

1 **Full Title: Impact of extrinsic incubation temperature on natural selection during Zika**  
2 **virus infection of *Aedes aegypti*.**

3 **Short Title: Temperature impacts on Zika virus population structure**

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23 **Abstract**

24 Arthropod-borne viruses (arboviruses) require replication across a wide range of temperatures  
25 to perpetuate. While vertebrate hosts tend to maintain temperatures of approximately 37°C -  
26 40°C, arthropods are subject to ambient temperatures which can have a daily fluctuation of >  
27 10°C. Temperatures impact vector competence, extrinsic incubation period, and mosquito  
28 survival unimodally, with optimum occurring at some intermediate temperature. In addition, the  
29 mean and range of daily temperature fluctuations influence arbovirus perpetuation and vector  
30 competence. The impact of temperature on arbovirus genetic diversity during systemic mosquito  
31 infection, however, is poorly understood. Therefore, we determined how constant extrinsic  
32 incubation temperatures of 25°C, 28°C, 32°C, and 35°C control Zika virus (ZIKV) vector  
33 competence and population dynamics within *Aedes aegypti* and *Aedes albopictus* mosquitoes.  
34 We also examined diurnally fluctuating temperatures which more faithfully mimic field conditions  
35 in the tropics. We found that vector competence varied in a unimodal manner for constant  
36 temperatures peaking between 28°C and 32°C for both *Aedes* species. Transmission peaked at  
37 10 days post-infection for *Aedes aegypti* and 14 days for *Aedes albopictus*. The effect of diurnal  
38 temperature was distinct and could not have been predicted from constant temperature-derived  
39 data. Using RNA-seq to characterize ZIKV population structure, we identified that temperature  
40 alters the selective environment in unexpected ways. During mosquito infection, constant  
41 temperatures more often elicited positive selection whereas diurnal temperatures led to strong  
42 purifying selection in both *Aedes* species. These findings demonstrate that temperature has  
43 multiple impacts on ZIKV biology within mosquitoes, including major effects on the selective  
44 environment within mosquitoes.

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47 **Author Summary**

48 Arthropod-borne viruses (arboviruses) have emerged in recent decades due to complex factors  
49 that include increases in international travel and trade, the breakdown of public health  
50 infrastructure, land use changes, and many other factors. Climate change also has the potential  
51 to shift the geographical ranges of arthropod vectors, consequently increasing the global risk of  
52 arbovirus infection. Changing temperatures may also alter the virus-host interaction, ultimately  
53 resulting in the emergence of new viruses and virus genotypes in new areas. Therefore, we  
54 sought to characterize how temperature (both constant and fluctuating) alters the ability of  
55 *Aedes aegypti* and *Aedes albopictus* to transmit Zika virus, and how it influences virus  
56 populations within mosquitoes. We found that intermediate temperatures maximize virus  
57 transmission compared to more extreme and fluctuating temperatures. Constant temperatures  
58 increased positive selection on virus genomes, while fluctuating temperatures strengthened  
59 purifying selection. Our studies provide evidence that in addition to altering VC, temperature  
60 significantly influences the selective environment within mosquitoes.

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## 69 **Introduction**

70 Arthropod-borne viruses (arboviruses) such as Zika virus (ZIKV, Flaviviridae, *Flavivirus*) are  
71 mainly RNA viruses that are transmitted by arthropod vectors among vertebrate hosts [1]. Thus,  
72 arboviruses are required to alternately replicate in hosts with drastically different body  
73 temperatures. This affects transmission dynamics, replication rates, and population structure.  
74 While replication in vertebrates generally occurs within 2-3 degrees of 38°C [2], infection in  
75 mosquitoes may occur at a much wider range of temperatures: Mosquito vectors are distributed  
76 throughout tropical and temperate climates and the geographical range of important species is  
77 increasing [3]. Climate variations such as heat waves, cold snaps, or daily temperature  
78 fluctuations change the host environment within which arboviruses replicate and are  
79 transmitted. Fluctuations in the temperature of the host environment are central to arbovirus  
80 biology [4] and virus-host interaction [5-7].

81 The impact of temperature on vector competence (VC), i.e. the ability of a mosquito to acquire,  
82 maintain, and transmit a pathogen, is well described. Temperature increases impact VC in a  
83 unimodal manner (having one clear peak), with extreme low (16°C) and high (38°C)  
84 temperatures having low VC while median temperatures (28°C -32°C) have higher VC [8, 9].

