Challenges in the Biotechnological Implementation of Oral RNA Interference as an Antiviral Strategy in *Aedes aegypti*

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Abstract

Mosquitoes, particularly *Aedes aegypti*, are critical vectors for globally significant pathogenic viruses. This study examines the limitations of oral RNA interference (RNAi) as a strategy to disrupt viral transmission by *Ae. aegypti*. We hypothesized that double-stranded RNA (dsRNA) targeting the Zika virus (ZIKV) or chikungunya virus (CHIKV) genomes produced by engineered bacterial symbionts could trigger an antiviral response. Mosquitoes mono-colonized with *Escherichia coli* producing dsZIK or dsCHIK did not display reduced viral titers following exposure to virus-contaminated bloodmeals and failed to generate dsZIK- or dsCHIK-derived small interfering RNAs. To address potential limitations of bacterial dsRNA release, we explored dsRNA inoculation via feeding and injection. While viral replication was impeded in mosquitoes injected with dsZIK or dsCHIK, no antiviral effect was observed in dsRNA-fed mosquitoes. These findings highlight complexities of implementing oral RNAi as an antiviral strategy in *Ae. aegypti* and warrant further exploration of local and systemic RNAi mechanisms.

Keywords

Aedes aegypti, arbovirus, oral RNA interference, small RNA, paratransgenesis

Introduction

Mosquitoes are vectors for several arthropod-borne viruses (or arboviruses), which are responsible for multiple outbreaks worldwide. Over the past decade, the emergence or re-emergence of viruses such as dengue (DENV), Zika (ZIKV), or chikungunya (CHIKV) viruses, together with the current lack of vaccines for most of these viruses and the emergence of insecticide resistant mosquito populations has necessitated the development of new strategies to control the spread of these pathogens and their vectors.

Mosquito vector competence (*i.e.*, the ability of a mosquito to acquire and transmit a virus) is dependent on efficient viral replication and dissemination from the gut to the salivary glands, where the virus must enter the saliva to be transmitted to the next vertebrate host during subsequent blood-feeding. One of the mechanisms that mosquitoes use to control viral replication is RNA interference (RNAi), which is triggered by viral double-stranded RNA (dsRNA) intermediates produced during RNA virus replication. The RNase Dicer-2 binds dsRNAs and processes them into 21 nucleotide (nt) small-interfering RNAs (siRNAs)[1]. These are used by Argonaute-2 as sequence-specific guides to target complementary viral RNAs, thereby hindering viral replication[2–5]. Key studies in *Aedes aegypti* mosquitoes showed that a protective RNAi-mediated antiviral response could be triggered by exposure to dsRNA molecules corresponding to portions of the DENV or ZIKV genomes either by transgenic production of dsDENV or by injection of dsZIK[6,7], validating the potential use of exogenous dsRNA molecules to limit arbovirus infection.

During viral infection through a bloodmeal, the mosquito midgut represents the first mechanical and immunological barrier encountered by the virus and crossing this barrier is critical to establish an infection in the mosquito. Thus, we reasoned that blocking viral replication in the midgut could be an effective way to limit viral transmission and we hypothesized that bacterial symbionts in the mosquito gut could be used as a platform to produce and deliver dsRNA to mosquitoes and stimulate a prophylactic RNAi-based antiviral response. Indeed, a recent study in honeybees showed the possibility of reducing viral replication through colonization with an antiviral dsRNA-producing symbiont[8]. When it comes to mosquitoes, numerous studies have demonstrated the potential for gene silencing by introducing dsRNA through feeding or using microorganisms that produce dsRNA[9–14]. While a handful of studies have pointed out challenges like low efficiency and variability in oral RNA interference in mosquitoes[15,16], it's noteworthy that none of these investigations have explored the concept of microbiota-mediated oral delivery of antiviral dsRNA.

Paratransgenesis is the genetic modification of a symbiotic microorganism that is associated with a disease vector with the aim of disrupting the transmission of pathogens carried by the vector. This approach offers the advantage of potential self-sustainability, as the targeted microorganism is typically selected to undergo effective horizontal (between individuals) and vertical (from parents to progeny) transmission[17]. Various paratransgenic methods have been proposed to reduce *Plasmodium* transmission by *Anopheles* mosquitoes, including the use symbiotic bacteria engineered to produce anti-*Plasmodium* molecules or mosquito immune factors[18–20]. Additionally, other paratransgenic strategies have focused on interfering with mosquito development or

65 fertility[10,12,21]. However, there are currently no developed strategies to impede virus transmission
66 by *Aedes* mosquitoes through manipulation of their microbiota.

67 Here, we investigated the potential targeted induction of antiviral RNAi against ZIKV and CHIKV in
68 *Ae. aegypti* mosquitoes through colonization with bacteria capable of producing antiviral dsRNA.
69 We used *Escherichia coli* as a proof-of-concept and found that mosquitoes colonized by dsZIK- and
70 dsCHIKV-expressing bacteria did not exhibit reduced viral titers when infected with the
71 corresponding virus through a bloodmeal. Additionally, there was no discernible production of
72 siRNAs induced by the ingestion of bacteria-produced dsRNA. When we fed mosquitoes with naked
73 dsRNA, we did not observe enhanced antiviral immunity, likely due to the absence of an effective
74 antiviral RNAi-based response in the midgut and insufficient activation of systemic RNAi.

75 **Results**

76 *Colonization of mosquitoes with dsRNA-producing* Escherichia coli

77 To generate *E. coli* strains expressing antiviral dsRNA, we took advantage of the HT115 strain, which
78 has historically been used in *Caenorhabditis elegans* to induce RNAi. This strain is suitable for dsRNA
79 production as it possesses an isopropylthio-β-galactoside (IPTG)-inducible *T7 polymerase* gene and
80 has a mutation in the *rnc* allele encoding RNase III, resulting in reduced dsRNA degradation[22]. This
81 allows the synthesis of the target sequence by transcription from a DNA template containing the T7
82 promoter. To induce the production of dsRNA for ZIKV or CHIKV, we cloned the target viral
83 sequences between two directionally opposed T7 promoters and terminators in the T444T plasmid
84 to produce dsRNA by convergent transcription[23] (**Figure 1A, Supplementary Figure 1A**). To test
85 whether a unidirectionally-transcribed RNA with a hairpin secondary structure would result in a
86 more stable and effective dsRNA production, an inverted repeat of the CHIKV target sequence was
87 cloned in a pUC18 plasmid downstream from a T7 promoter. The inverted repeat consisted of the
88 CHIKV target sequence in both polarities separated by a non-viral spacer sequence (**Supplementary**
89 **Figure 1B**). The target viral sequences were selected for dsRNA production based on the following
90 criteria: (i) we excluded sequences that could produce 21mers with high similarity to any sequence
91 in the *Ae. aegypti* genome in order to avoid potential interference of endogenous gene expression;
92 (ii) we targeted regions corresponding to only partial coding regions of viral structural genes to
93 prevent translation of the target sequences into functional viral proteins; (iii) we avoided sequences
94 corresponding to noncoding subgenomic flaviviral RNA or viral proteins shown to repress mosquito
95 immunity[24–26](**Figure 1B, Supplementary Figure 1C**). A T444T plasmid containing a portion of the
96 GFP coding sequence instead of a target viral sequence was produced as a control.

97 To confirm the production of dsRNA by bacteria, RNA was extracted from overnight cultures of *E.*
98 *coli* HT115-T444T-ZIK1 induced or not with IPTG for 24 h. Northern blot with ZIK1 sense and
99 antisense probes was performed on bacterial total RNA and on dsZIK1 synthesized *in vitro* as a
100 positive control. The agarose gel showed a major 480 nt band for *in vitro* synthesized dsZIK1,
101 although a higher ~900 nt band was visible (**Supplementary Figure 2A**). In the corresponding
102 northern blot, ZIK1 sense and antisense probes bound to both 480 and ~900 nt bands
103 (**Supplementary Figure 2B**), suggesting that the larger band is composed by dsRNA multimers or

104 higher order assemblies. These molecules are commonly found in both *in vitro* and *in vivo*
105 synthesized dsRNA with unknown effect on RNAi activity[27,28]. A signal at ~350 nt was detected by
106 sense and antisense probes in uninduced bacteria (**Supplementary Figure 2B**). This might represent
107 a truncated RNA transcribed by the T7 RNA Polymerase, whose transcription is directed by *lac*
108 operon control elements. This system is commonly known to be leaky and to show basal
109 transcription in absence of IPTG induction, especially in stationary phase cultures[29,30]. Bacteria
110 induced with IPTG showed a major ~650 nt band in the agarose gel (**Supplementary Figure 2A**),
111 which was recognized by both ZIK1 probes together with other transcription products spanning
112 from ~250 to ~900 nt (**Supplementary Figure 2B**). The major band at ~650 nt represents the main
113 ZIK1 transcript from the T444T-ZIK1 plasmid, which is 186 nt longer than the *in vitro* transcript due
114 to additional plasmid sequences flanking the *Not*I and *Age*I cloning sites. The ZIK1 sense probe
115 showed high specificity for the target ZIKV sequence, while no significant signal was visible in a
116 northern blot of total RNA extracted from overnight cultures of *E. coli* HT115-T444T-CHIK1
117 (**Supplementary Figure 2C-D**). Altogether the northern blot analysis revealed abundant production
118 of dsRNA by the modified bacteria.

119 To test the mosquito colonization properties of *E. coli* HT115, the transstadial transmissibility of *E.*
120 *coli* HT115 (from larvae to adults), and the stability of the T444T plasmid *in vivo* in the absence of
121 antibiotic selection, we produced *Ae. aegypti* larvae mono-colonized by *E. coli* HT115-T444T-ZIK1
122 (**Figure 1C**). Mono-colonization at the larval stage was used to maximize the exposure of mosquitoes
123 to dsRNA. *E. coli* HT115 was added to axenic larvae obtained from surface sterilized eggs[31]. The
124 HT115 strain was able to support standard larval development, as previously described for other *E.*
125 *coli* strains[31,32]. To assess the transstadial transmission of *E. coli* HT115, we measured bacterial loads
126 in adult mosquitoes originating from mono-colonized larvae kept in non-sterile conditions after
127 adult emergence. When mosquitoes were provided only with sterile sucrose, *E. coli* HT115 carrying
128 the T444T-ZIK1 plasmid could not be isolated from whole mosquitoes collected one or four days
129 post-emergence, showing the low transstadial transmissibility of this strain (**Figure 1D**). When
130 mosquitoes were provided with a sucrose solution contaminated with *E. coli* HT115 carrying the
131 T444T-ZIK1 plasmid, bacteria could be isolated from whole mosquitoes at both tested time points,
132 with 100 % of mosquitoes colonized four days post-emergence. Bacterial loads were comparable
133 when measured on antibiotic-free plates or plates containing ampicillin to select for bacteria
134 carrying the T444T-ZIK1 plasmid (Wilcoxon test: sterile sugar day 1, p = NA; sterile sugar day 4, p =
135 0.38; bacteria supplemented day 1, p = 1; bacteria supplemented day 4, p = 0.71), indicating that *E.*
136 *coli* HT115 could stably colonize adult mosquitoes, it represented most of the bacteria present at
137 the adult stage, and it could maintain the plasmid *in vivo* for at least four days.

138 *Mosquitoes colonized with dsRNA-producing bacteria do not show reduced viral titers or production*
139 *of dsRNA-specific siRNAs*

140 To assess the effect of the bacterial-produced dsRNA on viral infections, we exposed mosquitoes to
141 a ZIKV- or CHIKV-containing infectious bloodmeal. We reasoned that mosquitoes mono-colonized
142 at the larval stage with the dsRNA-producing bacteria would be the best model to work with, as
143 they were exposed to the dsRNA during their entire development. We produced larvae mono-

144 colonized with *E. coli* HT115 carrying the T444T-GFP, T444T-ZIK1, T444T-CHIK1, or pUC18-CHIK1
145 plasmids and we provided the same bacteria to the adult mosquitoes that developed from these
146 larvae (**Figure 2A, Supplementary Figure 1D**). To activate transcription of the dsRNA from the T7
147 promoter(s), IPTG was added to the bacterial culture medium, the larval water, and the sucrose
148 solution provided to adults. Mosquitoes were offered bacteria-contaminated sucrose from their
149 emergence onward. Four- to five-day-old mosquitoes were blood-fed with a ZIKV- or CHIKV-
150 containing infectious bloodmeal and provided with bacteria-contaminated sucrose after the
151 bloodmeal. For ZIKV, viral titers were measured in the midguts, carcasses, and heads of mosquitoes
152 at six and ten days post-infection to assess infection and dissemination rates. We could not detect
153 a significant difference in ZIKV titers for mosquitoes colonized by dsZIK1-producing *E. coli* compared
154 to mosquitoes colonized by dsGFP-producing *E. coli* in any tissue at any time-point (**Figure 2B, C,**
155 Wilcoxon test on log transformed viral titers: day 6 midguts $p = 0.54$, carcasses $p = 0.72$, heads $p =$
156 0.87; day 10 midguts $p = 0.09$, carcasses $p = 0.29$, heads $p = 0.16$). The prevalence of infection was
157 also similar between the two conditions (Wilcoxon test: day 6 midguts p = NA, carcasses $p = 0.71$,
158 heads $p = 0.63$; day 10 midguts $p = 0.55$, carcasses $p = 0.35$, heads $p = 0.29$). These data indicate that
159 bacteria-produced dsRNA did not induce a protective RNAi-based antiviral response.

160 For CHIKV, viral titers were measured in the bodies and heads of mosquitoes at two and five days
161 post-infection. The results indicated that CHIKV titers were not specifically reduced by the exposure
162 of mosquitoes to dsCHIKV. Contrary to our hypothesis that dsRNA-producing bacteria could induce
163 a sequence-specific prophylactic antiviral response, CHIKV titers were higher in the bodies and
164 heads of mosquitoes colonized with bacteria carrying the T444T-CHIK1 or pUC18-CHIK1 plasmids
165 than those of mosquitoes colonized with bacteria carrying the T444T-GFP plasmid at day two post-
166 infection, while viral loads were similar in mosquitoes colonized with the three types of bacteria at
167 day five post-infection (**Supplementary Figure 1E-F**, Kruskal-Wallis test on log transformed viral
168 titers: day 2 body $p < 0.001$; day 2 head p = 0.004; day 5 body $p = 0.3$, ns; day 5 head $p = 0.8$, ns;
169 Kruskal-Wallis test on prevalence day 2 body p = NA; day 2 head p = 0.003; day 5 body p = NA; day
170 5 head $p = 0.5$, ns; see Supplementary Table 2 for individual comparisons). Taken together, these
171 results suggest that colonization of mosquitoes with dsRNA-producing bacteria did not trigger an
172 efficient antiviral response against flaviviruses or alphaviruses.

173 To verify whether the mosquito RNAi machinery could produce siRNAs from the dsRNA produced
174 by bacteria, we sequenced small RNAs from adult mosquitoes mono-colonized for two days with *E.*
175 *coli* HT115 carrying the T444T-ZIK1 plasmid (**Figure 3A**). Reads mapping to the ZIKV target sequence
176 on both positive and negative strands were obtained, with an enrichment of positive strand-reads
177 (**Figure 3B**). This further confirmed that dsRNA is effectively transcribed *in vivo* by *E. coli* HT115.
178 Most of the reads mapping to the target viral sequence fell within the expected range of 20 to 28
179 nt, but no enrichment at 21 nt was observed, suggesting that siRNAs were not produced from the
180 bacterial-produced dsRNA (**Figure 3C**, Z-score positive sense reads: 1.67, $p = 0.09$, ns; Z-score
181 negative sense reads: 1.67, $p = 0.10$, ns). As our mosquito colony is persistently infected with the
182 insect specific virus Phasi-Charoen Like Virus (PCLV), we used this natural infection as a quality
183 control for our small RNA sequencing. We observed that 21 nt reads were significantly enriched
184 among all small RNA reads from 18 to 33 nt, suggesting that PCLV-specific siRNAs were produced in

185 these mosquitoes and were detected by our small RNA sequencing pipeline (**Supplementary Figure**
186 **3A-F**).

187 To assess whether different cloning strategies would result in an improved dsRNA-induced
188 response, we colonized mosquitoes with *E. coli* HT115 bacteria carrying the pUC18 hairpin-
189 producing plasmid containing either the 481 bp ZIK1 sequence (**Figure 3D**) or a shorter (175 bp) ZIK1
190 sequence (**Figure 3G**). We did not find evidence for siRNA production from dsRNA derived from
191 either the long (**Figure 3E-F**, Z-score positive sense reads: 0.86, p = 0.39, ns; Z-score negative sense
192 reads: 0.33, p = 0.74, ns) or short construct (**Figure 3H-I**, Z-score positive sense reads: 1.12, p = 0.26,
193 ns; Z-score negative sense reads: -0.08, p = NA).

194 To assess whether the observed lack of an antiviral RNAi-based response was due to poor release
195 of dsRNA from live bacteria, we sequenced small RNAs from mosquitoes supplemented with the
196 same *E. coli* strains heat-killed before supplementation, as heat-killed bacteria were previously used
197 to induce oral RNAi in mosquitoes[21]. We did not find evidence for target-specific siRNA production
198 in any of the samples produced from mosquitoes supplemented with heat-killed bacteria
199 (**Supplementary Figure 4A-I**, T444T-ZIK1: Z-score positive sense reads: 0.31, p = 0.76, ns; Z-score
200 negative sense reads: -0.43, p = NA; pUC18-ZIK1: Z-score positive sense reads: 0.29, p = 0.77, ns; Z-
201 score negative sense reads: -0.31, p = NA; pUC18-ZIK1-short: Z-score positive sense reads: 2.27, p =
202 0.02; Z-score negative sense reads: 0.60, p = 0.55, ns). We conclude that dsRNA produced by
203 bacteria failed to interact with the mosquito RNAi machinery and to induce the production of target-
204 specific siRNAs.

205 *Oral supplementation of* in vitro *synthesized dsRNA does not induce an antiviral response in adult*
206 *mosquitoes*

207 The lack of an interaction between the bacterial-produced dsRNA and the mosquito RNAi machinery
208 could be due to several factors, such as lack of internalization of dsRNA by mosquito gut cells or low
209 stability of dsRNA in the mosquito gut. To investigate whether dsRNA could be internalized by the
210 mosquito gut epithelium and to overcome the potential limiting factor of insufficient release of
211 dsRNA from bacteria, we produced Cy3-labeled dsZIK1 (dsZIK1-Cy3) *in vitro*. Due to the low quantity
212 of dsZIK1-Cy3 that we obtained, we tested whether mosquitoes could feed on a 200 µL sucrose
213 solution provided on a microtube cap (to prevent dilution of dsZIK1-Cy3 in a standard feeding
214 volume). To determine the rate of feeding with this setup, we provided mosquitoes with a sucrose
215 solution containing a blue dye. Mosquitoes that fed on the solution showed a readily visible blue
216 abdomen (**Figure 4A**). We dissected these mosquitoes after one or two days of feeding and verified
217 the presence of blue dye in the crop (where the sugar is stored before digestion) and midgut. While
218 after one day of feeding 67 % (10/15) of mosquitoes showed a blue crop and 80 % (12/15) a blue
219 midgut, after two days all mosquitoes (15/15) were found to have fed on the sucrose solution and
220 showed both a blue crop and midgut. dsZIK1-Cy3 was thus provided to mosquitoes for 48 h in a
221 sucrose solution with the setup described above, but without the blue dye that could potentially
222 interfere with fluorescence detection. Adult mosquitoes fed with dsZIK1-Cy3 were dissected and
223 guts (including crop and hindgut) were observed under a confocal microscope. Mosquitoes fed on
224 sucrose containing dsZIK1-Cy3 showed bright red fluorescence in all gut compartments (**Figure 4B-**

225 **C**). Interestingly, cells displaying red fluorescence in both the cytoplasm and nucleus were observed
226 in the midgut, which is the region in which blood is stored during digestion and that is primarily
227 infected by viruses ingested with blood (**Figure 4D**). These data indicate that at least some mosquito
228 gut epithelial cells can internalize dsRNA.

