar et al., 2007), which is partly attributed to
47
48 354 the action of the ubiquitin-proteasome pathway (Choy et al., 2015).

49
50 355 In salivary glands, we detected DENV (+) RNA from day 7 and DENV (-) RNA from day 14 post
51
52 356 virus exposure. DENV (+) RNA significantly accumulated from day 7 to day 21 post virus exposure.
53
54 357 Together, this provides strong evidence that DENV replicates within the salivary glands, a key tissue
55
56 358 for virus transmission. Unlike the midgut samples, some salivary gland samples were positive for
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58 359 DENV genomic RNA in the absence of detectable (-) RNA. This discrepancy could reflect either a
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360 delay in DENV replication initiation, or insufficient sensitivity of the assay to detect initially low levels of
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2 361 replicative RNA. Interestingly, we noticed that the (+) / (-) RNA ratio was significantly lower in the
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4 362 midgut than in the salivary glands. This suggests that the dynamics of DENV replication may differ
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6 363 between mosquito tissues.

7
8 364 Direct evidence for DENV replication in *Ae. aegypti* salivary glands has important implications for
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10 365 DENV fitness and evolution. Despite anatomical barriers that result in population bottlenecks during
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12 366 mosquito infection, active replication in salivary glands likely replenishes DENV genetic diversity prior
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14 367 to transmission to the next human host. This helps to explain the relatively high level of DENV genetic
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16 368 diversity observed in salivary glands (Lambrechts and Lequime, 2016; Lequime et al., 2016), similar to
17
18 369 the levels of DENV genetic diversity measured in human plasma (Sim et al., 2015). Genetic diversity
19
20 370 generated by *de novo* mutations during replication, however, does not prevent genetic drift due to
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22 371 population bottlenecks. As a result, salivary glands from individual mosquitoes contain unique viral
23
24 372 populations (Lequime et al., 2016; Sim et al., 2015). For ethical reasons, evolutionary forces acting on
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26 373 DENV populations during mosquito-to-human transmission cannot be experimentally examined, but
27
28 374 DENV genetic diversity in salivary secretions collected *in vitro* could be a reasonable proxy.

29
30 375 Another underexplored area that deserves attention is the cellular biology of DENV-infected
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32 376 salivary glands. DENV tropism within mosquito salivary glands is heterogeneous (Linthicum et al.,
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34 377 1996; Raquin et al., 2012; Salazar et al., 2007) potentially due to the distribution of putative salivary
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36 378 gland receptors (Cao-Lormeau, 2009). Ultra structural studies of mosquito salivary glands infected by
37
38 379 DENV documented the absence of cytopathic effects, the lateral lobes being first infected with a
39
40 380 relative small number of viral particles localized in the cytoplasm and the apical cavities of acinar cells,
41
42 381 as well as in the salivary duct (Linthicum et al., 1996; Salazar et al., 2007; Sriurairatna and
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44 382 Bhamarapravati, 1977). Interestingly, the pattern of salivary gland infection depends on the specific
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46 383 vector/arbovirus association (Girard et al., 2005, 2007; Scott et al., 1984; Soekiman et al., 1986;
47
48 384 Sriurairatna and Bhamarapravati, 1977; Vega-Rúa et al., 2015). Further studies combining molecular
49
50 385 detection of DENV and ultra structural observations will be necessary to reach a more thorough
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52 386 understanding of intimate interactions between DENV and its mosquito vector.

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56 388 **Acknowledgments**

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17
18 398 the manuscript.

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23 24 401 **5. References**

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22 580 **Fig. S1. Immunofluorescence labelling of DENV E protein in *Ae. aegypti* salivary glands.** DENV

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24 581 E antigen was detected using Alexa488-coupled secondary antibody (green) in salivary glands on day

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26 582 7, day 14 and day 21 post DENV exposure. Nuclei in salivary gland cells were stained by DAPI (blue).

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28 583 Preparations were observed under an epifluorescence microscope at 10x magnification (scale bar =

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30 584 400 µm).