85 The extrinsic incubation temperature (EIT) also influences viral replication and dissemination  
86 within vectors [10-15], altering the extrinsic incubation period, i.e. the number of days between  
87 acquisition of an infection and infectiousness to a new host [5, 16]. Most studies examining the  
88 effects of temperature on VC have used single, constant temperatures to represent optimal  
89 conditions for mosquito colony survival [17-20]. However, diurnal temperature fluctuations more  
90 accurately model the environmental conditions encountered by mosquitoes in the field [21-24].  
91 Although temperature clearly exerts a strong selective pressure on RNA viruses [25, 26], little is  
92 known about how it may influence the composition of arbovirus populations during mosquito

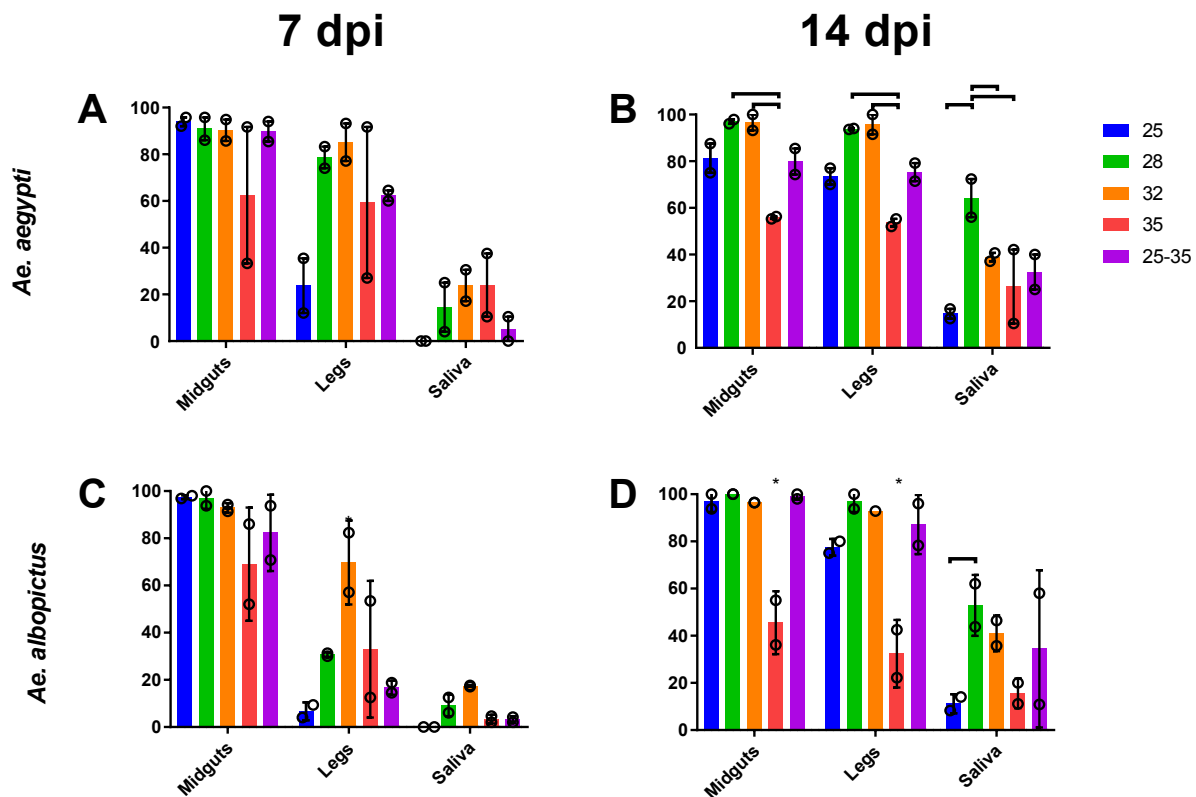
93 infection. Thus, while temperature clearly effects arbovirus transmission and epidemiology, its  
94 impact on arbovirus evolution remains unclear.

95 RNA viruses like ZIKV have the capacity to evolve rapidly in response to changing  
96 environments. This is due, in part, to short generation times and error-prone replication [27, 28].  
97 As a result, arboviruses, including ZIKV, exist within hosts as large populations of mixed  
98 haplotypes, which is critical to their perpetuation in nature [29-33]. While there have been  
99 numerous studies assessing ZIKV VC and viral ecology and some efforts focusing on the use of  
100 environmental data to predict virus spread, there is limited knowledge as to how environmental  
101 factors such as temperature impact the selective environments and mutational diversity of  
102 arboviruses within mosquitoes. Accordingly, we sought to determine whether ZIKV mutational  
103 diversity is altered by EIT during systemic infection of *Ae. aegypti* and *Aedes albopictus* (*Ae.*  
104 *albopictus*) vectors. We exposed mosquitoes to a Puerto Rican isolate of ZIKV and held them at  
105 constant temperatures of 25°C, 28°C, 32°C, 35°C; and a diurnal fluctuation from 25°C to 35°C.  
106 We then assessed VC and measured virus mutational diversity in different tissue compartments  
107 of each mosquito using next-generation sequencing (NGS). Our results suggest that the  
108 selective environment within mosquitoes is significantly modified by temperature, and that  
109 temperature fluctuations exert unique constraints upon arbovirus sequences.