229 To explore if *in vitro*-synthesized dsRNA could induce an antiviral RNAi-based response when orally
230 administered to mosquitoes, we fed mosquitoes with non-labeled dsZIK1 and, as a control, with
231 dsGFP (**Figure 4E**). To confirm that the same dsRNA could induce an antiviral effect when introduced
232 systemically into the mosquito body, we injected mosquitoes with dsZIK1 or dsGFP. Four days after
233 the oral supplementation or the injection, we provided mosquitoes with a ZIKV-containing
234 infectious bloodmeal and measured viral titers in whole mosquitoes by plaque assays at seven and
235 14-days post-infection. We found that oral supplementation of dsZIK1 did not decrease viral titers
236 in mosquitoes collected at either time point, while injection of dsZIK1 completely blocked viral
237 replication at both timepoints (**Figure 4F-G**, Kruskal-Wallis test on log transformed viral titers: day
238 7 $p < 0.001$; day 14 $p < 0.001$; Kruskal-Wallis test on prevalence: day 7 $p < 0.001$; day 14 $p < 0.001$;
239 see Supplementary Table 2 for individual comparisons). In line with these results, mosquitoes fed
240 with dsCHIK1 did not show any significant reduction in viral titers after infection with CHIKV, while
241 mosquitoes injected with dsCHIK1 showed reduced viral titers in whole bodies two days post-
242 infection and marginally significant reduced titers five days post-infection (**Supplementary Figure**
243 **5A-C**, Kruskal-Wallis test on log transformed viral titers: day 2 $p < 0.001$; day 5 $p = 0.04$; Kruskal-
244 Wallis test on prevalence: day 2 $p = 0.18$; day 5 $p = 0.07$; see Supplementary Table 2 for individual
245 comparisons).

246 To verify that siRNAs were produced from dsRNA introduced into mosquitoes by injection or
247 feeding, we performed small RNA sequencing of RNA from whole mosquito samples. We found that
248 21 nt siRNAs were enriched in RNA from whole bodies of mosquitoes fed or injected with dsRNA
249 four days before sampling (**Figure 4H-K,** fed, Z-score positive sense reads: 2.35, $p = 0.02$; fed, Z-score
250 negative sense reads: 2.21, $p = 0.03$; injected, Z-score positive sense reads: 2.80, $p = 0.005$; injected,
251 Z-score negative sense reads: 2.94, $p = 0.003$).

252 To investigate the localization and integrity of dsRNA in fed and injected mosquitoes, RNA was
253 extracted from midguts, carcasses, and heads of mosquitoes collected two and four days after
254 feeding/injection (**Supplementary Figure 6A**). Northern blot analysis of samples collected at day
255 two indicated that RNA corresponding to the dsZIK1 sequence was detectable in the midgut,
256 carcass, and head of mosquitoes fed with dsRNA, while only in the carcass and head of mosquitoes
257 injected with dsRNA (**Supplementary Figure 6C**). At day four, a signal from dsZIK1 was detected in
258 the carcass of both fed and injected mosquitoes, and in the midgut of fed mosquitoes
259 (**Supplementary Figure 6C**). It is important to mention that, while the crop was not included in any
260 sample because of the presumable high concentration of dsRNA in this organ, carcass samples
261 contained Malpighian tubules and hindguts, and head samples included the anterior part of the
262 digestive system. Therefore, we cannot determine whether the dsZIK1 signal visible in RNA samples
263 extracted from fed mosquitoes derives from dsRNA that is circulating in the haemocoel and entering

264 other organs or if it represents what is ingested by mosquitoes that fed on dsRNA. However, these
265 results confirmed that dsRNA was not degraded by midgut RNases in fed mosquitoes.

266 **Discussion**

267 In mosquitoes, targeted RNAi can be activated by introduction of dsRNA corresponding to target
268 sequences. This stimulates the production of target-specific siRNAs from the introduced dsRNA,
269 which are used to guide degradation of complementary RNA. When the introduced dsRNA is
270 designed to target viral RNA, introduction of dsRNA prior to infection can limit subsequent viral
271 replication in a sequence-specific manner. In this paper we tested whether bacteria colonizing the
272 mosquito midgut could be used as a platform to produce virus-specific dsRNA and stimulate an
273 enhanced antiviral response against arboviruses. We showed that the *E. coli* HT115 strain, originally
274 used in *C. elegans* to induce RNAi, is poorly transmitted from mono-colonized larvae to adult
275 mosquitoes but can stably colonize the mosquito gut when provided to adult mosquitoes through
276 sucrose supplementation (**Figure 1D**). However, we did not detect any significant reduction on ZIKV
277 or CHIKV titers in mosquitoes colonized by dsZIK- or dsCHIK-producing bacteria (**Figure 2B-C** and
278 **Supplementary Figure 1E-F**). Moreover, our small RNA sequencing results did not show evidence
279 for the production of 21 nt siRNAs specific for the viral target sequence in mosquitoes provided with
280 live or heat-killed dsRNA-producing bacteria (**Figure 3**, **Supplementary Figure 4**). We identified at
281 least three potential issues that may have limited the interaction of the bacterial-produced dsRNA
282 with the mosquito RNAi machinery: (i) insufficient production or release of dsRNA by bacteria in the
283 mosquito gut, (ii) low stability of dsRNA in the mosquito gut, and/or (iii) limited uptake of dsRNA
284 from mosquito gut cells.

285 Although we confirmed dsRNA production *in vivo* by bacteria (**Supplementary Figure 2**), we cannot
286 confirm whether dsRNA was released by bacteria in the mosquito gut. In previous experiments in *C.*
287 *elegans*, *E. coli* HT115 was used as a source of food for the nematode and undergoes cell lysis during
288 ingestion and release dsRNA[22,33]. It is unknown to what extent bacterial cells are lysed in the
289 mosquito gut. Live bacteria are needed at the larval stage to support mosquito development and
290 are found in the larval gut by two hours post-hatching, but dead bacteria can be found in the larval
291 gastric caeca and midgut by 16 hours post-hatching[34]. This suggests that, at least at the larval stage,
292 some bacteria undergo cell lysis in the mosquito gut and potentially release dsRNA. Live, culturable
293 bacteria have been isolated from the adult gut from both lab-reared and field-collected
294 mosquitoes[35–37] and an expansion of some members of the microbiota is observed 24 h after a
295 bloodmeal[38,39]. However, the relative proportions of live and dead bacteria in adult mosquitoes are
296 unknown.

297 To test whether production or release of dsRNA from bacteria was a limiting factor in our system,
298 we decided to orally administer naked dsRNA to mosquitoes. Northern blot analysis of different
299 mosquito tissues confirmed that dsRNA molecules were, at least in part, intact after being provided
300 to mosquitoes via oral supplementation for two days, indicating that orally acquired dsRNA is not
301 totally degraded by gut RNases (**Supplementary Figure 6C**). Although oral acquisition of dsZIK
302 induced production of dsZIK-derived siRNAs in mosquitoes (**Figure 4H-I**), it failed to trigger an
303 antiviral RNAi response and limit ZIKV replication (**Figure 4F-G**). In contrast, the same dsZIK provided

304 systemically though injection completely blocked viral replication (**Figure 4F-G**). This suggests that
305 dsRNA provided via oral supplementation is not able to induce an RNAi response capable of
306 inhibiting viral replication. Downstream from siRNA production, RNAi has been shown to have a
307 limited role in restricting DENV or ZIKV infection in the *Ae. aegypti* midgut, predominantly due to
308 the lack of expression of the dsRNA-binding protein, Loqs2, in this tissue[40]. This may explain why
309 siRNA produced after oral administration of dsRNA failed to produce an effective RNAi-based
310 response. To overcome this limitation, further applications involving oral RNAi should explore the
311 co-expression of Loqs2 in the mosquito midgut.

312 Cy3-labeled dsRNA provided orally to mosquitoes could be visualized in the crop, diverticula, and
313 some midgut cells, where fluorescence could be detected in both the cytoplasm and nucleus (**Figure**
314 **4B-D**). However, we did not investigate whether orally supplemented dsRNA could be found in other
315 tissues distal from the digestive system. A recent study described a similar strong fluorescence signal
316 in the alimentary canal of *Ae. aegypti* larvae and adults fed with fluorescently-labeled dsRNA[41].
317 Interestingly, this study showed that adult mosquitoes fed with fluorescently-labeled dsRNA did not
318 display fluorescence in other tissues, while mosquitoes intrathoracically injected with dsRNA
319 showed strong fluorescence in hemocytes, pericardial cells, and ovaries. Uptake of dsRNA and
320 amplification of the RNAi signal by hemocytes is essential for effective antiviral RNAi in *Drosophila*
321 *melanogaster*[42]. We can therefore speculate that the failure of orally acquired dsRNA to inhibit viral
322 replication in *Ae. aegypti* may be due to incorrect processing of this dsRNA, which may be
323 internalized by midgut cells, but not hemocytes or cells in other tissues relevant for an antiviral
324 response.

325 The only previous report of successful microbiota-mediated antiviral RNAi in insects was in
326 honeybees infected with deformed wing virus, where bees colonized by viral dsRNA-producing
327 microbiota were found to exhibit longer lifespans and reduced viral replication compared to bees
328 carrying dsGFP-producing bacteria[8]. However, mosquitoes and bees might have physiological
329 differences that could limit this application against arboviruses. For instance, the honeybee
330 microbiota almost exclusively colonizes the hindgut[43], while mosquitoes have high bacterial loads
331 mainly in the crop and midgut[44]. Differences in viral infection dynamics and/or dsRNA uptake by
332 different tissues might explain the different results obtained with *Apis millifera* and *Ae. aegypti*.

333 Over the last ten years, oral RNAi targeting essential mosquito genes has been proposed as a vector
334 control strategy to inhibit or block mosquito development and/or reproduction. Several methods
335 have been developed to deliver dsRNA to mosquito larvae or adults, including feeding of naked
336 dsRNA, colonization with microorganisms producing dsRNA, or feeding with nanoparticle-dsRNA
337 complexes (reviewed in [21]). However, some studies have reported high variability or low gene
338 silencing efficiency when dsRNA was provided via oral feeding[15,16] and it is likely that other studies
339 with contradictory or negative results were left unpublished. As emphasized in a recent discussion
340 by Devang Mehta, the importance of showcasing negative results for the advancement of science
341 cannot be overstated[45]. It is key to acknowledge that when negative results go unpublished, other
342 scientists can't learn from them and end up repeating failed experiments, leading to a waste of
343 public funds and a delay in genuine progress. Our results point to clear limitations of oral RNAi as a

344 strategy to disrupt viral transmission by mosquitoes and should be considered when developing
345 new strategies for vector control.

346

347 **Materials and Methods**

348 *Bacteria, viruses, cells and mosquitoes*

349 *E. coli* HT115(DE3, Genotype: F-, mcrA, mcrB, IN(rrnD-rrnE)1, rnc14::Tn10) was obtained from the
350 Caenorhabditis Genetics Center (CGC, University of Minnesota). For all experiments, bacteria were
351 inoculated from fresh colonies in Luria-Bertani (LB) broth supplemented with 20 µg/mL tetracycline.
352 The growth medium was supplemented with 100 µg/mL ampicillin for bacteria carrying the T444T
353 or pUC18 plasmids.

354 The ZIKV strain used in this study is the prototype African MR766 strain, derived from a previously
355 described infectious clone[46]. The CHIKV strain used is the Caribbean strain, Asian genotype originally
356 described in[47]. Viral stocks were produced in Vero E6 cells.

357 Vero E6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies)
358 supplemented with 10 % fetal bovine serum (Invitrogen) and 1 % Penicillin/Streptomycin (P/S, Life
359 Technologies) at 37 °C with 5 % CO_2.

360 *Ae. aegypti* mosquitoes belonged to the 28th–31st generation of a colony created in 2013 from
361 mosquitoes collected in the Kamphaeng Phet Province, Thailand[48]. They were maintained under
362 standard insectary conditions (27 ˚C, 70 % relative humidity, 12h:12h light/dark cycle). Mosquitoes
363 are routinely blood-fed with rabbit blood with an Hemoteck system (Hemotek Ltd). Conventionally
364 reared larvae were reared in 30x40 cm plastic trays with 1.5 L of dechlorinated tap water and ground
365 TetraMin fish food. Pupae were transferred in plastic cages (BugDorm) for emergence and adults
366 were provided with autoclaved 10 % sucrose solution.

367 *Selection of viral target sequences for dsRNA production*

368 Sequences of the ZIKV (MR766 strain) or CHIKV (Caribbean strain) genome spanning regions of the
369 polyprotein coding sequence corresponding to different mature proteins were selected and used as
370 a blastn[49] query against the *Ae. aegypti* genome in Vectorbase and all *Aedes* sequences in Genebank
371 to verify that they did not target any *Aedes* sequence. To exclude any interference by mosquito
372 small RNAs on the target viral sequence, small RNA library data previously produced in the lab from
373 *Ae. aegypti* uninfected mosquitoes were mapped on the ZIKV and CHIKV sequences. Any mosquito
374 small RNA was found to map on the selected viral sequences.

375 *Cloning*

376 The T444T plasmid was a gift from Tibor Vellai (Addgene plasmid #113081; RRID:
377 Addgene_113081[23]). GFP and target viral sequences were cloned into T444T using *Not*I and *Age*I
378 restriction sites and standard cloning techniques. The NEBuilder® HiFi DNA Assembly Kit (New
379 English Biolabs) was used to insert the following elements into the pUC18 plasmid: the T7 promoter,

380 *Not*I and *Age*I restriction sites, a spacer sequence corresponding to a 300 bp portion of an intron of
381 the *Drosophila melanogaster actin* gene (GenBank: M18829.1), *Nhe*I and *Kpn*I restriction sites and
382 the T7 terminator sequence. The final pUC18-spacer plasmid sequence and map are available upon
383 request. The same primers used for the cloning in T444T were used to clone GFP and target viral
384 sequences in pUC18-spacer using *Not*I and *Age*I restriction sites. To clone the inverted repeat, *Nhe*I
385 and *Kpn*I restriction sites were used. A shorter ZIK1 sequence (175 bp) was also cloned in pUC18-
386 spacer using the same cloning strategy. All primers used are listed in Supplementary Table 1. After
387 cloning, plasmid sequences were confirmed via Sanger sequencing from both sides of the insert.

388 *Mono-colonization of* Ae. aegypti *larvae with* E. coli *HT115*

389 Egg sterilization and larval mono-colonization were performed in sterile conditions working inside
390 a microbiological safety cabinet. *Ae. aegypti* eggs were surface sterilized with subsequent 5-minute
391 washes in 70 % ethanol, 1 % bleach, and 70 % ethanol. After three rinses in sterile Milli-Q water,
392 eggs were transferred to a 25 mL cell culture flask with vented cap overnight for hatching. On the
393 same day, *E. coli* HT115 carrying the selected plasmid was used to inoculate LB supplemented with
394 40 µM IPTG, 20 µg/mL tetracycline and 100 µg/mL ampicillin, and grown for ~ 16 h at 37 ˚C, 200 rpm
395 shaking. The following day, the bacterial culture was centrifuged and resuspended in sterile Milli-Q
396 water at a final concentration of 10^8 CFUs/mL. First instar larvae were placed in plastic boxes cleaned
397 with ethanol (200 larvae per box), together with 500 mL of bacterial suspension, 40 µM IPTG and 5
398 mL of sterile fish food (TetraMin baby, 50 mg/mL). Boxes were closed with filtered covers and kept
399 at 27 ˚C, 12h:12h light/dark cycle. Pupae were transferred into sterile boxes for emergence as in[32].
400 Larval water was plated on LB agar and LB agar supplemented with 100 µg/mL ampicillin to confirm
401 the mono-colonization of mosquitoes. The same number of colonies and the same colony
402 morphology were observed in both media.

403 To monitor the transstadial transmission of *E. coli* HT115-T444T-ZIK1 from larvae to adults, adult
404 mosquitoes originating from mono-colonized larvae were either provided with 10 % sterile sucrose
405 or with bacteria resuspended in 10 % sterile sucrose at a final concentration of 10^9 CFUs/mL. At days
406 one and four post-emergence, mosquitoes were collected, surface sterilized for 3 minutes in 70 %
407 ethanol, rinsed in sterile PBS, and individually homogenized in 200 µL LB. Mosquito homogenates
408 were serially diluted in LB and plated on LB agar and LB agar supplemented with 100 µg/mL
409 ampicillin. Plates were placed overnight at 37 ˚C and the number of colonies was counted to
410 estimate whole mosquito bacterial loads.

411 *Infection of mono-colonized mosquitoes with ZIKV or CHIKV*

412 Larvae mono-colonized by the selected strain of *E. coli* HT115 were produced as described above.
413 Larval water was supplemented with 40 µM IPTG to induce the transcription of the dsRNA by
414 bacteria. Adult mosquitoes were provided with a 10 % sterile sucrose solution supplemented with
415 10^9 CFUs/mL bacteria and 40 µM IPTG for four days. The day before the infectious bloodmeal,
416 mosquitoes were deprived of the sucrose/bacteria/IPTG solution for starvation. Mosquitoes were
417 transferred to a biosafety level 3 insectary and offered an infectious bloodmeal on a Hemoteck
418 system (Hemotek Ltd) consisting of washed rabbit blood supplemented with 10^6 PFUs/mL of ZIKV

419 or CHIKV and 10 mM ATP. Fully engorged mosquitoes were kept at 27 ˚C, 70 % relative humidity,
420 12h:12h light/dark cycle until sampling and provided with fresh bacteria and sucrose every three
421 days. At the selected time points, mosquitoes were dissected and tissues were placed in 200 µL PBS.
422 Samples were kept at -80 °C until processing.

423 *Plaque assay*

424 The day before the assay, Vero-E6 cells were seeded in 24-well plates at a density of $1.75*10^5$
425 cells/well in DMEM with 10 % FBS and 1 % P/S. The day of the assay, mosquito samples were
426 homogenized in a bead beater for 30 sec at 5000 rpm. Ten-fold dilutions of mosquito homogenates
427 or of an aliquot of the bloodmeal were prepared in DMEM. Two-hundred µL of each dilution were
428 added to the cell monolayer and incubated for 1 h at 37 °C with 5 % CO_2. Cells were covered with
429 DMEM with 2 % FBS, 1 % P/S and 0.8 % agarose. After seven (ZIKV) or three (CHIKV) days, cells were
430 fixed with 4 % paraformaldehyde. Plaques were counted after 0.2 % crystal violet staining.

431 *Small RNA sequencing*

432 RNA was extracted from pools of five mosquitoes using TRIzol. Small RNAs of 18-33 nt in length were
433 purified from a 15% acrylamide/bisacrylamide (37.5:1), 7 M urea gel as described previously[50].
434 Libraries were prepared using the NEBNext® Small RNA Library Prep Set for Illumina® (New England
435 BioLabs), with the Universal miRNA Cloning Linker (New England BioLabs) as the 3' adaptor and in-
436 house designed indexed primers. Libraries were diluted to 4 nM and sequenced using an Illumina
437 NextSeq 500 High Output kit v2 (75 cycles) on an Illumina NextSeq 500 platform.