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Figure1
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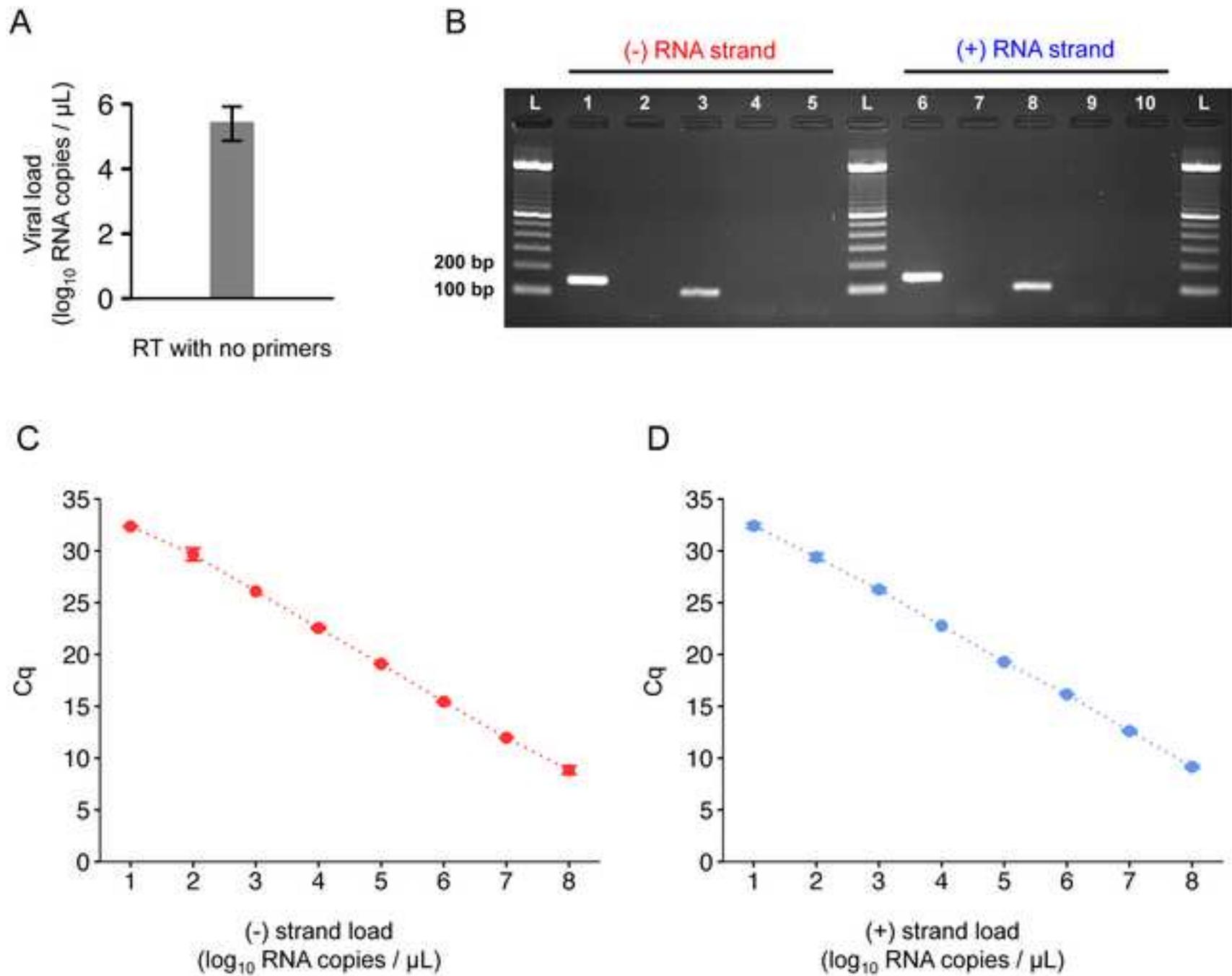
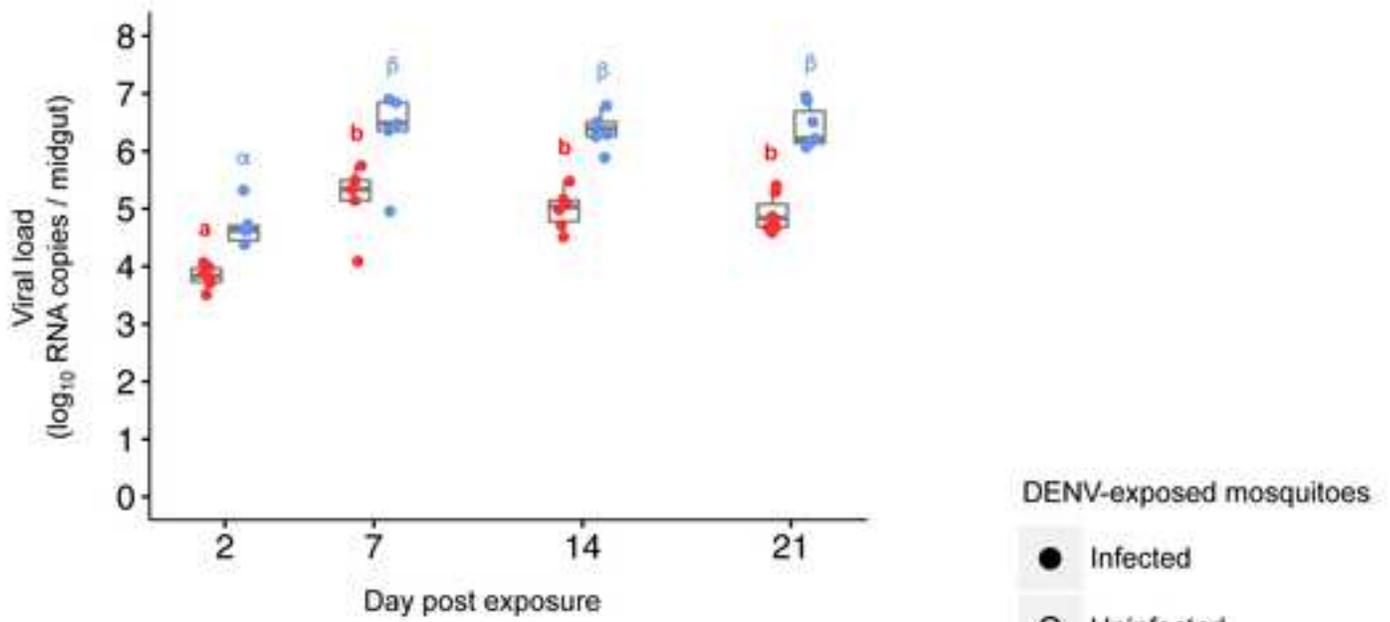