## 110 **Results**

111 To assess how extrinsic incubation temperature affects vector competence for ZIKV we  
112 exposed *Ae. aegypti* and *Ae. albopictus* to ZIKV (n=72-108), held them at 25°C, 28°C, 32°C,  
113 35°C, and alternating diurnal temperature that fluctuated between 25°C-35°C. Infection rates  
114 were high in all mosquitoes except those held at 35°C (Fig 1). In both *Aedes* species, moderate  
115 temperatures (28°C and 32°C) significantly ( $p < 0.05$ , Two-tailed Fisher's exact test) increased  
116 dissemination and transmission at 7 days post-infection (Fig 1A & 1C). The difference in  
117 dissemination was most notable in *Ae. albopictus*, which was ~30% at 28°C and ~80% at 32°C

118 (Fig 1A). Our diurnal temperature group did not fit with the expected unimodal distribution given  
119 the mean daily temperature in this group (30°C). Instead, infection in this temperature group  
120 was lower, and most closely resemble infection rates of the 25°C and 28°C temperatures or  
121 32°C and 35°C temperatures. Mosquitoes experiencing diurnal temperatures also had  
122 significantly lower dissemination and transmission ( $p < 0.05$ , Two-tailed Fisher's exact test)  
123 compared to the standard laboratory colony temperature of 28°C, which is used for most VC  
124 studies. [diurnal temperature depressed aedes vector competence compared to the optimal,  
125 mean temperature.]



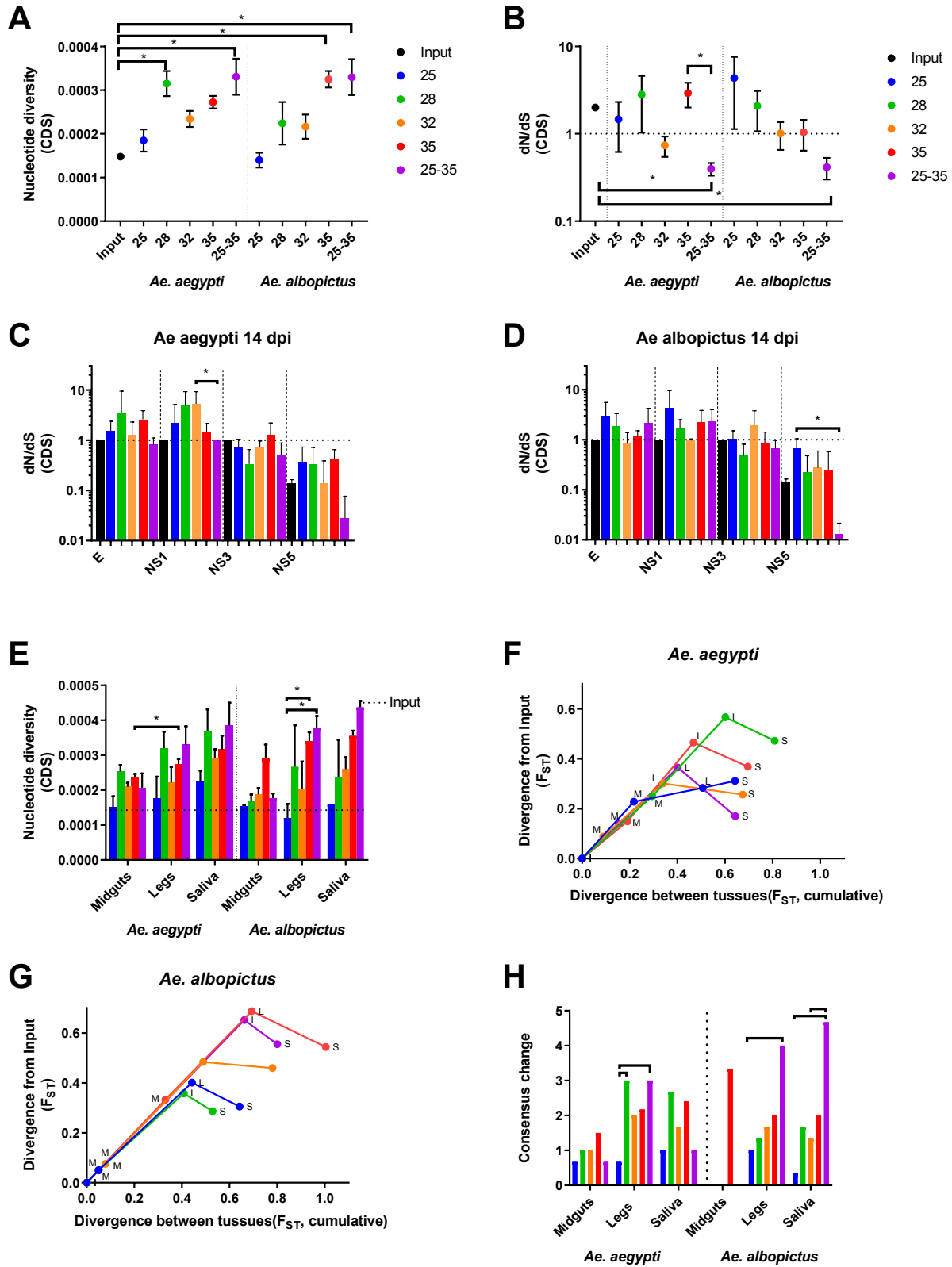
126

127 **Fig 1. Extrinsic incubation temperature alters ZIKV transmission efficiency in *Aedes***  
128 **mosquitoes.** Percent of *Ae. aegypti* (A & B) and *Ae. albopictus* (C & D) with ZIKV in midgut,  
129 legs, and saliva at 7 (A & C) and 14 (B&D) days post feeding. The bar represents the mean and  
130 the open circles represent the value of each experiment with SEM shown with error bars.