438 *Bioinformatics analysis of small RNA libraries*

439 FastQC[51] was used to assess the quality of fastq file. Low quality portions and adaptors were
440 trimmed from each read using cutadapt[52]. Only reads with a phred score ≥20 were kept. FastQC was
441 used on the fastq files created by cutadapt to evaluate the overall quality. Mapping was performed
442 using Bowtie[53] with the -v 1 option (one mismatch between the read and its target) using as
443 reference sequences the ZIKV, CHIKV, or GFP target sequences, or the PCLV genome. Bowtie
444 generates results in sam format. All sam files were analyzed by different tools of the package
445 samtools[54] to produce bam indexed files. To analyze these bam files, different kind of graphs were
446 generated using home-made R scripts with several Bioconductor libraries such as Rsamtools and
447 Shortreads[55].

448 *Synthesis and labelling of dsRNA*

449 RNA corresponding to the same ZIK1, CHIK1 and GFP sequences cloned in the T444T plasmid was
450 transcribed *in vitro* using the T7 MEGAscript Kit (Invitrogen) following the manufacturer's
451 instructions. A PCR amplicon generated with both forward and reverse primers containing the T7
452 promoter sequence (Supplementary Table 1) was used as a template for the *in vitro* transcription.
453 A DNase treatment was performed after transcription to eliminate the DNA template. The annealing
454 of the two strands of dsRNA was allowed by heating the RNA at 98 °C for 5 min and leaving it to cool
455 down slowly to room temperature. After quantification with Nanodrop, RNA was stored at -80 °C

456 until use. The Silencer™ siRNA Labeling Kit with Cy™3 dye (Invitrogen) was used to label dsRNA
457 following the manufacturer's instructions. An aliquot of the labeled dsRNA was run on a 1 % agarose
458 gel to visualize a shift in the migration and thus confirm the labeling.

459 *Oral feeding of mosquitoes with labeled dsRNA, sample collection and visualization*

460 One-to-three day old conventionally reared female mosquitoes were starved for one day and then
461 provided with a 10 % sucrose solution containing 200 ng/µL of the labeled dsRNA. After two days of
462 feeding, midguts were dissected and fixed overnight in 4 % methanol-free paraformaldehyde in PBS.
463 After three washes with PBS, samples were permeabilized in 1 % BSA, 0.1 % Triton X-100 in PBS for
464 1 h at room temperature. After three washes with PBS, midguts were incubated for 30 minutes with
465 DAPI and Oregon Green 488 Phalloidin (Invitrogen) dyes to stain DNA and actin, respectively. After
466 three additional washes in PBS, midguts were mounted on slides with Vectashield H-1000 (Vector
467 Laboratories) and visualized on a Zeiss LSM 780 confocal microscope at 100X magnification. To
468 reconstruct mosquito full digestive tract images, multiple flanking images were acquired using the
469 "tile" function and subsequently assembled using the ZEN Microscopy Software.

470 *Infection of mosquitoes fed or injected with dsRNA*

471 One-to-three day old conventionally reared female mosquitoes were starved for one day and then
472 provided with a 10 % sucrose solution containing 200 ng/µL of dsRNA and 1 mg/mL of the blue dye,
473 Erioglaucine disodium salt (Sigma-Aldrich). The control consisted of mosquitoes fed on blue-dyed
474 sucrose without dsRNA. All mosquitoes were allowed to feed on the sucrose solution for three days
475 and then only mosquitoes showing a blue abdomen were selected for infection. In parallel, one-to-
476 three day old conventionally reared female mosquitoes were intrathoracically injected with 250 ng
477 of dsRNA using a Nanoject III system (Drummond). They were left to recover for three days. The day
478 before the infectious bloodmeal, mosquitoes were deprived of sucrose for starvation. Mosquitoes
479 were infected with 10^6 PFUs/mL of ZIKV MR766 or CHIKV as described above. At the selected time
480 points, whole mosquitoes were collected in 2 mL screwcap tubes containing 200 µL of sterile PBS
481 and glass beads. Samples were kept at -80 °C until processing. Viral titers in whole mosquitoes were
482 measured by plaque assay as described above.

483 *Northern blot*

484 ZIK1 sense and antisense RNA probes labeled with digoxigenin were synthesized using the DIG
485 Northern Starter Kit (Roche) following the manufacturer's instructions. The template consisted of a
486 PCR product obtained with primers containing the T7 (sense) or T3 (antisense) promoter sequence
487 (Supplementary Table 1). Serial dilutions of the two probes were visualized on a dot blot for probe
488 quantification.

489 *E. coli* HT115-T444T-ZIK1 or -CHIK1 (probe specificity control) was inoculated in 5 mL LB
490 supplemented with 20 µg/mL tetracycline and 100 µg/mL ampicillin and grown overnight at 37 °C
491 shaking at 200 rpm. The following day, 100 µL of the overnight culture were used to inoculate 5 mL
492 of LB with 20 µg/mL tetracycline and 100 µg/mL ampicillin with or without 40 µM IPTG. Bacteria
493 were grown overnight at 37 °C shaking at 200 rpm. After 24 h, 1 mL of bacterial culture was

494 centrifuged and the bacterial pellet was resuspended in 100 µL of 0.1 % SDS. Bacterial extracts were
495 incubated for 2 min at 95 °C for cell lysis and RNA was extracted using TRIzol.

496 Mosquitoes fed or injected with dsRNA were dissected after two or four days of feeding with 200
497 ng/µL dsZIK or after injection with 250 ng/mosquito of dsZIK1. For each time-point/condition, 15 to
498 28 mosquitoes were dissected and their midguts, carcasses (excluding crop, including hindgut and
499 Malpighian tubules), and heads were pooled and stored at -80 °C until processing. Total RNA was
500 extracted from each sample pool using TRIzol.

501 For northern blot analysis, RNA samples were mixed with a glyoxal-containing loading dye,
502 incubated at 50 °C for 30 min for RNA denaturation, and loaded on a 1.2 % agarose gel. Five µg of
503 total RNA were loaded on the gel for all samples (including *in vitro* synthesized dsRNA) except for
504 midguts at day 4 (injected) and heads (fed/injected) at day 4, for which 1.5 µg of RNA were loaded
505 due to low RNA yields. After gel electrophoresis, RNA was transferred to a Nytran Super Charge
506 membrane (Whatman) using the NorthernMax^{TM}-Gly kit (Invitrogen) following the manufacturer's
507 instructions. After transfer and crosslinking with UV light, the membrane was incubated overnight
508 with 0.2 ng/µL of the selected probe. Membranes were incubated with an Alkaline Phosphatase-
509 conjugated Anti-Digoxigenin antibody and the chemiluminescent signal was revealed using a
510 ChemiDoc MP imaging system (Biorad).

511 *Statistics*

512 All graphs were created using GraphPad Prism (version 9.5.1) or R (version 4.2.1). Statistical analyses
513 were performed in R (version 4.2.1). The normality of data was tested with a Shapiro test. A
514 Wilcoxon Signed Rank test was used to compare mosquito bacterial loads, prevalence and $\log_{10}$
515 transformed viral titers of mosquitoes colonized by dsZIK-producing bacteria. A Kruskal-Wallis one-
516 way analysis of variance followed by a Dunn test with Bonferroni correction was used to compare
517 $\log_{10}$ transformed viral titers in mosquitoes colonized by dsCHIK producing bacteria, or in
518 mosquitoes fed or injected with dsZIK/dsCHIK. To determine if 21 nt siRNAs were significantly
519 enriched in our small RNA sequencing, a Z-score and a *p* value were calculated to compare the
520 amount of 21 nt reads to the population average of all 18 to 33 nt reads. Detailed results of statistical
521 analyses are shown in Supplementary Table 2. R codes are available upon request.

522

523 **Data availability**

524 The small RNA sequencing data were deposited to the Sequence Read Archive under accession
525 number PRJNA1034089.

526 **Acknowledgments**

527 We are grateful to Jared Nigg and Louis Lambrechts for critically reading of the manuscript. We
528 thank Catherine Lallemand for technical assistance in mosquito rearing, and Madeleine Lausted for
529 assistance in dsRNA feeding.
530 This work was supported by funding from the French Government's Investissement d'Avenir
531 program, Laboratoire d'Excellence Integrative Biology of Emerging Infectious Diseases (grant ANR-
532 10-LABX-62-IBEID), Agence Nationale de la Recherche (grant ANR-22-CE35-0001, MAMMAMIA),
533 and Fondation iXcore - iXlife - iXblue Pour La Recherche to M.C.S., the Pasteur-Roux-Cantarini
534 fellowship of Institut Pasteur to O.R. We acknowledge the kind financial support to the Photonic
535 BioImaging (UTechS PBI) platform by the Institut Pasteur (Paris), the France–BioImaging
536 infrastructure network supported by the Agence Nationale de la Recherche (ANR-10–INBS–04,
537 Investments for the future), and the Région Ile-de-France (program DIM-Malinf).

538 **Authors contributions**

539 O.R. and M.-C.S. designed the experiments; O.R., A.H.-L., and H.B. performed the experiments; O.R.
540 and L.F. analyzed the data; O.R. wrote the paper with input from co-authors. All authors reviewed
541 and approved the final version of the manuscript.

542

543 **Declaration of interests**

544 The authors declare no competing interests.

545

546 **Figures legends**

547 **Figure 1. Colonization of mosquitoes with dsRNA producing *E. coli*.** (**A**) Representation of the T444T
548 plasmid. The target viral sequence was cloned between two directionally opposed T7 promoters to
549 allow convergent transcription of a sense and antisense RNAs that form dsRNA upon annealing. (**B**)
550 Position of the target viral sequence on the ZIKV genome. The selected sequence is 481 nt and spans
551 the coding sequences for the Capsid (Cap) and the Pre-membrane (Pr) protein coding genes. The
552 other components of the ZIKV genome are: M (Membrane), E (Envelope) NS1-5 (Non-Structural
553 proteins). (**C**) Experimental set up for mosquito mono-colonization with *E. coli* HT115. Sterile eggs
554 were obtained by subsequent washes in 70 % ethanol and 1 % bleach. An overnight culture of *E. coli*
555 HT115 carrying the T444T-ZIK1 plasmid was added to axenic larvae hatched from sterile eggs to
556 obtain mono-colonized larvae. Adults emerging from mono-colonized larvae were provided with
557 either sterile sucrose or sucrose supplemented with *E. coli* HT115. Colony forming units (CFUs) were
558 determined in whole adults to evaluate the efficiency of bacterial colonization. (**D**) Bacterial loads

559 (mean) and plasmid stability *in vivo*. CFUs were determine one and four days-post-emergence in
560 adult mosquitoes mono-colonized with *E. coli* HT115-T444T-ZIK1 at the larval stage, fed with either
561 sterile (black) or bacterial supplemented (yellow) sucrose. To determine plasmid stability in bacteria
562 during mosquito colonization, CFUs were determined on LB plates supplemented (full dots) or not
563 (empty dots) with ampicillin. Bacterial loads were compared using a Wilcoxon test.

564 **Figure 2. Mosquitoes colonized with dsZIK-producing bacteria do not show reduced viral titers.**
565 (**A**) Experimental protocol for ZIKV infection of mosquitoes colonized by dsRNA-producing *E. coli*.
566 Mosquitoes originating from mono-colonized larvae were kept on bacteria-supplemented sucrose
567 for four days. After one day of starvation, they were offered a ZIKV infectious bloodmeal and
568 subsequently kept with bacteria-supplemented sucrose until sampling at six and ten days post-
569 infection. At each time point midguts, carcasses, and heads of individual mosquitoes were collected.
570 (**B-C**) Viral titers (mean ± SEM) in midguts, carcasses, and heads of individual mosquitoes colonized
571 by *E. coli* HT115 expressing dsGFP (grey) or dsZIK1 (yellow) and collected six (**B**) or ten (**C**) days post-
572 infection. Each plot shows the log of plaque forming units (PFUs) measured in individual mosquitoes
573 in each tissue. Pie charts show the proportion of infected (grey/yellow) and uninfected (white)
574 mosquitoes. Numbers below pie charts represent the number of infected over uninfected
575 mosquitoes per tissue/time-point. Viral loads were compared using a Wilcoxon test.

576 **Figure 3. Mosquitoes colonized with dsZIK-producing bacteria do not show production of dsZIK-**
577 **specific siRNAs.** Small RNAs were sequenced from pools of whole mosquitoes colonized for two
578 days with the *E. coli* HT115 strain carrying either the T444T-ZIK1 plasmid (**A**), or the pUC18-ZIK1
579 plasmid with the 481 nt (**D**) or 175 nt (**G**) ZIKV sequence. For each condition, the number of reads
580 mapping to each position of the positive strand (grey) or negative strand (black) of the target
581 sequence is displayed (**B**, **E**, **H**). The size distribution of the reads shown in (**B, E, H**) is shown in (**C**, **F**,
582 **I**), respectively. Z-scores were calculated for the number of 21 nt reads compared to the mean of all
583 18-33 nt reads.

584 **Figure 4. A protective antiviral response is induced by systemic injection of dsRNA in adult**
585 **mosquitoes, but not by feeding.** (**A**) A blue sucrose solution containing dsRNA was provided to
586 mosquitoes for two days. Blue dye in the crop and midgut is visible through the cuticle and allows
587 selection of fed mosquitoes. (**B**-**D**) Confocal images of the digestive tract of a mosquito fed for 48 h
588 with dsZIK1-Cy3. In (**B**) different dyes indicate DNA (blue, DAPI), actin (green, Phalloidin), and
589 dsZIK1-Cy3 (red, Cy3), while in (**C**) only the signal of dsZIK1-Cy3 is shown. In (**C**) the different
590 components of the mosquito digestive tract are indicated. (**D**) represents a higher magnification
591 image of a region of the same midgut shown in (**B**-**C**). Scale bars correspond to 500 µm (**B**-**C**) or 50
592 µm (**D**). (**E**) Experimental protocol for ZIKV infection of mosquitoes fed or injected with dsZIK1 or
593 dsGFP. At day 0, mosquitoes were fed with a 200 ng/µL solution of dsZIK1 or dsGFP, or injected with
594 250 ng dsZIK1 or dsGFP/mosquito. After four days, they were offered a ZIKV infectious bloodmeal.
595 At seven and 14 days after infection, whole individual mosquitoes were collected. (**F**-**G**) Viral titers
596 (mean ± SEM) in individual mosquitoes (whole bodies) fed on sucrose (black) or sucrose containing
597 dsGFP (full grey) or dsZIK1 (full yellow), or injected with dsGFP (empty grey) or dsZIK1 (empty
598 yellow). Mosquitoes were collected seven (**F**) or 14 (**G**) days post-infection. Each plot shows the log

599 of plaque forming units (PFUs) measured in individual mosquitoes. Pie charts show the proportion
600 of infected (black/grey/yellow) and uninfected (white) mosquitoes. Numbers below pie charts
601 represent the number of infected over uninfected mosquitoes per tissue/time-point. Viral loads
602 were compared using a Kruskal-Wallis one-way ANOVA followed by a Dunn test with Bonferroni
603 correction. The global ANOVA *p* value is displayed in the top-right corner of each plot and
604 significance groups ($p < 0.05$) are denoted by letters. (**H**-**K**) Small RNAs were sequenced from pools
605 of five whole mosquitoes fed with dsZIK1 for two days (**H**, **I**) or injected with dsZIK1 two days before
606 sampling (**J**, **K**). (**H**, **J**) For each condition, the number of reads mapping to each position of the
607 positive strand (grey) or negative strand (black) of the target sequence is displayed. The size
608 distribution of the reads shown in (**H**, **J**) is shown in (**I**, **K**), respectively. Z-scores were calculated for
609 the number of 21 nt reads compared to the mean of all 18-33 nt reads.

610

611 **Supplementary figures and tables legends**

612 **Supplementary Figure 1. Mosquitoes colonized with dsCHIK-producing bacteria do not show**
613 **reduced viral titers.** (**A**) Representation of the T444T plasmid. The target viral sequence was cloned
614 between two directionally opposed T7 promoters to allow convergent transcription of a sense and
615 antisense RNAs that form dsRNA upon annealing. (**B**) Representation of the pUC18 plasmid. An
616 inverted repeat of the target viral sequence was cloned downstream from a T7 promoter, allowing
617 the formation of an RNA hairpin after transcription. (**C**) Position of the target viral sequence on the
618 CHIKV genome. The selected sequence is 557 nt long and spans the coding sequences for the Capsid
619 (Cap) and the Envelope (E3) peptide coding genes. The other components of the CHIKV genome are
620 NSPs (Non-Structural Proteins). (**D**) Experimental protocol for CHIKV infection of mosquitoes
621 colonized by dsRNA-producing *E. coli*. Mosquitoes originating from mono-colonized larvae were
622 kept on bacteria-supplemented sucrose for four days. After one day of starvation, they were offered
623 a CHIKV-containing infectious bloodmeal and subsequently kept with bacteria-supplemented
624 sucrose until sampling at two and five days post-infection. At each time point, bodies and heads of
625 individual mosquitoes were collected. (**E-F**) Viral titers (mean $\pm$ SEM) in bodies and heads of
626 individual mosquitoes colonized by *E. coli* HT115 carrying the T444T-GFP (grey), T444T-CHIK1 (dark
627 blue), or pUC18-CHIK1 (light blue) plasmid and collected two (**E**) or five (**F**) days post-infection. Each
628 plot shows the log of plaque forming units (PFUs) measured in individual mosquitoes in each tissue.
629 Pie charts show the proportion of infected (grey/blue) and uninfected (white) mosquitoes. Numbers
630 below pie charts represent the number of infected over uninfected mosquitoes per tissue/time-
631 point. Viral loads were compared using a Kruskal-Wallis one-way ANOVA followed by a Dunn test
632 with Bonferroni correction. The global ANOVA *p* value is displayed in the top-right corner of each
633 plot and significance groups ($p < 0.05$) are denoted by letters.

634 **Supplementary Figure 2. Northern blot analysis of *E. coli* HT115-T444T-ZIK1 total RNA.** (**A**, **B**)
635 Ethidium bromide-stained agarose gel (**A**) and northern blot (**B**) of dsZIK1 synthesized *in vitro* and
636 total RNA extracted from a 24 h bacterial culture without and with 40 µM IPTG induction. For (**B**),
637 the same samples were loaded in duplicate on the same agarose gel and transferred on the same
638 membrane. The membrane was cut and incubated with either a sense (left) or antisense (right) 345

639 nt probe annealing to the ZIK1 sequence. (**C**, **D**) Specificity of the ZIK1 sense probe. Ethidium
640 bromide-stained agarose gel (**C**) and northern blot (**D**) of the same samples showed in (**A**, **B**) loaded
641 together with dsCHIK1 synthesized *in vitro* and total RNA extracted from a 24 h culture of *E. coli*
642 HT115-T444T-CHIK1 without and with 40 µM IPTG induction. Northern blot membrane was
643 incubated with the sense ZIK1 probe to show binding specificity to dsZIK1 RNA.

644 **Supplementary Figure 3. Sequencing quality control of small RNAs mapping on the Phasi-Charoen**
645 **Like Virus (PCLV) genome.** Small RNAs were sequenced from the pools of whole mosquitoes shown
646 in Figure 3 and mapped to the PCLV genomic segments S (**A**-**B**), M (**C**-**D**), and L (**E**-**F**). The number of
647 reads mapping to each position of the positive strand (grey) or negative strand (black) of the PCLV
648 genome is shown (**A**, **C**, **E**). The size distribution of the reads shown in (**A, C, E**) is shown in (**B**, **D**, **F**),
649 respectively. Z-scores were calculated for the number of 21 nt reads compared to the mean of all
650 18-33 nt reads.