Figure 2

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B

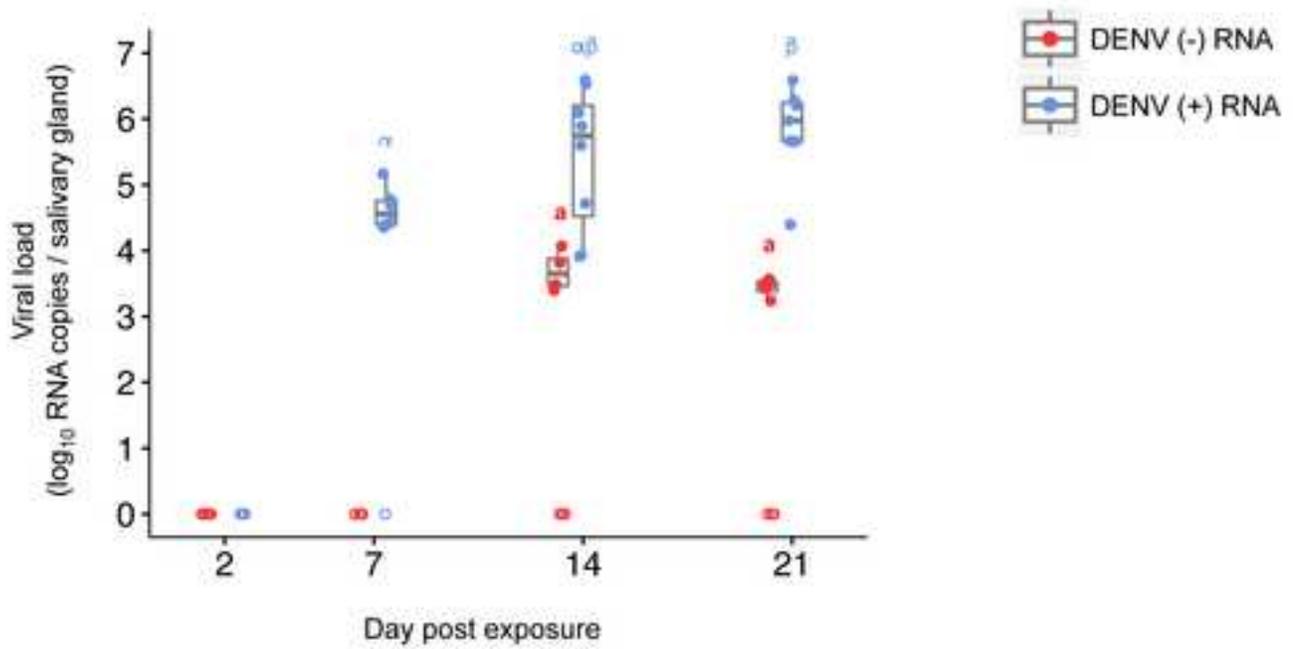
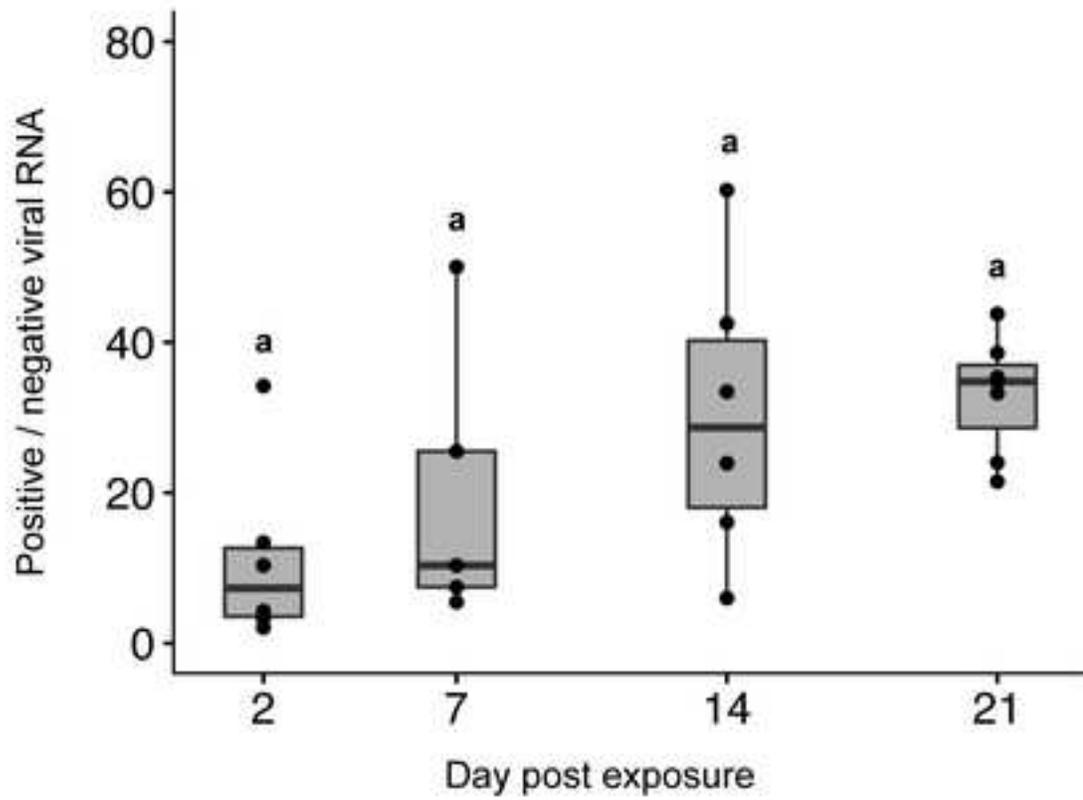