131 Brackets above vertical bars indicate statistical significance between indicated groups, asterisks  
132 indicate significance from the entire species-tissue group ( $p < 0.05$ , Two-tailed Fisher's exact  
133 test).

134 Consensus-level changes were rarely observed in ZIKV in these experiments. We therefore  
135 assessed the effect of temperature and mosquito species on ZIKV mutational diversity at the  
136 intrahost level by sequencing virus collected from 3 biological replicates of tissues (midgut, legs,  
137 and saliva) harvested from *Ae. aegypti* and *Ae. albopictus* at 14 days post exposure. Nucleotide  
138 diversity across the coding sequence was lowest in mosquitoes held at 25°C and highest in  
139 mosquitoes held at 35°C or under a diurnally fluctuating temperature regime. Minimal  
140 differences were observed when comparing *Ae. aegypti* to *Ae. albopictus* (Fig 2A).  $d_N/d_S$  was  
141 estimated across the coding sequence to assess selection acting upon viral genomes. In both  
142 *Aedes* species,  $d_N/d_S$  was significantly lower in ZIKV from diurnal-exposed mosquitoes  
143 compared to those held at constant temperatures, and solely virus from these mosquitoes had  
144  $d_N/d_S$  much lower than 1 (Fig 2B). Similar levels of richness, complexity, and divergence were  
145 observed in all mosquitoes and EIT groups (Not Shown).





147 **Fig 2. Temperature alters virus diversification, selection and divergence during mosquito**  
148 **infection.** ZIKV population diversity at varying temperatures was determined using measures of  
149 nucleotide diversity (A) and natural selection ( $d_N/d_S$ ) (B).  $d_N/d_S$  was also examined by virus  
150 coding region for virus that replicated in *Ae. aegypti* (C), and *Ae. albopictus* (D). Results for E,  
151 NS1, NS3, and NS5 protein coding regions are shown.  $d_N/d_S$  was near 1 for C, prM NS2A,  
152 NS2B, NS4A, NS4B (not shown. Nucleotide diversity (E) and divergence (F-G) were determined  
153 for ZIKV in different mosquito compartments. Divergence from input population (y-axis) and  
154 cumulative divergence between tissues (x-axis) (F-G) is presented. Midguts (M), legs (L), Saliva  
155 (S). Consensus change counts also are presented (H) by temperature and mosquito species.,  
156 and majority variants accumulated (H), as markers of population diversity. Significance was  
157 tested using the Kruskal-Wallis test with Dunn's correction (A-D, H, \*  $p < 0.05$ ) or 2-way ANOVA  
158 with Tukey's (E,  $p$ -value  $< 0.05$ ). Figures present the mean and SEM (A-B, E) or 95% CI (C-D).

159 To assess coding region-specific signatures of selection, we analyzed  $d_N/d_S$  for each viral  
160 protein coding sequence independently. In both mosquito species exposed to diurnal  
161 temperatures,  $d_N/d_S$  was much less than 0.1 only within the NS5 coding sequence (Fig 2C-D  
162 mean  $d_N/d_S$  0.027 in *Ae. aegypti* and 0.0132 in *Ae. albopictus*). E and NS1 coding sequences  
163 had  $d_N/d_S$  greater than 1 when temperatures were constant, ranging from a mean  $d_N/d_S$  of 1.298  
164 at 32°C in E to 5.325 at 32°C in NS1 in *Ae. aegypti*. *Ae. albopictus* had a mean low  $d_N/d_S$  of  
165 1.154 at 35°C in E and a high of 4.267 at 25°C in NS1, the exception being 32°C in *Ae.*  
166 *albopictus* where  $d_N/d_S$  was 0.0853 (E) and 0.9804 (NS1) respectively (Fig 2C-D).