651 **Supplementary Figure 4. Mosquitoes colonized with heat-killed dsZIK-producing bacteria do not**
652 **show production of dsZIK-specific siRNAs.** Small RNAs were sequenced from pools of whole
653 mosquitoes supplemented for two days with sucrose containing heat-killed *E. coli* HT115 (heated
654 for 5 min at 98 °C) carrying either the T444T-ZIK1 plasmid (**A**), or the pUC18-ZIK1 plasmid with the
655 481 nt (**D**) or 175 nt (**G**) ZIKV sequence. For each condition, the number of reads mapping to each
656 position of the positive strand (grey) or negative strand (black) of the target sequence is displayed
657 (**B**, **E**, **H**). The size distribution of the reads shown in (**B, E, H**) is shown in (**C**, **F**, **I**), respectively. Z-
658 scores were calculated for the number of 21 nt reads compared to the mean of all 18-33 nt reads.

659 **Supplementary Figure 5. Effect of oral supplementation or injection of *in vitro* synthesized dsRNA**
660 **on CHIKV infection.** (**A**) Experimental protocol for CHIKV infection of mosquitoes fed or injected
661 with dsCHIK1 or dsGFP. At day 0, mosquitoes were fed with a 200 ng/µL solution of dsCHIK1 or
662 dsGFP, or injected with 250 ng dsCHIK1 or dsGFP/mosquito. After four days, they were offered a
663 CHIKV infectious bloodmeal. At two and five days after infection, whole individual mosquitoes were
664 collected. (**B-C**) Viral titers (mean ± SEM) in individual mosquitoes (whole bodies) fed on sucrose
665 (black) or sucrose containing dsGFP (full grey) or dsCHIK1 (full blue), or injected with dsGFP (empty
666 grey) or dsCHIK1 (empty blue). Mosquitoes were collected two (**B**) or five (**C**) days post-infection.
667 Each plot shows the log of plaque forming units (PFUs) measured in individual mosquitoes. Pie
668 charts show the proportion of infected (black/grey/blue) and uninfected (white) mosquitoes.
669 Numbers below pie charts represent the number of infected over uninfected mosquitoes per
670 tissue/time-point. Viral loads were compared using a Kruskal-Wallis one-way ANOVA followed by a
671 Dunn test with Bonferroni correction. The global ANOVA p value is displayed in the top-right corner
672 of each plot and significance groups ($p < 0.05$) are denoted by letters.

673 **Supplementary Figure 6. Northern blot analysis of total RNA extracted from mosquitoes fed or**
674 **injected with dsZIK1.** (**A**) Experimental protocol for sample collection of mosquitoes fed or injected
675 with dsZIK1. At day 0, mosquitoes were fed with a 200 ng/µL solution of dsZIK1 or injected with 250
676 ng dsZIK1/mosquito. After two and four days, midguts, carcasses, and heads of individual
677 mosquitoes were collected and pooled. RNA was extracted from pools of 15 to 28 mosquitoes. (**B**-
678 **C**) Ethidium bromide-stained agarose gel (**B**) and northern blot (**C**) of RNA samples extracted from

679 pools of midguts (M), carcasses (C), or heads (H) of mosquitoes fed or injected with dsZIK1 collected
680 two or four days after feeding/injection. The membrane was incubated with a sense 345 nt probe
681 annealing to the ZIK1 sequence. dsZIK1 synthesized *in vitro* was loaded on the same gel as a control.

682 **Supplementary Table 1. List of primers used in this study.** For each primer, the following
683 information is indicated: name, sequence (5'-3'), GenBank reference sequence, position on the
684 reference sequence, amplicon length and application.

685 **Supplementary Table 2. Results of statistical analyses.**

686

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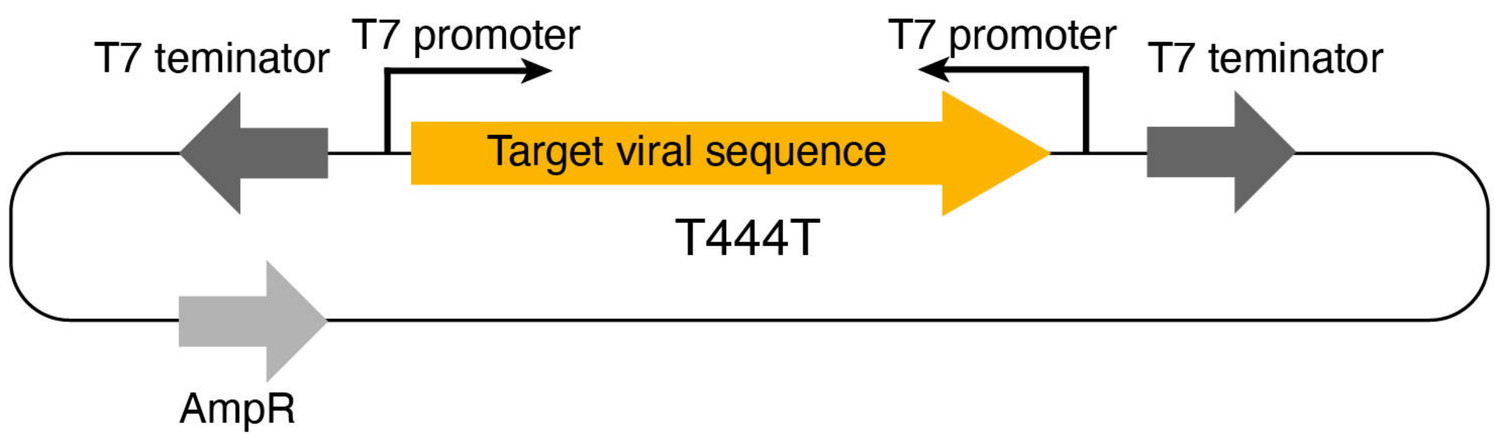
811

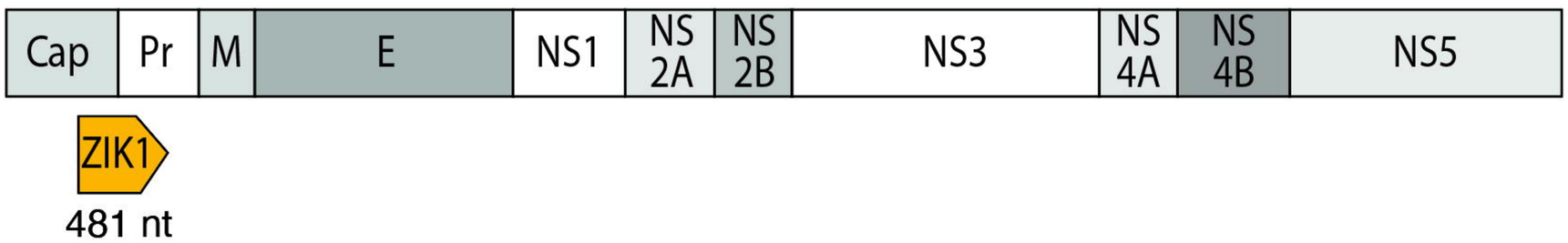
A

Cloning strategy

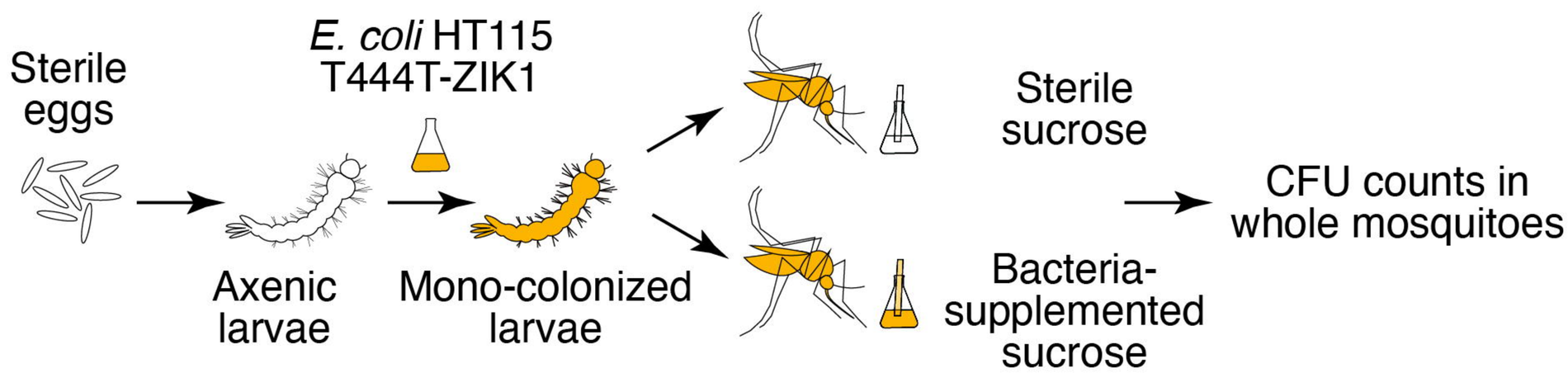


B

Target viral sequence

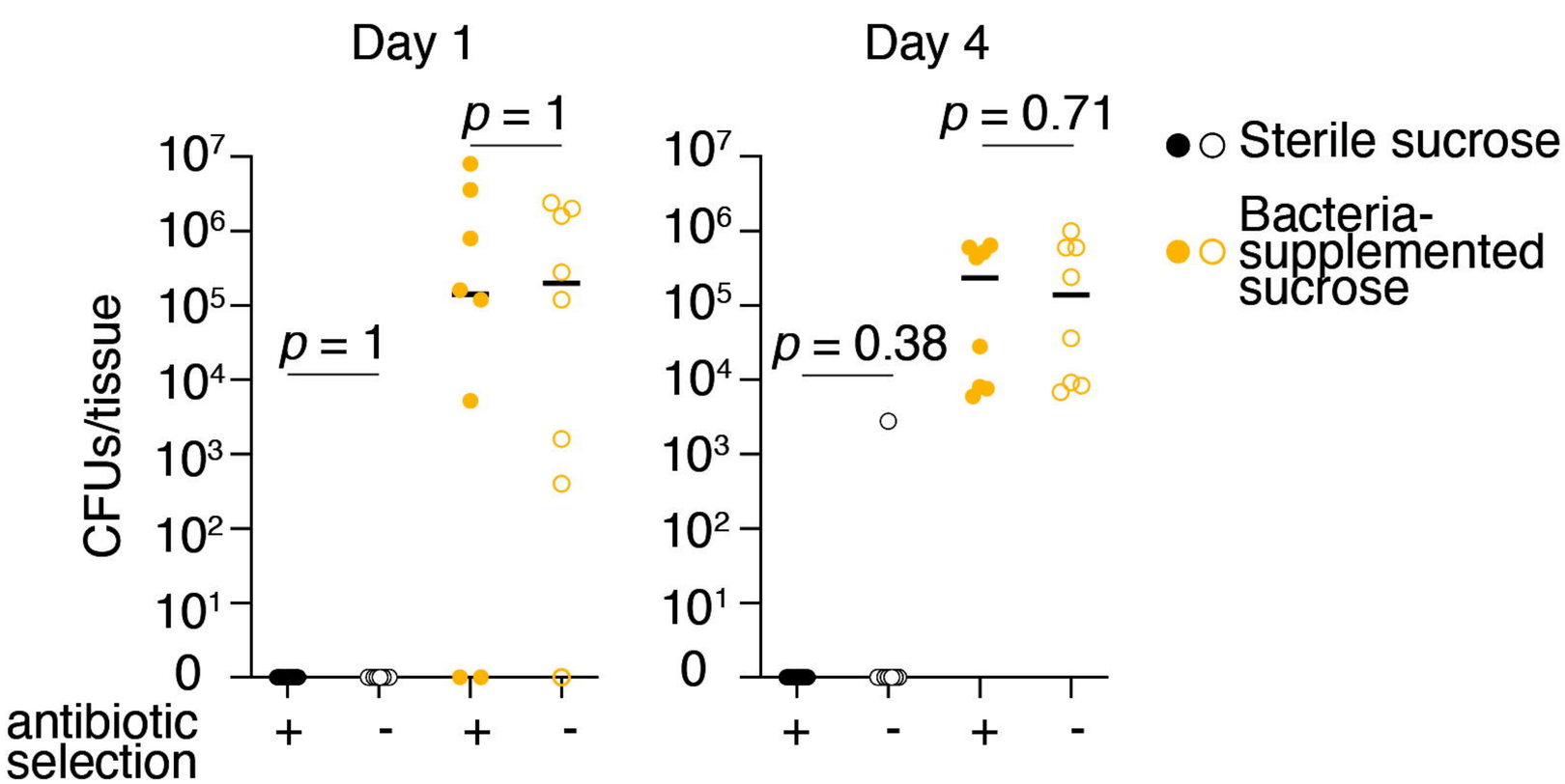


C



D

Bacterial colonization



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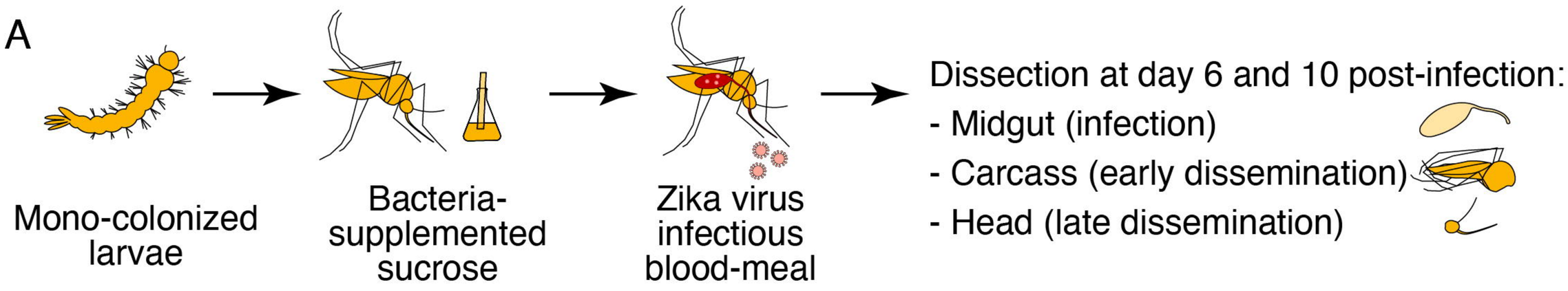
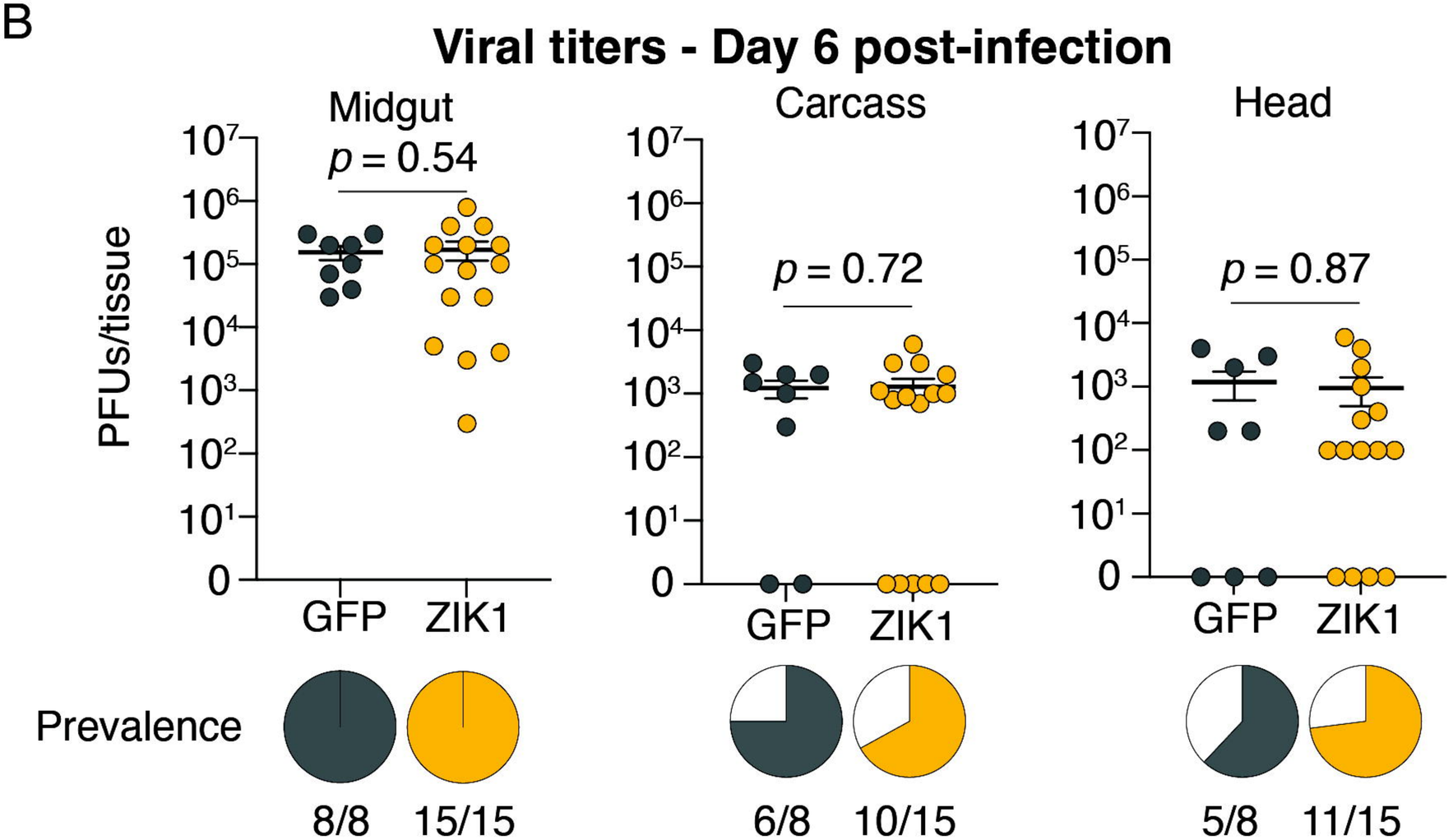
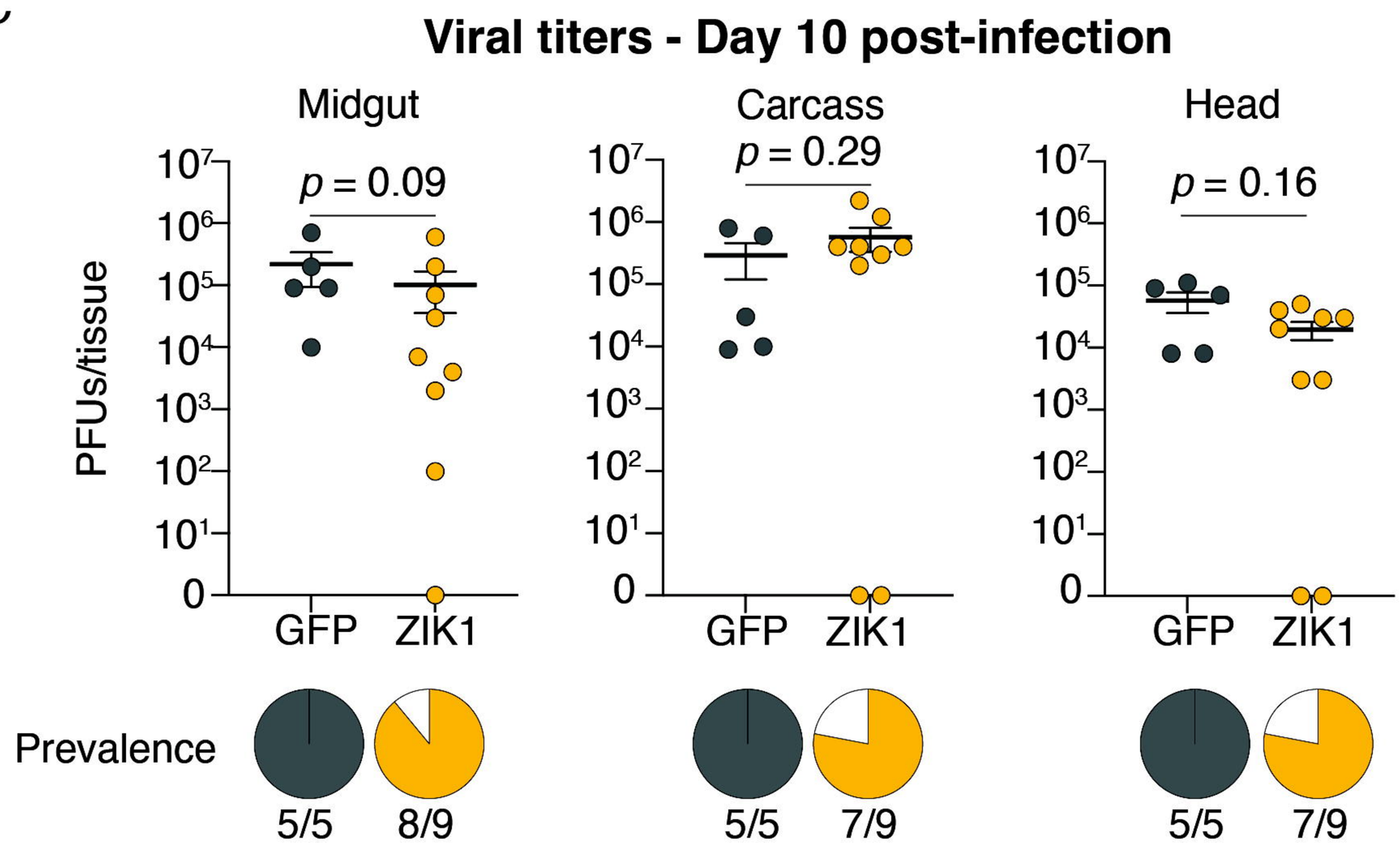