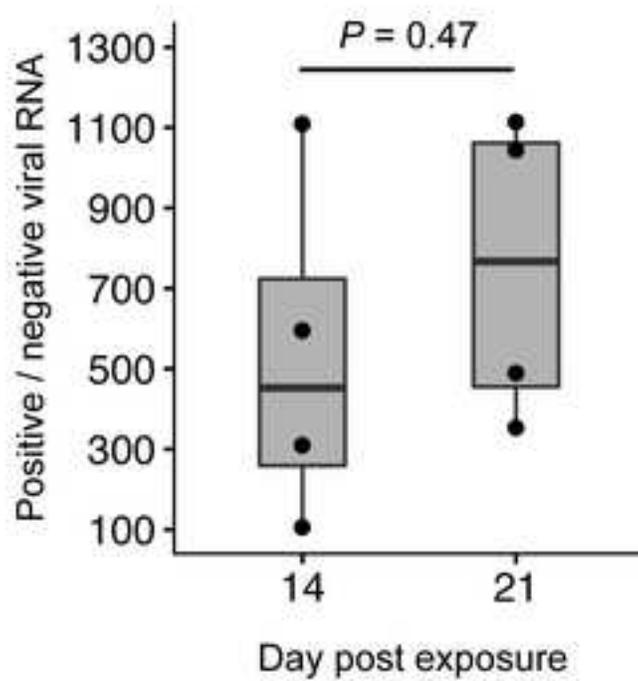


Figure3
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A



B



FigureS1
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