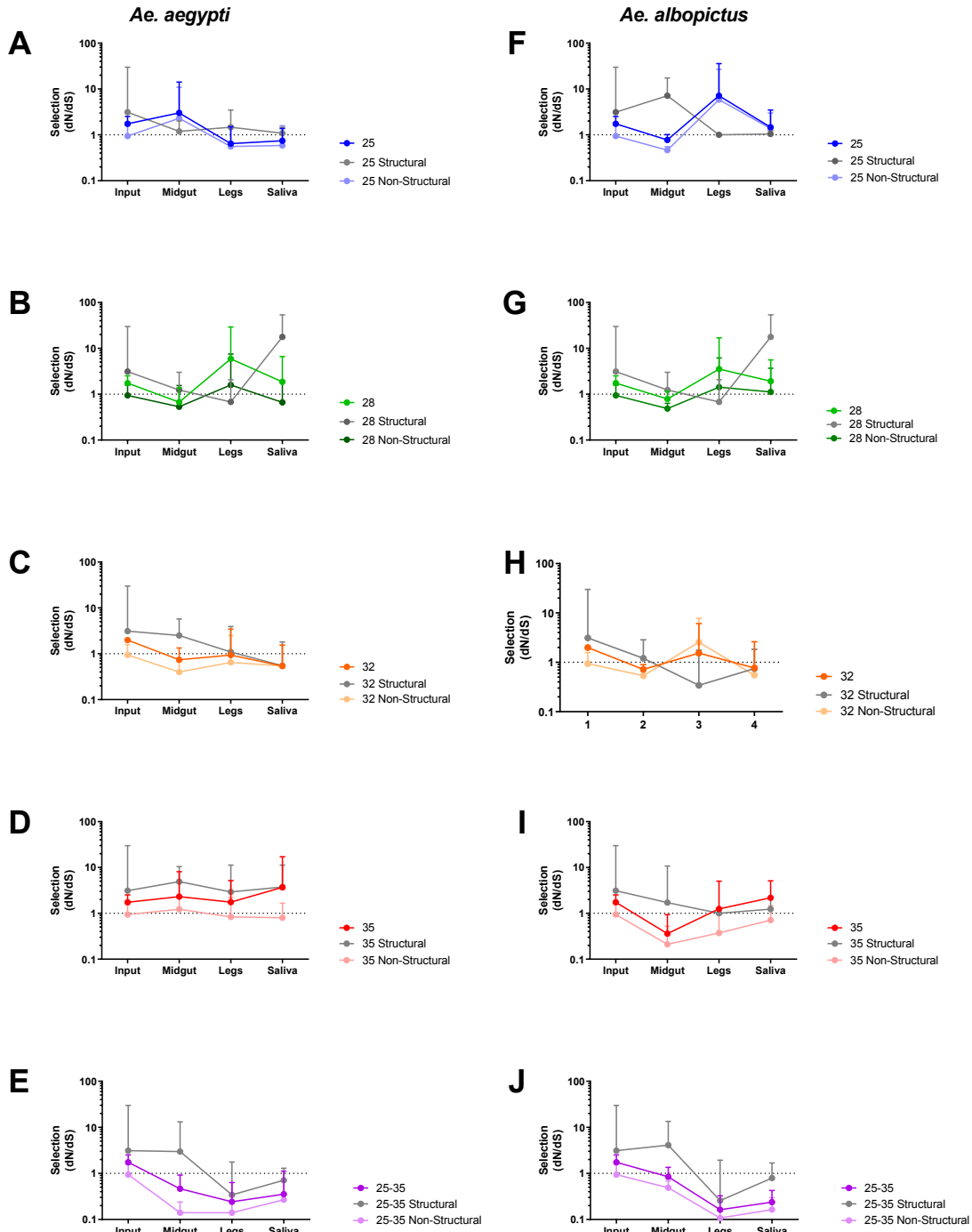
167 Since arboviruses encounter multiple replication environments and barriers during systemic  
168 mosquito infection, we next assessed intrahost population diversity in the midguts, legs, and  
169 saliva of mosquitoes held at varying temperatures (Fig 2E-H). In all tissue compartments of both  
170 species tested, nucleotide diversity tended to increase with increasing temperature (Fig 2E),  
171 with diurnally-exposed mosquitoes having lower diversity during midgut infection but increased

172 genetic diversity during systemic infection that resulted in some of the highest levels of  
173 nucleotide diversity in leg and saliva-associated virus (Fig 2E). Analysis of the fixation index  
174 ( $F_{ST}$ ), during systemic infection revealed temperature- and species-specific patterns of  
175 divergence from the input population (Fig 2F-G). Generally, ZIKV diverged more in the midguts  
176 of *Ae. aegypti* than *Ae. albopictus*. The 28°C EIT group diverged more than any other EIT group  
177 in *Ae. aegypti* (Fig 2F). Whereas in *Ae. albopictus*, exposure to higher temperatures of 32°C  
178 and 35°C promote divergence. In both species, divergence was greatest when the population  
179 disseminated from the midgut to the legs and decreased from legs to saliva. These data provide  
180 evidence that divergence from the founding population was increased in the midgut and legs of  
181 both species and reduced as virus moved from legs to saliva, potentially due to stochastic  
182 reductions caused by bottlenecks and/or purifying selection.

183 Patterns of ZIKV population diversity during systemic infection are temperature dependent. This  
184 also is reflected in the number of changes to the consensus virus sequence (Fig 2H). Increasing  
185 temperature tended to increase the number of consensus changes, with 28°C somewhat of an  
186 outlier. Significantly more consensus changes were observed in *Ae. albopictus* for diurnal  
187 temperatures than any constant temperature group (Fig 2H).

188 Since ZIKV population diversity is influenced by the tissues of origin, as well as the constant and  
189 diurnal EIT, we assessed  $d_N/d_S$  during systemic infection for each EIT group across the entire  
190 CDS, and for the structural and nonstructural regions independently (Fig 3). Our input  
191 population had a  $d_N/d_S$  ratio of 1.75 for the CDS, 3.11 for the structural region, and 0.95 for the  
192 non-structural regions. This indicates that the structural regions of our input population were  
193 under positive selection ( $d_N/d_S$  greater than 1) during its propagation and preparation, whereas  
194 the non-structural regions were not. Interestingly, when ZIKV was exposed to diurnal fluctuating  
195 temperatures, it was under strong purifying selection ( $d_N/d_S$  less than 1) in both *Aedes* species  
196 (Fig 3E & 3J), whereas all constant temperatures caused ZIKV  $d_N/d_S$  generally near or above 1

197 (Fig 3). In the saliva, 25°C and 32°C EIT groups had a  $d_N/d_S$  that neared 1, decreasing from the  
 198 input in both *Aedes* species (Fig 3A, 3C, 3F, 3H). Conversely, 28°C and 35°C EIT groups  
 199 maintained or increased  $d_N/d_S$  when compared to input (Fig 3B, 3D, 3G, 3I).