Mono-colonized larvae
Bacteria-supplemented sucrose
Zika virus infectious blood-meal
Dissection at day 6 and 10 post-infection:
- Midgut (infection)
- Carcass (early dissemination)
- Head (late dissemination)

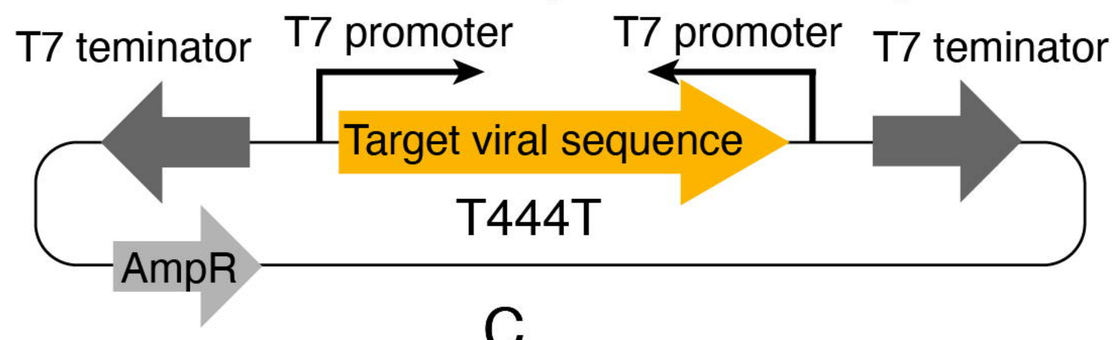
B


Viral titers - Day 6 post-infection
Midgut
Carcass
Head
PFUs/tissue
p = 0.54
p = 0.72
p = 0.87
GFP
ZIK1
Prevalence
8/8
15/15
6/8
10/15
5/8
11/15

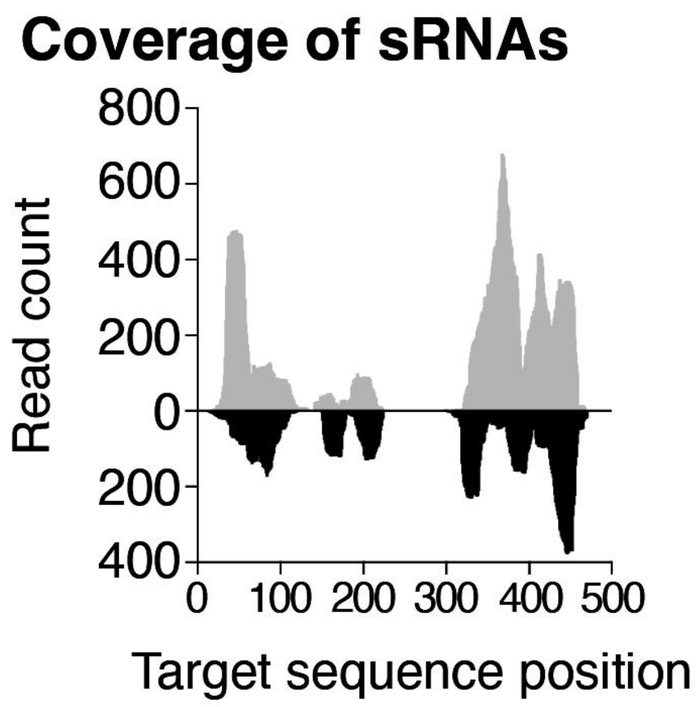
C


Viral titers - Day 10 post-infection
Midgut
Carcass
Head
PFUs/tissue
p = 0.09
p = 0.29
p = 0.16
GFP
ZIK1
Prevalence
5/5
8/9
5/5
7/9
5/5
7/9

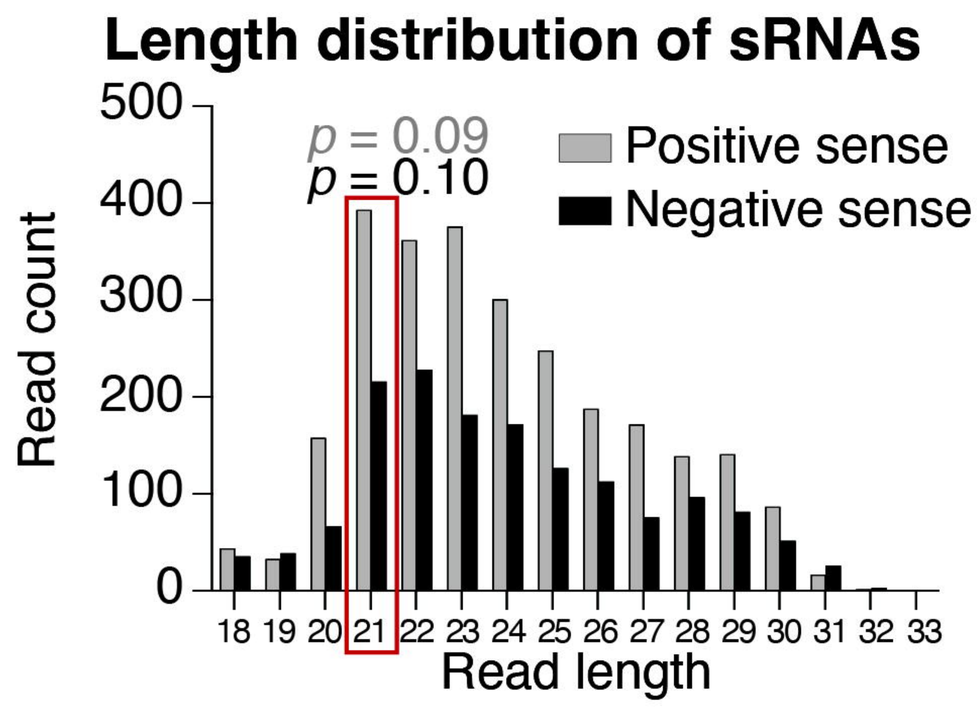
A


T444T-ZIK1 (live bacteria)
T7 teminator
T7 promoter
T7 promoter
T7 teminator
Target viral sequence
T444T
AmpR

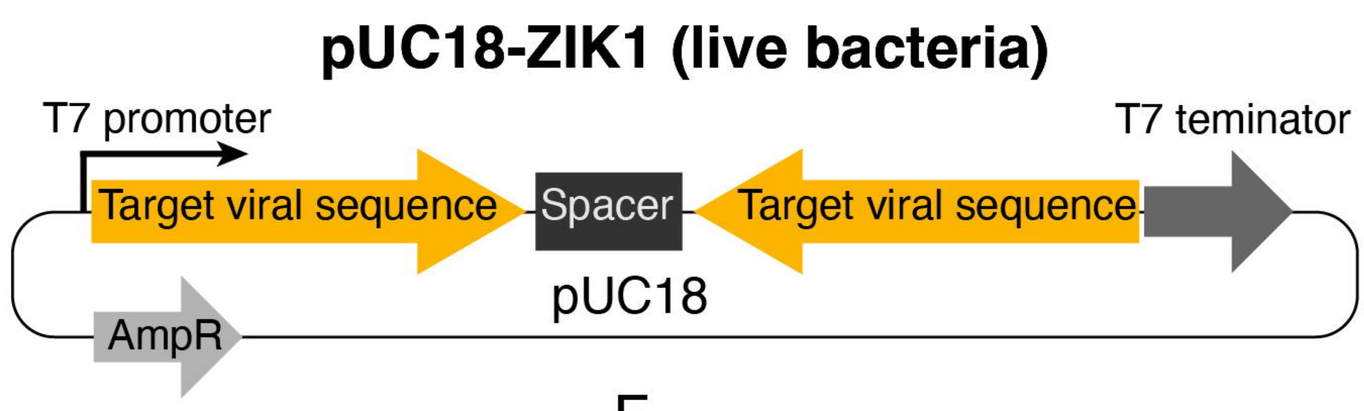
B


Coverage of sRNAs
Read count
800
600
400
200
0
200
400
0 100 200 300 400 500
Target sequence position

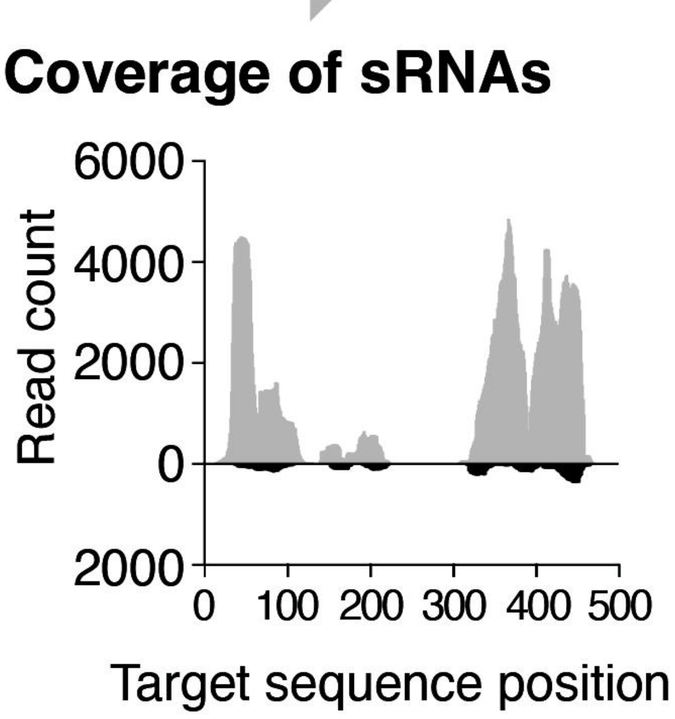
C


Length distribution of sRNAs
Read count
500
400
300
200
100
0
p = 0.09
p = 0.10
Positive sense
Negative sense
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
Read length

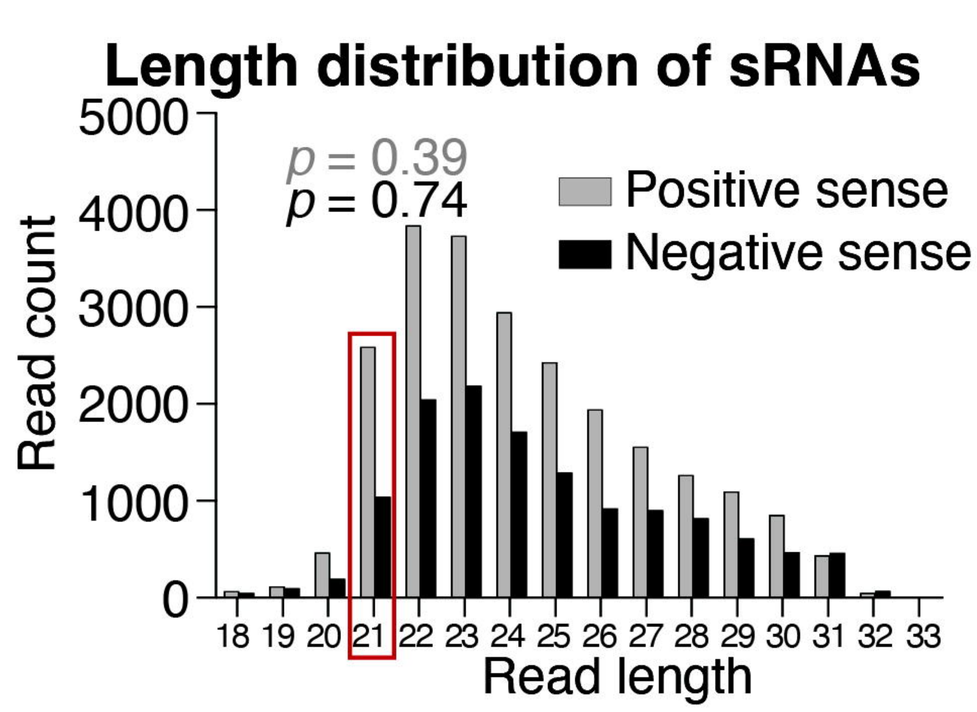
D


pUC18-ZIK1 (live bacteria)
T7 promoter
T7 teminator
Target viral sequence
Spacer
Target viral sequence
pUC18
AmpR

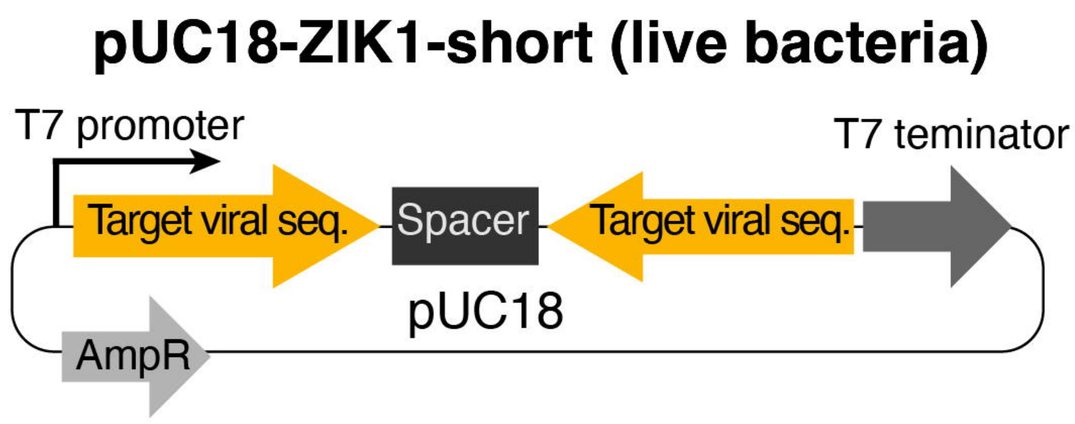
E


Coverage of sRNAs
Read count
6000
4000
2000
0
2000
0 100 200 300 400 500
Target sequence position

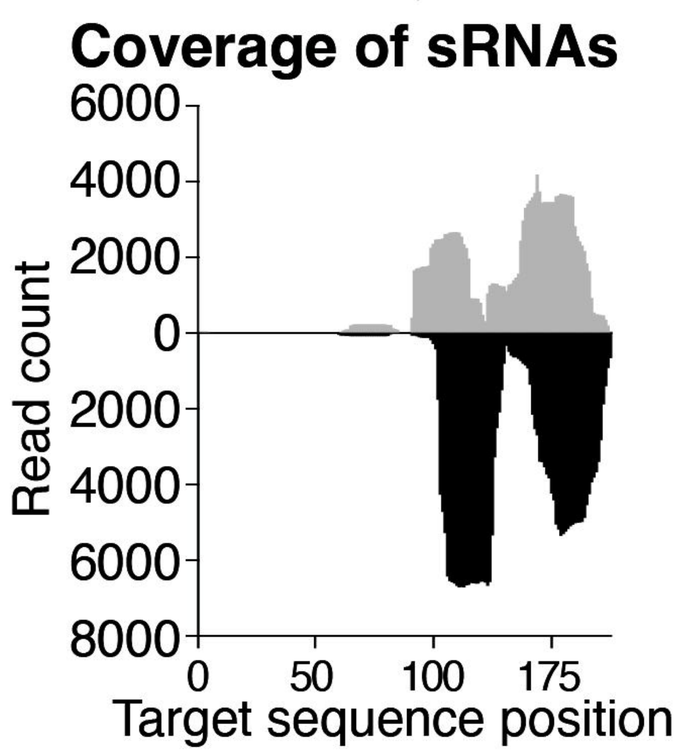
F


Length distribution of sRNAs
Read count
5000
4000
3000
2000
1000
0
p = 0.39
p = 0.74
Positive sense
Negative sense
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
Read length

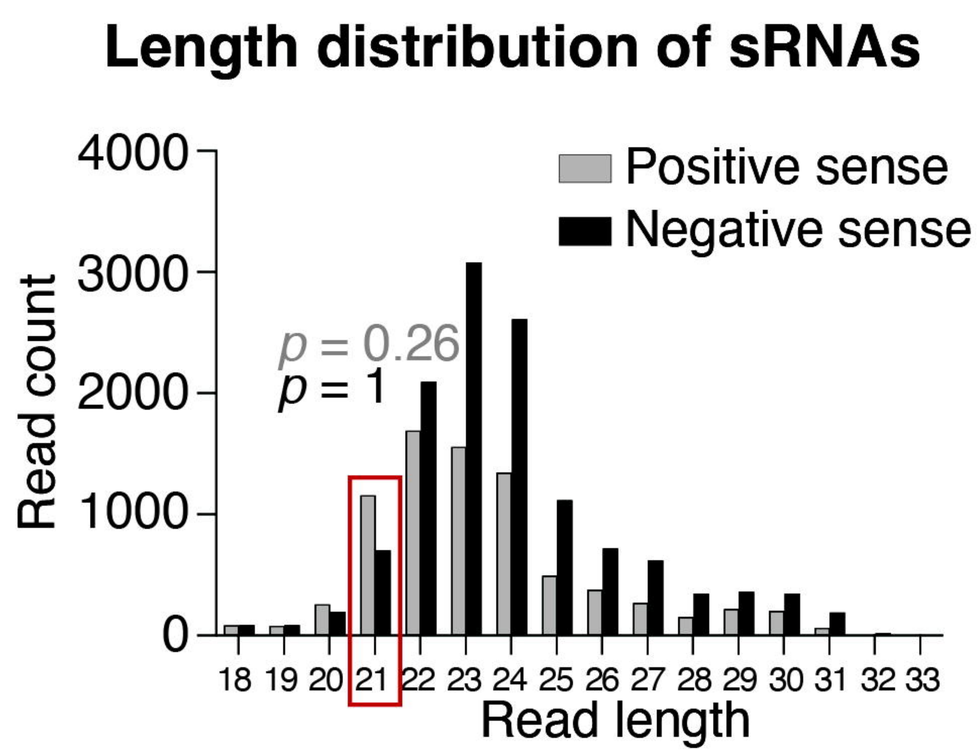
G

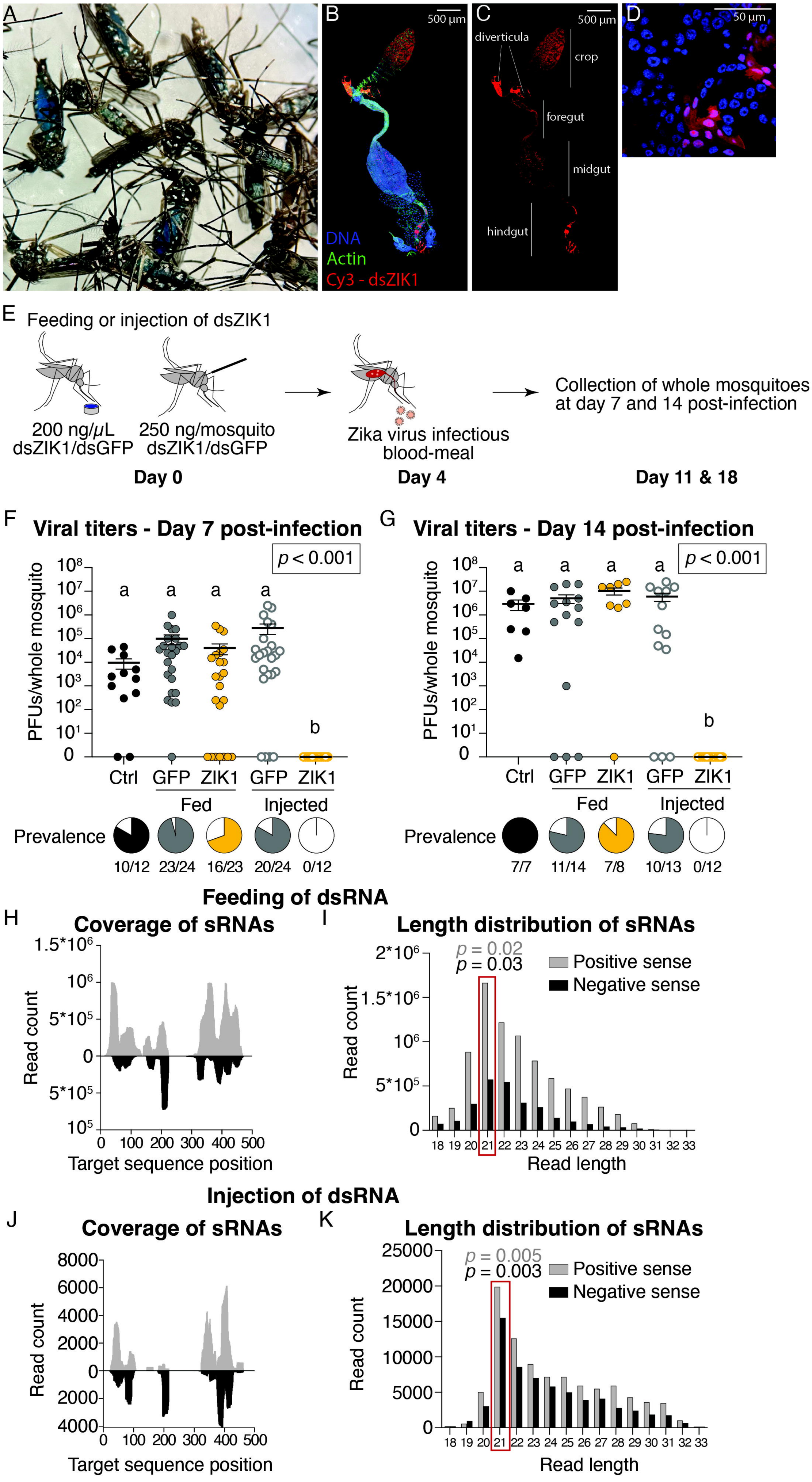

pUC18-ZIK1-short (live bacteria)
T7 promoter
T7 teminator
Target viral seq.
Spacer
Target viral seq.
pUC18
AmpR

H


Coverage of sRNAs
Read count
6000
4000
2000
0
2000
4000
6000
8000
0 50 100 175
Target sequence position

I

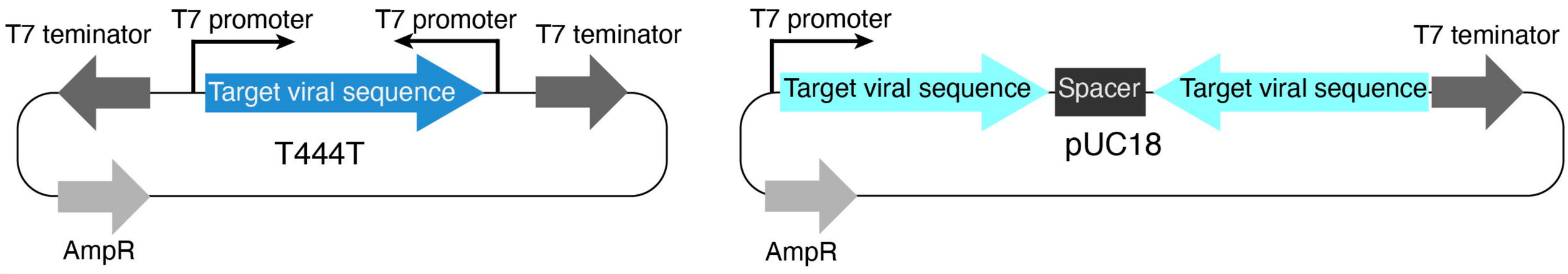

Length distribution of sRNAs
Read count
4000
3000
2000
1000
0
p = 0.26
p = 1
Positive sense
Negative sense
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
Read length


A
B
500 µm
DNA
Actin
Cy3 - dsZIK1
C
500 µm
diverticula
crop
foregut
midgut
hindgut
D
50 µm
E
Feeding or injection of dsZIK1
200 ng/µL dsZIK1/dsGFP
250 ng/mosquito dsZIK1/dsGFP
Day 0
Zika virus infectious blood-meal
Day 4
Collection of whole mosquitoes at day 7 and 14 post-infection
Day 11 & 18
F
Viral titers - Day 7 post-infection
p < 0.001
PFUs/whole mosquito
Ctrl
GFP
ZIK1
Fed
Injected
Prevalence
10/12
23/24
16/23
20/24
0/12
G
Viral titers - Day 14 post-infection
p < 0.001
7/7
11/14
7/8
10/13
0/12
Feeding of dsRNA
H
Coverage of sRNAs
Read count
Target sequence position
I
Length distribution of sRNAs
p = 0.02
p = 0.03
Positive sense
Negative sense
Read length
Injection of dsRNA
J
Coverage of sRNAs
Target sequence position
K
Length distribution of sRNAs
p = 0.005
p = 0.003
Positive sense
Negative sense
Read length