200

201 **Fig 3. Fluctuating diurnal temperatures impose purifying selection on ZIKV during**  
 202 **systemic mosquito infection.**  $d_N/d_S$  (mean with 95% CI) for ZIKV CDS (closed circles),  
 203 structural sequence (Boxes). And Non-structural sequence (open circles), at indicated  
 204 temperatures, including diurnal fluctuating temperatures, in *Ae. aegypti* (A-E) and *Ae. albopictus*  
 205 (F-J).

206 Although relatively few consensus level changes occurred in ZIKV after replication in  
 207 mosquitoes, we identified a handful of changes to the ZIKV genome that occurred  
 208 independently in several mosquito tissues (Table 1). Of these consensus changes, 3  
 209 nonsynonymous and 1 synonymous SNVs were found in both *Ae. aegypti* and *Ae. albopictus*  
 210 (L330V E, W98G NS1, M220T NS1, and G83 NS5) samples. The remaining 4 consensus  
 211 changes were comprised of 1 non-synonymous mutation (T315I E) unique to *Ae. aegypti* and 3  
 212 mutations unique to *Ae. albopictus*: 1 non-synonymous mutation and 2 synonymous mutations  
 213 (K146E NS1, I94 NS2A and F682 NS5). All were present as minority variants in the input virus  
 214 population, and several have been documented in ZIKV genomic epidemiologic studies. (Table  
 215 1). These consensus changes tended to rise or fall in frequency in a species- and temperature-  
 216 dependent manner (Figure 4). In general, high and/or diurnal temperature tended to drive  
 217 variants to higher frequency in the population, while at the low temperature (25°C) variants  
 218 tended to remain closer to their input frequency. The frequency of the synonymous variant G83  
 219 in NS5 fluctuated in frequency similarly to M220T, suggesting linkage on the viral genome.

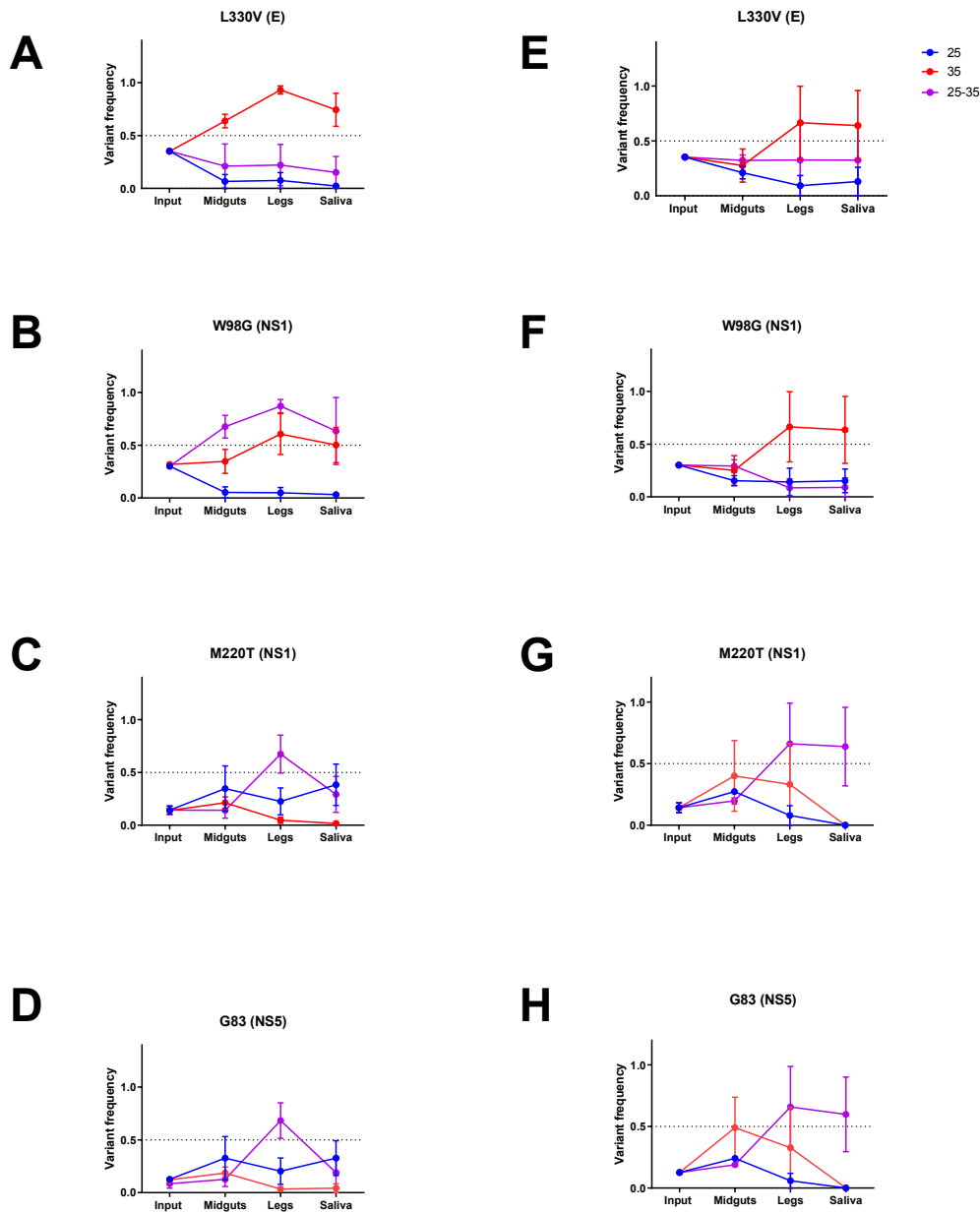
Species	Coding Sequence	AA	Input Freq <sup>a</sup>	Freq found <sup>b</sup> in Nature	Temperature Obs. <sup>c</sup>	Tissue <sup>d</sup> Obs.
<i>Aedes spp.</i>	E	L330V	0.35	100%	28, 32, 35, 25-35	M, L, S
	NS1	W98G	0.30	0%	28, 32, 35, 25-35	M, L, S
	NS1	M220T	0.14	0%	25, 28, 32, 35, 25-35	M, L, S
	NS5	G83	0.13	0%	25, 28, 32, 35, 25-35	M, L, S
<i>Ae. aegypti</i>	E	T315I	0.05	0%	28, 35	M, L, S
<i>Ae. albopictus</i>	NS1	K146E	0.01	2%	25, 28, 32, 25-35	L, S
	NS2A	I94	0.03	0.7%	35, 25-35	M, L, S
	NS5	F682	0.03	0%	35, 25-35	M, L, S