A

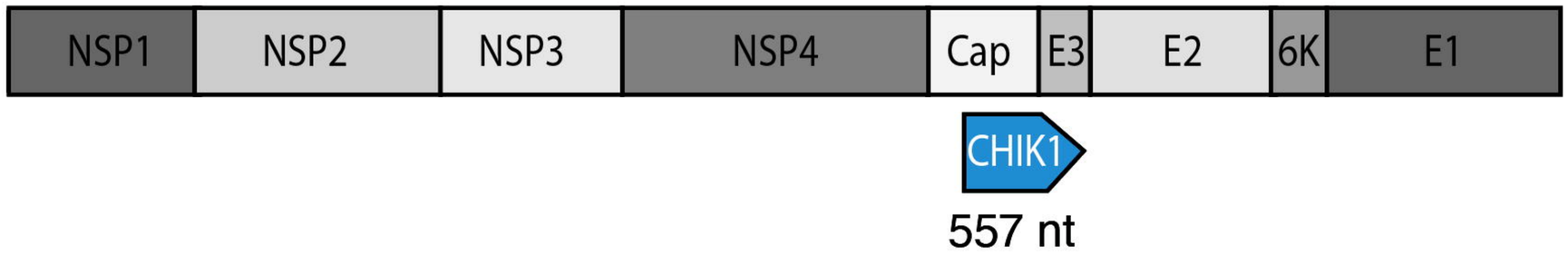
B

Cloning strategies

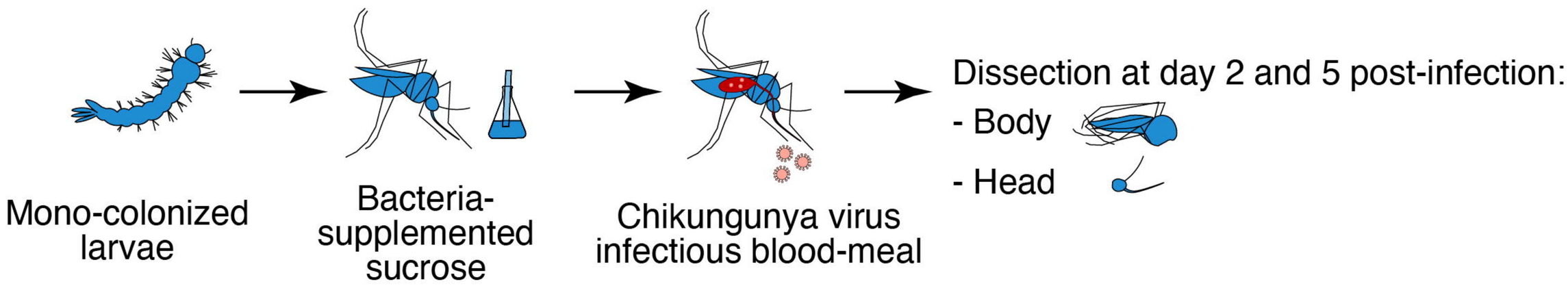


C

Target viral sequence

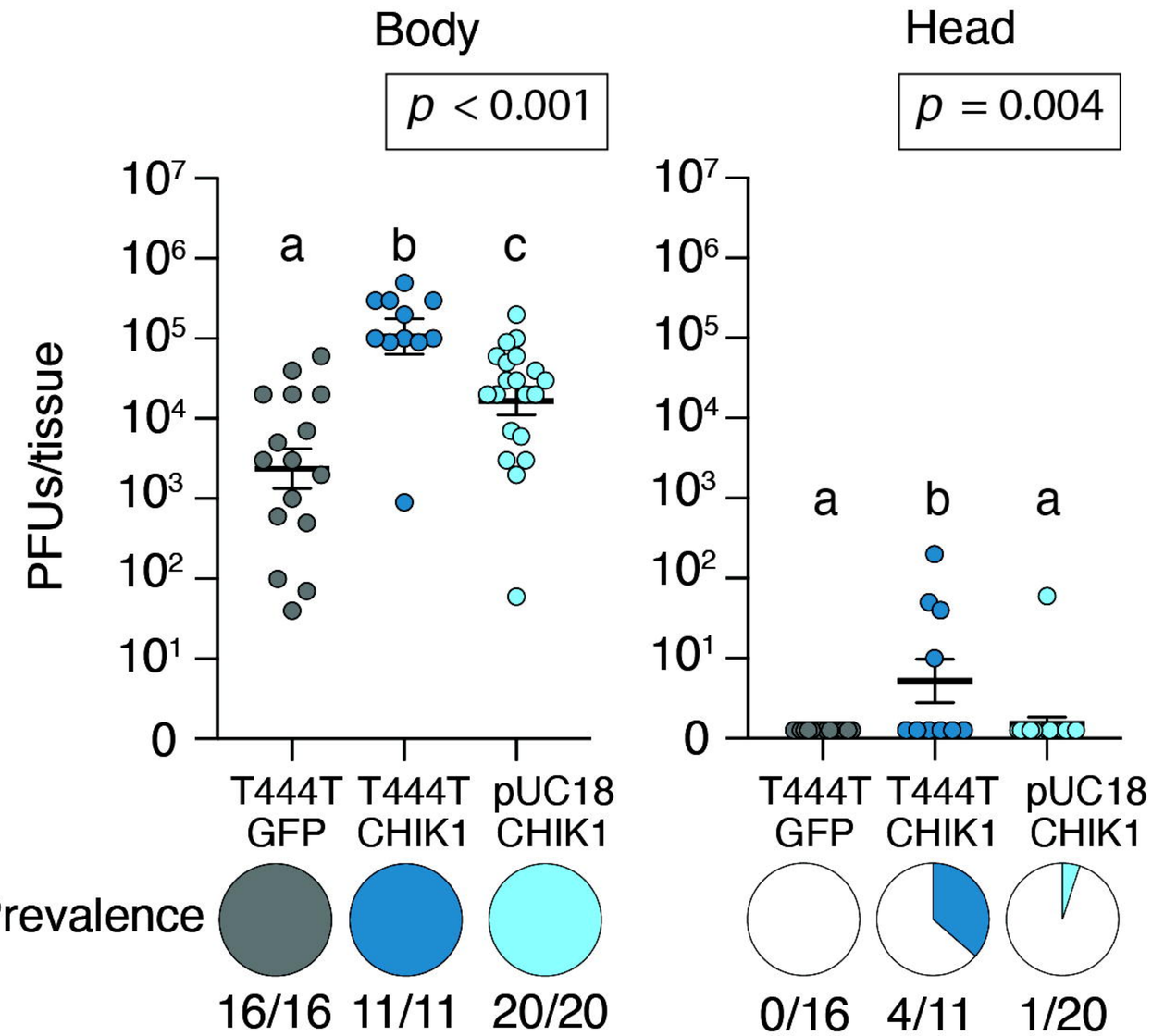


D



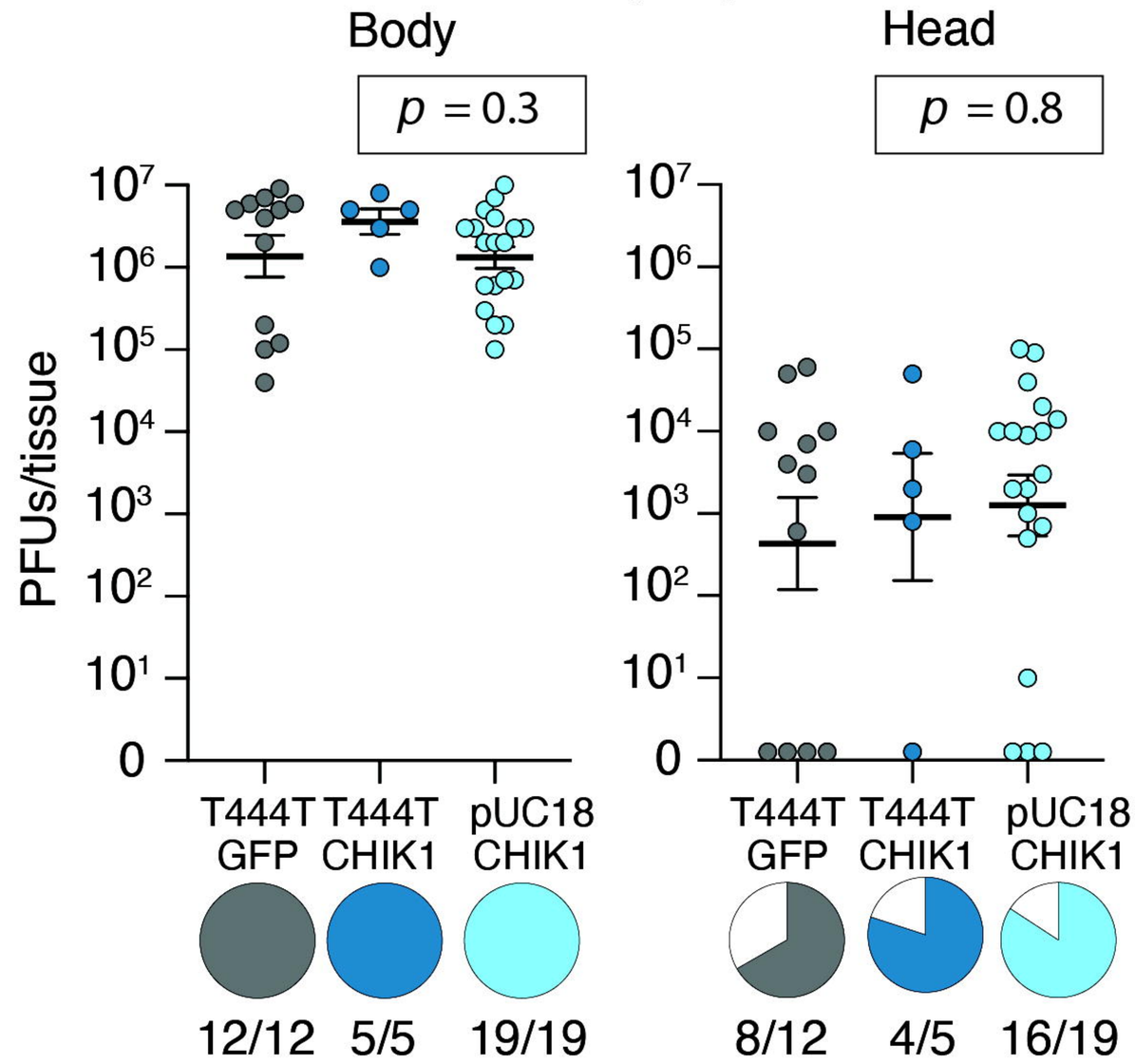
E

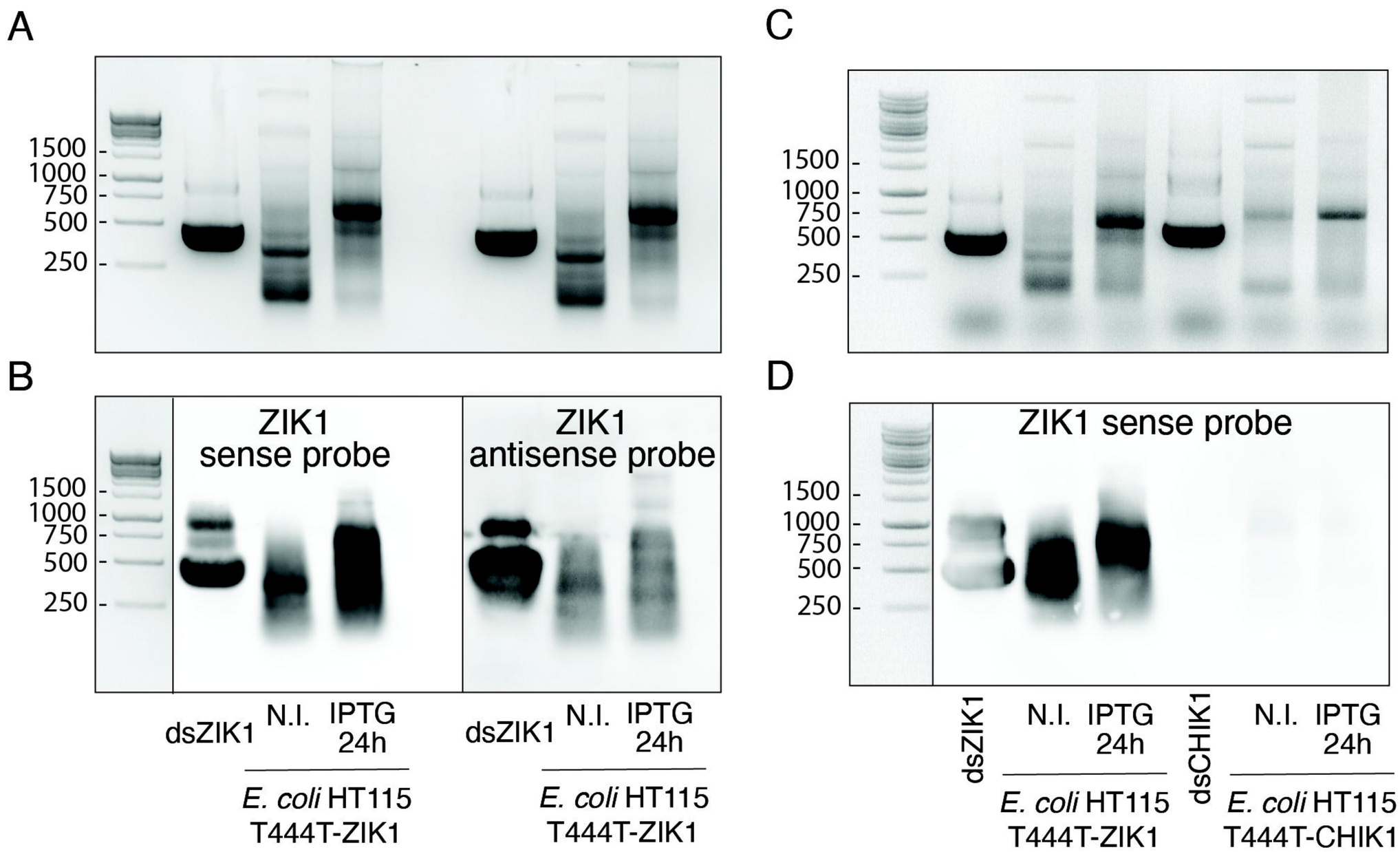
Viral titers - Day 2 post-infection

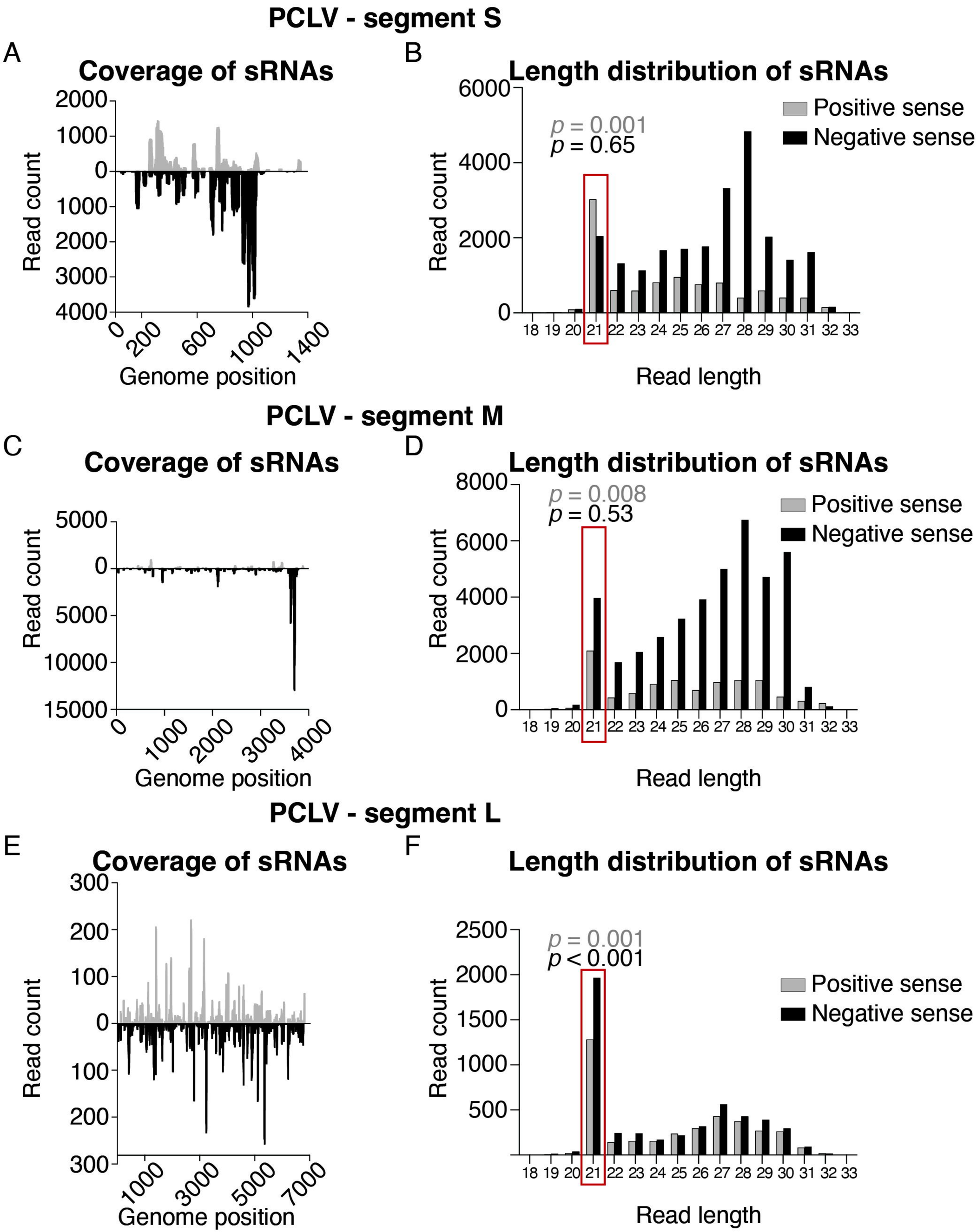


F

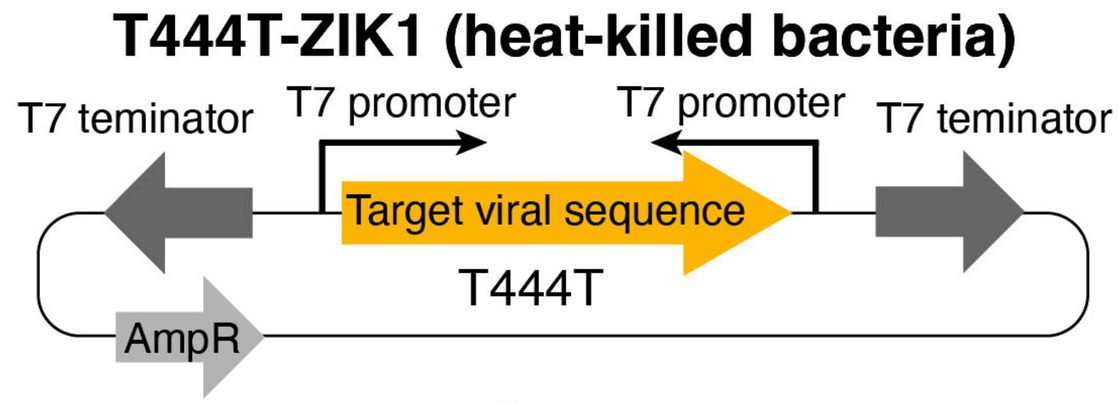
Viral titers - Day 5 post-infection



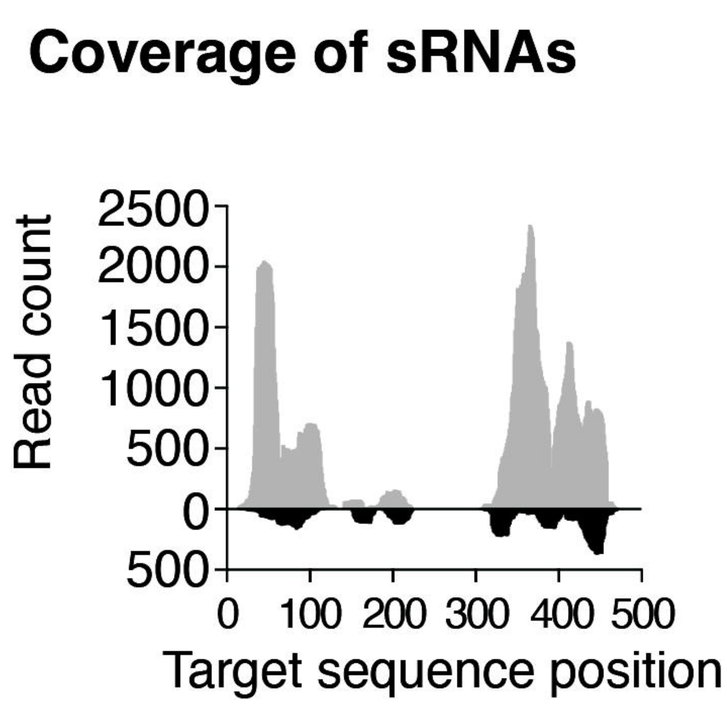

A
1500
1000
750
500
250
B
ZIK1
sense probe
ZIK1
antisense probe
1500
1000
750
500
250
dsZIK1
N.I.
IPTG
24h
dsZIK1
N.I.
IPTG
24h
E. coli HT115
T444T-ZIK1
E. coli HT115
T444T-ZIK1
C
1500
1000
750
500
250
D
ZIK1 sense probe
1500
1000
750
500
250
dsZIK1
N.I.
IPTG
24h
dsCHIK1
N.I.
IPTG
24h
E. coli HT115
T444T-ZIK1
E. coli HT115
T444T-CHIK1


PCLV - segment S
A
Coverage of sRNAs
Read count
Genome position
B
Length distribution of sRNAs
p = 0.001
p = 0.65
Positive sense
Negative sense
Read count
Read length
PCLV - segment M
C
Coverage of sRNAs
Read count
Genome position
D
Length distribution of sRNAs
p = 0.008
p = 0.53
Positive sense
Negative sense
Read count
Read length
PCLV - segment L
E
Coverage of sRNAs
Read count
Genome position
F
Length distribution of sRNAs
p = 0.001
p < 0.001
Positive sense
Negative sense
Read count
Read length

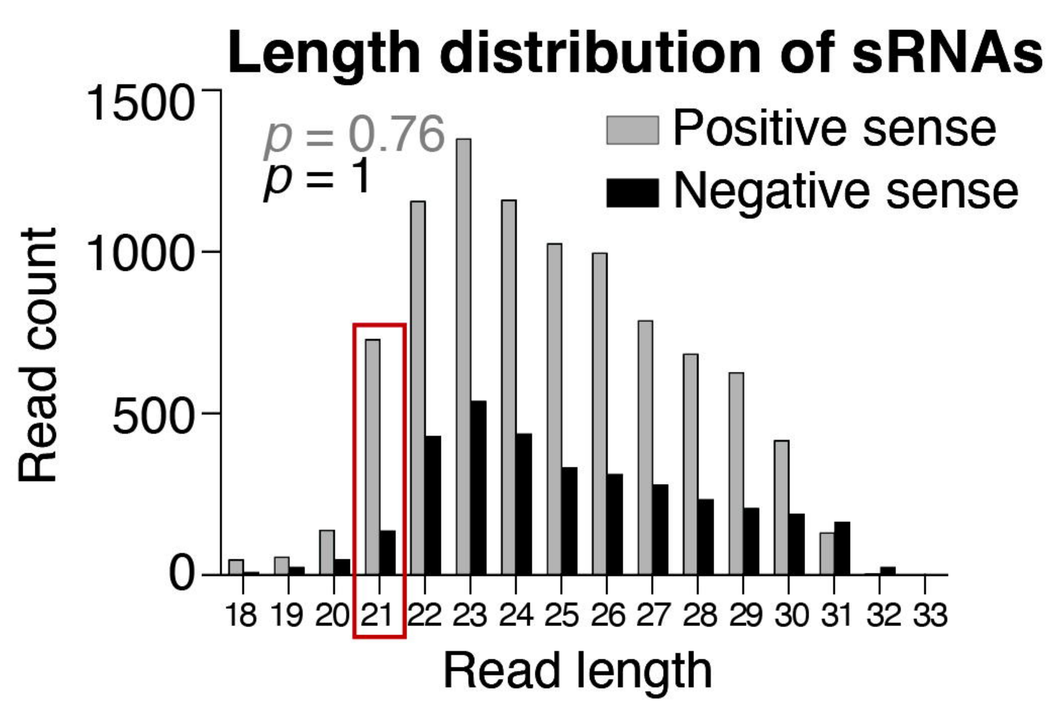
A


T444T-ZIK1 (heat-killed bacteria)
T7 teminator
T7 promoter
T7 promoter
T7 teminator
Target viral sequence
T444T
AmpR

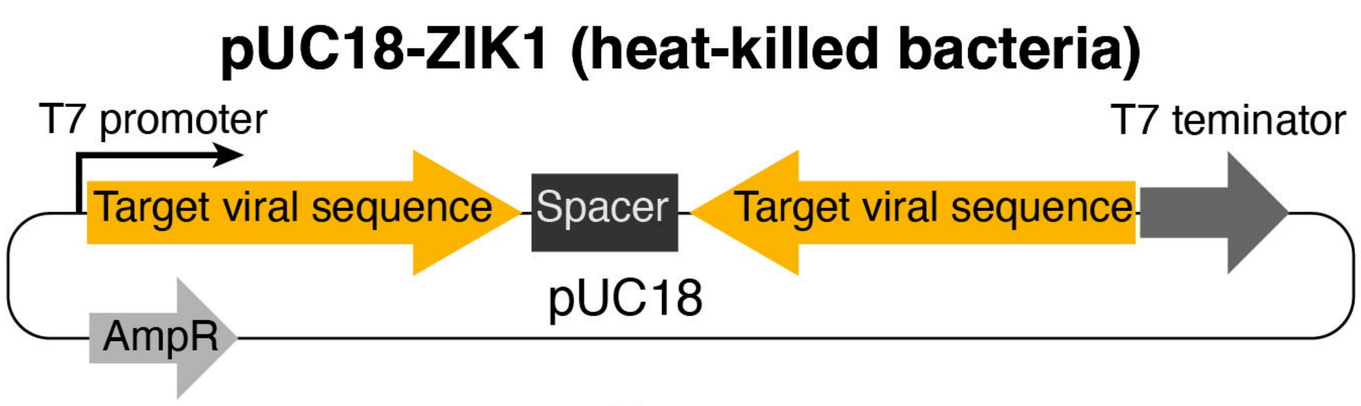
B


Coverage of sRNAs
Read count
2500
2000
1500
1000
500
0
500
0 100 200 300 400 500
Target sequence position

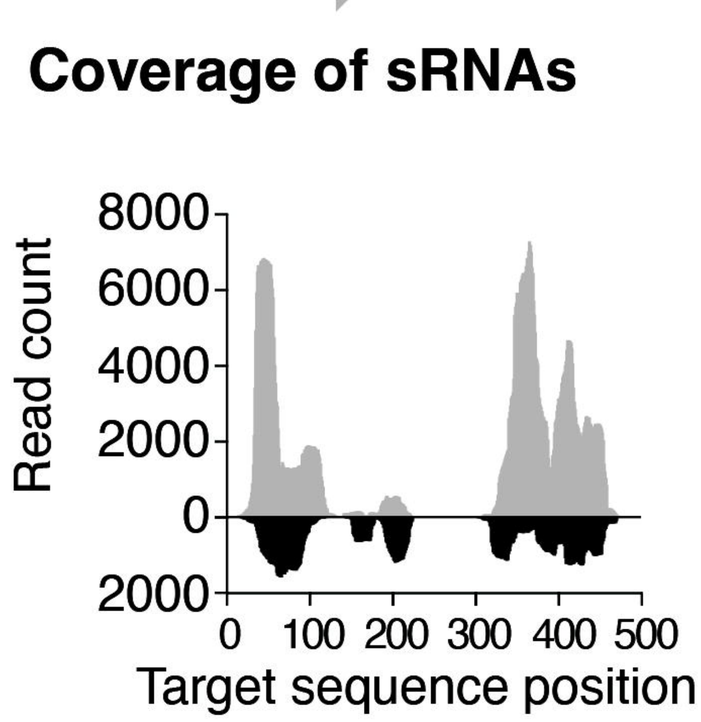
C


Length distribution of sRNAs
Positive sense
Negative sense
p = 0.76
p = 1
Read count
1500
1000
500
0
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
Read length

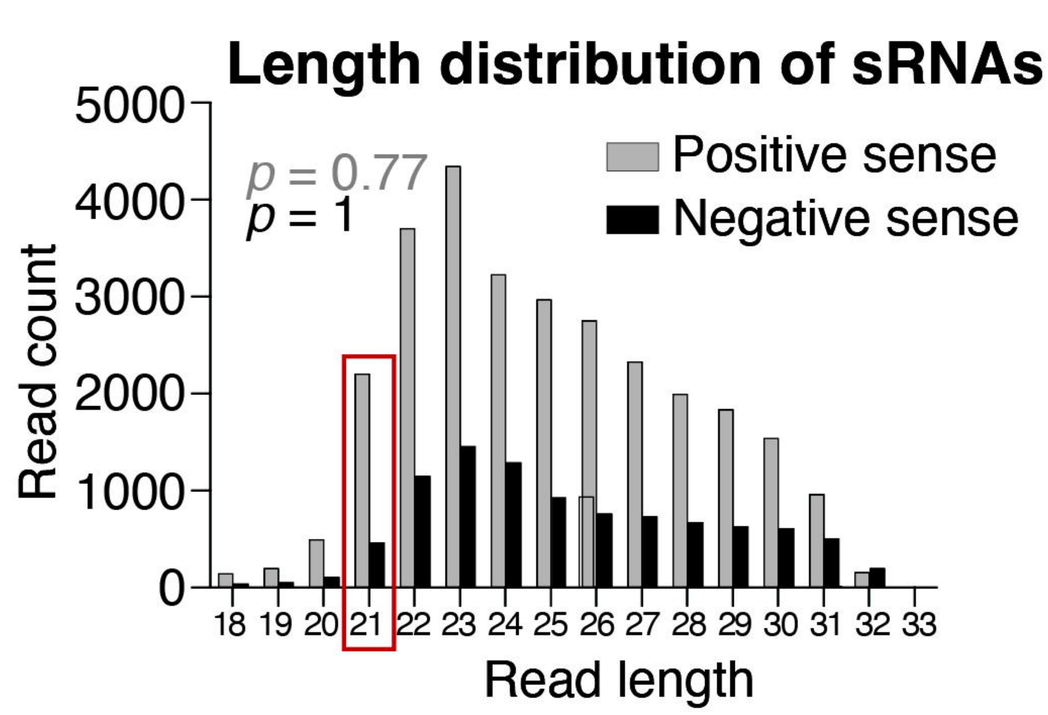
D


pUC18-ZIK1 (heat-killed bacteria)
T7 promoter
T7 teminator
Target viral sequence
Spacer
Target viral sequence
pUC18
AmpR

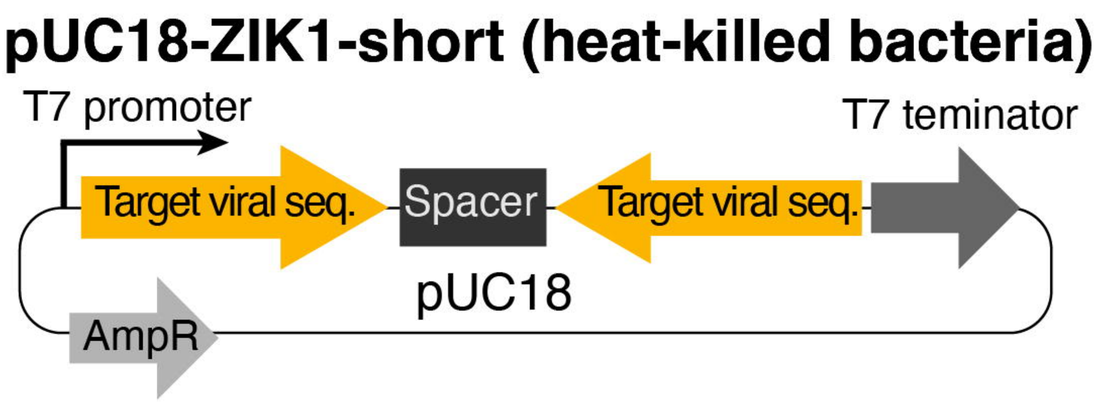
E


Coverage of sRNAs
Read count
8000
6000
4000
2000
0
2000
0 100 200 300 400 500
Target sequence position