220

221 **Table 1. Multiple ZIKV variants found in across biological samples and temperatures go**  
222 **to consensus.** <sup>a</sup> Variant frequency found in the stock input ZIKV population. <sup>b</sup> The percent  
223 sequence identity observed in nature when aligned to 150 complete ZIKV genomes. <sup>c</sup> Extrinsic  
224 incubation temperatures at which each variant was observed, <sup>d</sup> and tissues that each variant  
225 was observed. Black= both species, Blue= *Ae. aegypti* only, Red= *Ae. albopictus* only. M,  
226 midguts; L, legs; S, saliva.

## *Ae. aegypti*

## *Ae. albopictus*



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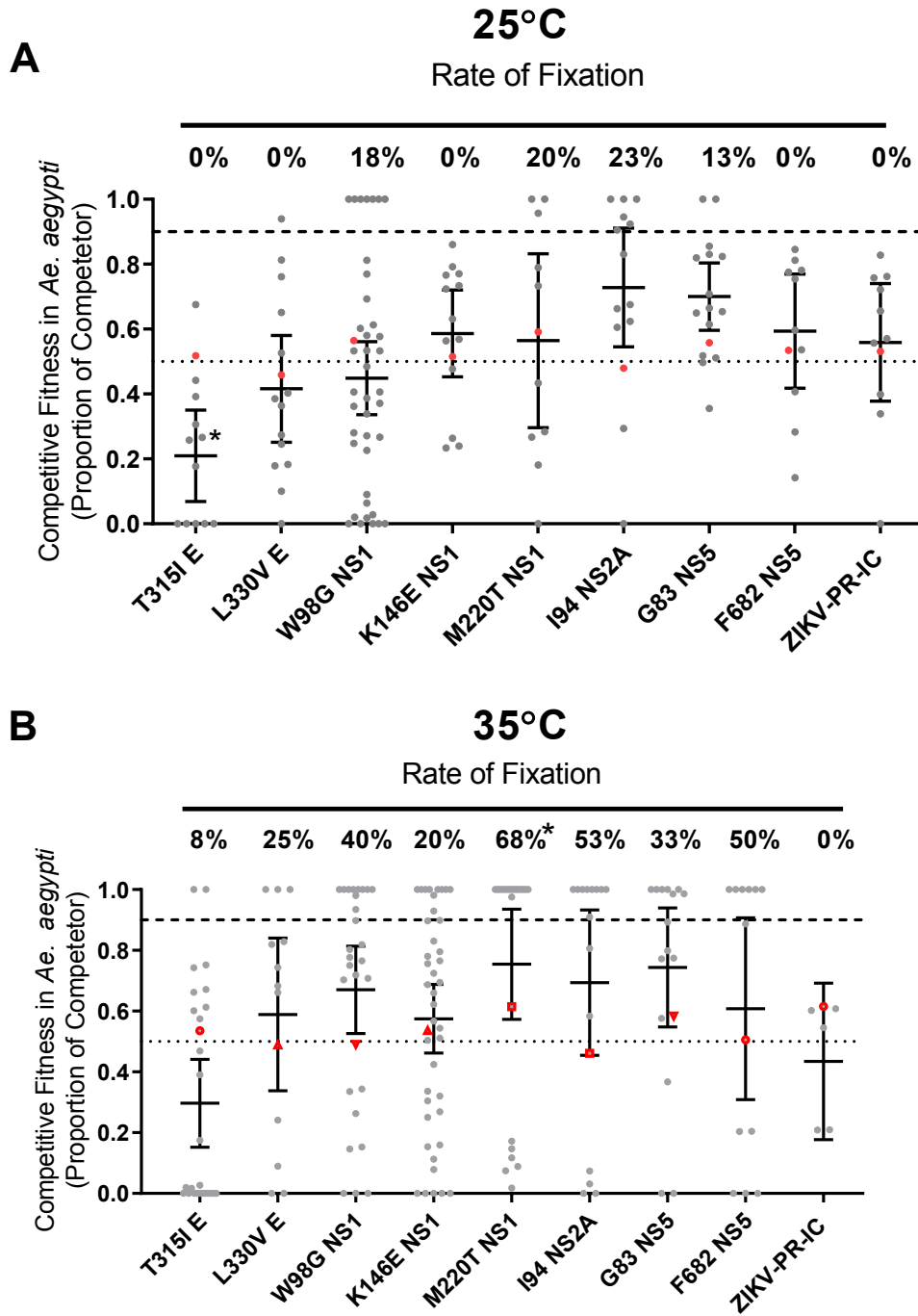
228 **Fig 4. EIT and species control ZIKV variant frequency during systemic infection.**