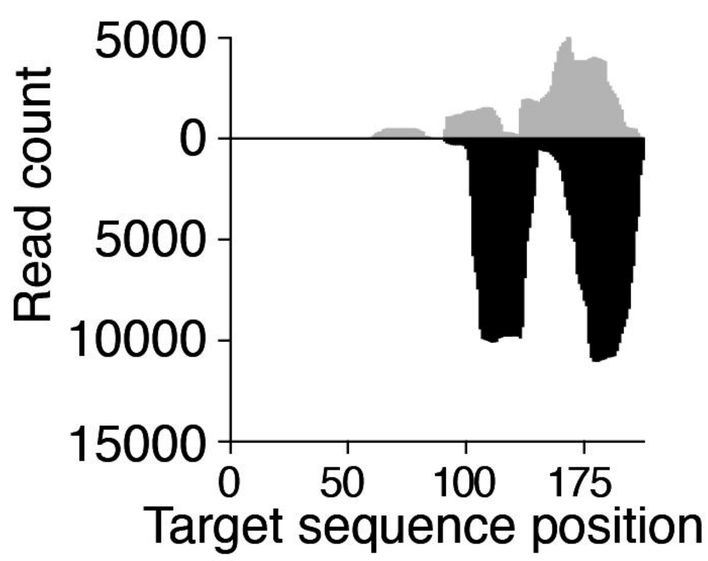
F


Length distribution of sRNAs
Positive sense
Negative sense
p = 0.77
p = 1
Read count
5000
4000
3000
2000
1000
0
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
Read length

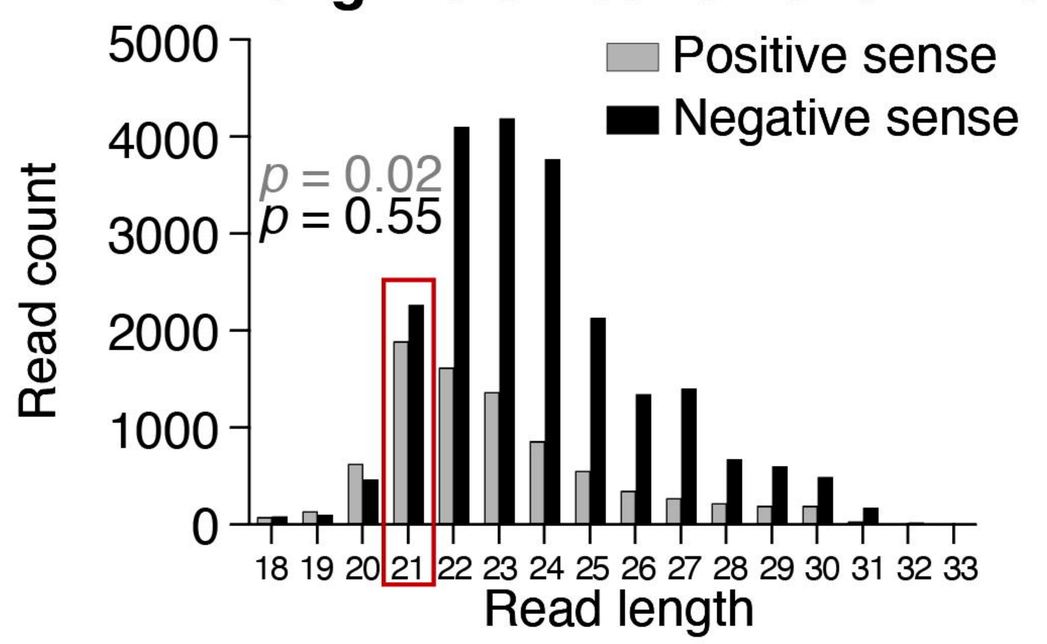
G


pUC18-ZIK1-short (heat-killed bacteria)
T7 promoter
T7 teminator
Target viral seq.
Spacer
Target viral seq.
pUC18
AmpR

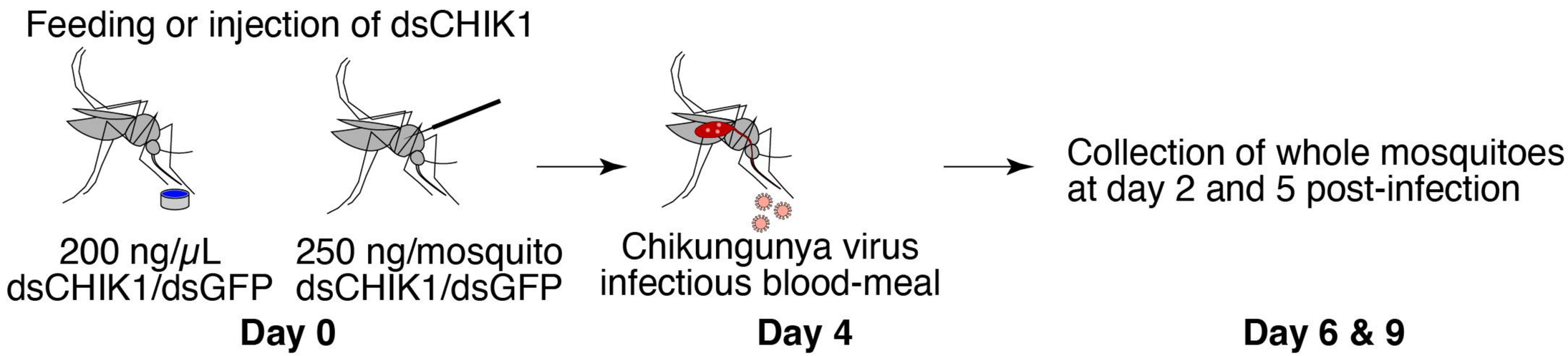
H


Coverage of sRNAs
Read count
5000
0
5000
10000
15000
0 50 100 175
Target sequence position

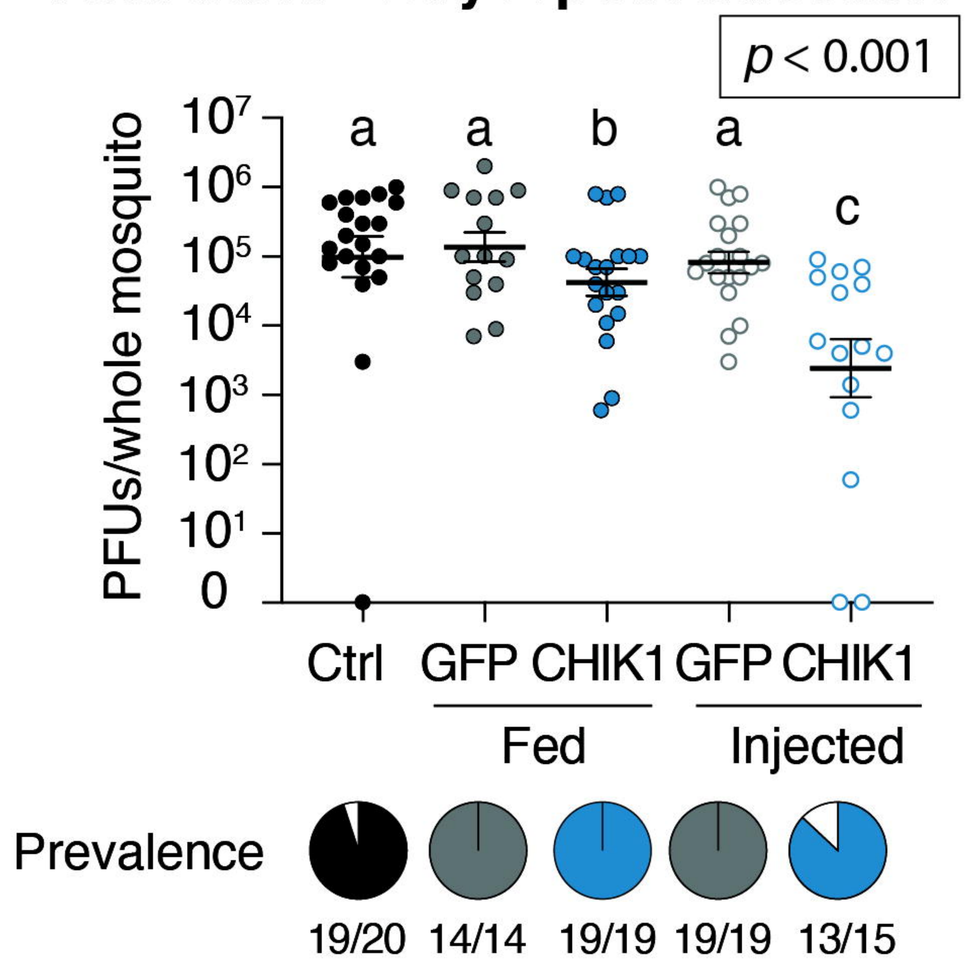
I


Length distribution of sRNAs
Positive sense
Negative sense
p = 0.02
p = 0.55
Read count
5000
4000
3000
2000
1000
0
18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
Read length

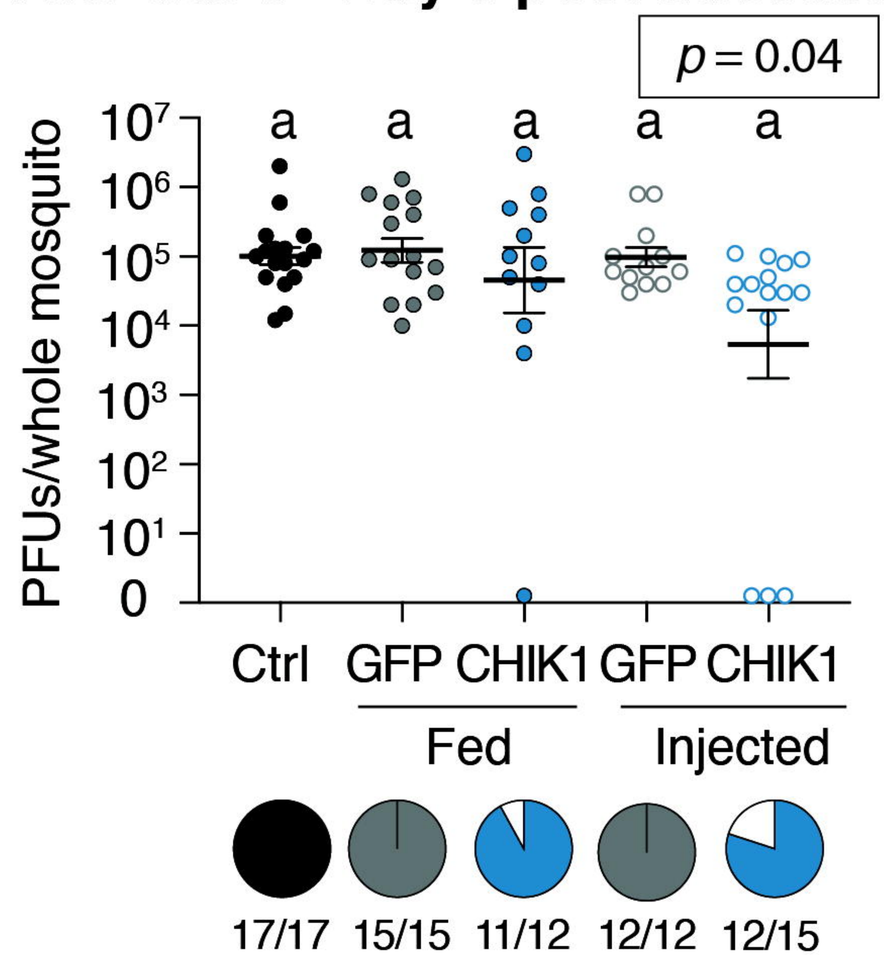
A


Feeding or injection of dsCHIK1
200 ng/μL dsCHIK1/dsGFP
250 ng/mosquito dsCHIK1/dsGFP
Chikungunya virus infectious blood-meal
Collection of whole mosquitoes at day 2 and 5 post-infection
Day 0
Day 4
Day 6 & 9

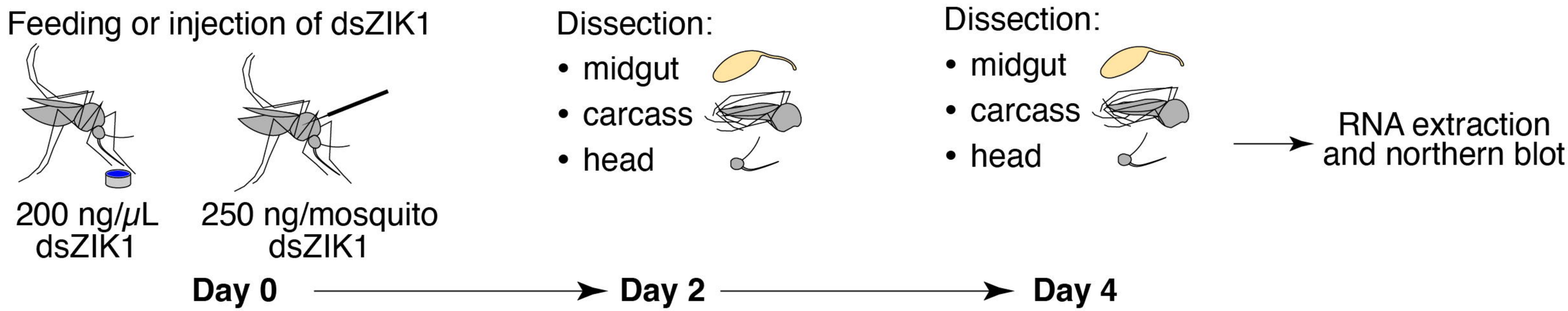
B


Viral titers - Day 2 post-infection
p < 0.001
PFUs/whole mosquito
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
0
a
a
b
a
c
Ctrl
GFP
CHIK1
GFP
CHIK1
Fed
Injected
Prevalence
19/20
14/14
19/19
19/19
13/15

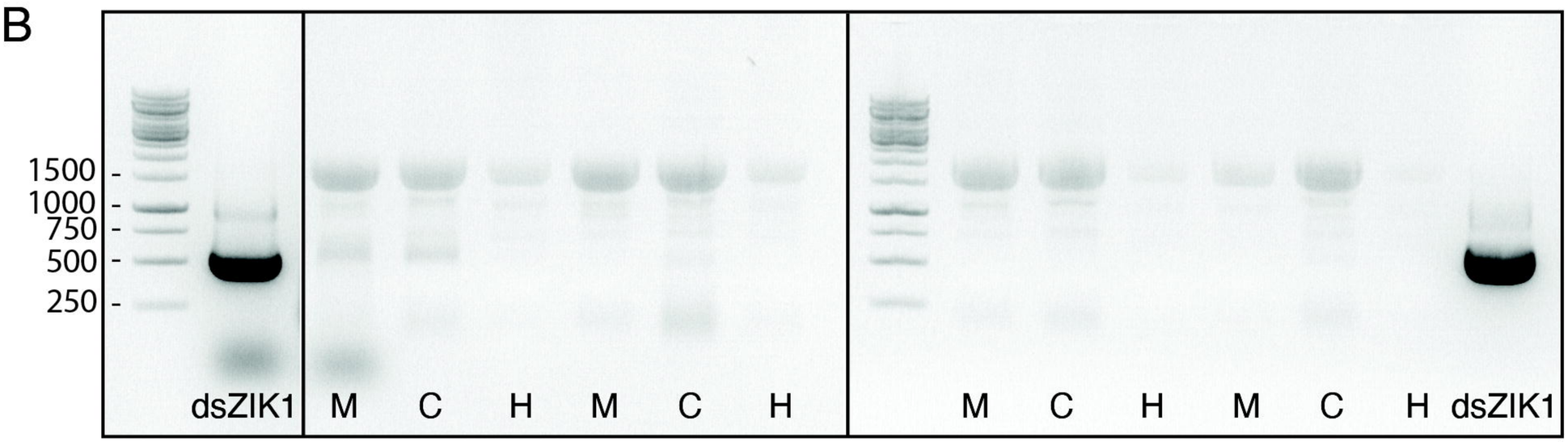
C


Viral titers - Day 5 post-infection
p = 0.04
PFUs/whole mosquito
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
0
a
a
a
a
a
Ctrl
GFP
CHIK1
GFP
CHIK1
Fed
Injected
17/17
15/15
11/12
12/12
12/15

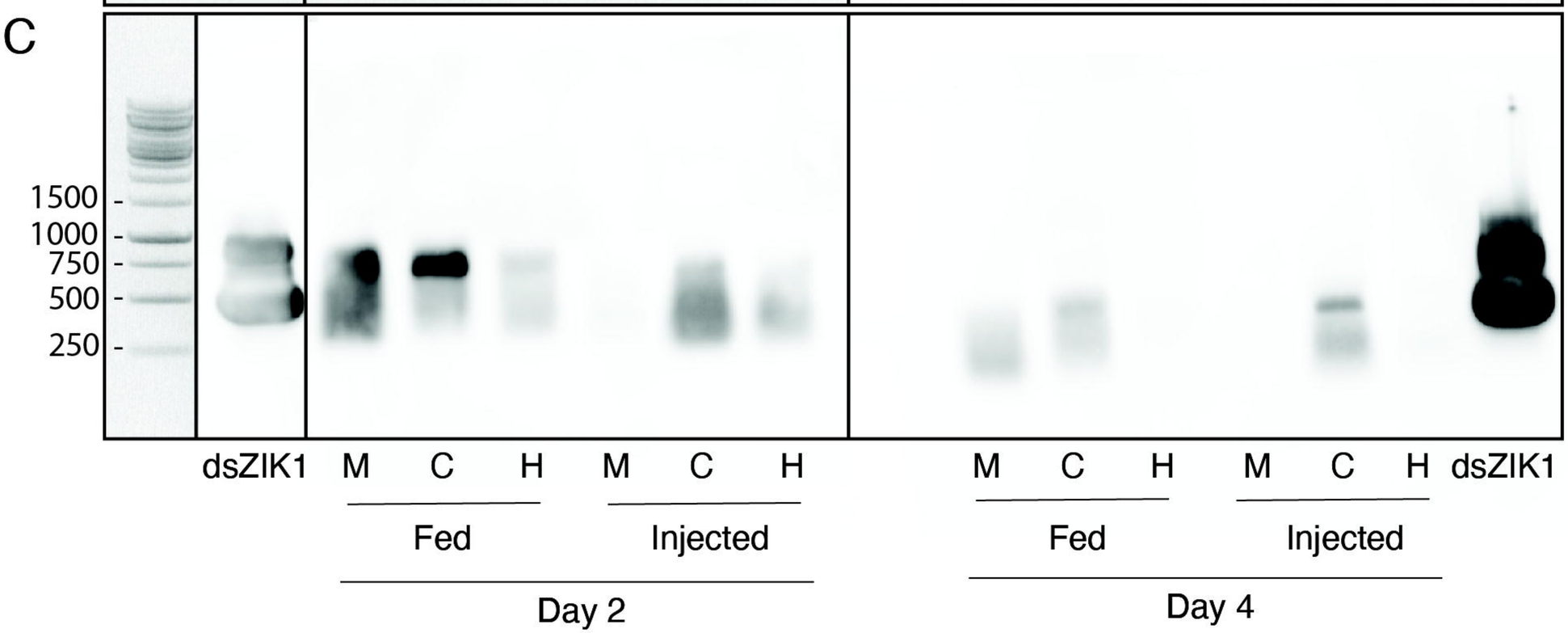
A


Feeding or injection of dsZIK1
200 ng/μL dsZIK1
250 ng/mosquito dsZIK1
Dissection:
• midgut
• carcass
• head
Dissection:
• midgut
• carcass
• head
RNA extraction and northern blot
Day 0
Day 2
Day 4

B


1500
1000
750
500
250
dsZIK1
M
C
H
M
C
H
M
C
H
M
C
H
dsZIK1

C


1500
1000
750
500
250
dsZIK1
M
C
H
M
C
H
M
C
H
M
C
H
dsZIK1
Fed
Injected
Fed
Injected
Day 2
Day 4