229 Frequencies of L330V E (A & E), W98G NS1 (B & F), M220T NS1 (C & G), and G83 NS5 (D &

230 H) in input, midgut, legs and saliva shown at 25, 35 and diurnal 25-35C in *Ae. aegypti* (A-D) and

231 *Ae. albopictus* (E-H). Mean and SEM of competitions from three biological replicates shown. We  
232 then competed engineered ZIKV mutants containing the eight consensus-changing mutations  
233 that arose during systemic infection (Table 1) in mosquitoes under low (25°C) and high (35°C)  
234 EITs (Figure 5) to assess whether the fitness of these variants may be temperature-  
235 dependent. Control competitions with marked and unmarked clones of the PRVABC59 virus  
236 were unremarkable, with no significant changes in test to reference virus detected at either EIT.  
237 Several mutants tended to rise in frequency at both 25°C and 35°C, with the overall rate of  
238 fixation (mutants that reached 100% frequency as measured by our assay) higher in  
239 mosquitoes held at 35°C ( $p < 0.001$ , Two-tailed Fisher's exact test). The fitness implications of  
240 these mutants tended to be variable. For example, in orally exposed *Ae. aegypti* bodies, the  
241 clone bearing the NS1 M220T mutant had a significant ( $p < 0.05$ , Kruskal-Wallis and Dunn's)  
242 fitness advantage 14 days after blood feeding compared to wildtype. Conversely, the  
243 envelope T315I mutant had significantly decreased ( $p < 0.05$ , Kruskal-Wallis and Dunn's)  
244 fitness compared to ZIKV-PR-IC at 25°C.





245

246 **Fig 5. High temperature increases variant fixation in *Aedes aegypti*.**

247 Indicated mutations were engineered into a ZIKV-PR-IC and mixed with a ZIKV-REF virus. The

248 proportion of each competitor (grey, mean with 95% CI, \*p-value < 0.05 compared with ZIKV-

249 PR-IC , Kruskal-Wallis and Dunn's) and rate of fixation (\*p-value < 0.05 compared with ZIKV-  
250 PR-IC , Two-tailed Fisher's exact test) was determined from mosquito bodies at 14-dpi for *Ae.*  
251 *aegypti* mosquitoes held at constant EIT's 25°C (A) & 35°C (B). Fixation indicate that 100% of  
252 the sequenced nucleotides were from the competitor virus. Initial viral inoculum (ratio of  
253 competitor virus to reference) is shown in red symbols.

## 254 **Discussion**

### 255 **Extrinsic incubation temperature alters vector competence**

256 Vector competence is largely determined by barriers to infection and escape from mosquito  
257 midgut and salivary glands [4, 34]. Our results support the extensive existing literature [8, 9, 35]  
258 that EIT controls infection and escape mechanisms, resulting in a unimodal distribution of VC  
259 values: Extreme low 25°C and high 35°C temperatures had the lowest VC, while moderate  
260 temperatures of 28°C and 32°C had peak VC (Fig 1). These results agree with previous  
261 studies [8] and mechanistic models predicting the ZIKV thermal optimal limit of 29°C for *Ae.*  
262 *aegypti* [8, 36]. However, the VC of mosquitoes held at diurnal temperatures were  
263 consistently lower than the moderate temperatures of 28°C and 32°C despite having a similar  
264 mean temperature. The reasons for this are unlikely caused by direct effects of temperature  
265 on virus replication because ZIKV readily undergoes replication in vertebrates that commonly  
266 maintain temperatures of 37°C, higher than any temperature tested here. It seems more  
267 likely that the depressed VC observed at higher temps is due to indirect alterations to some  
268 aspect of the mosquito environment caused by thermal stress. While our study does not  
269 capture behavioral and physiological adaptations of mosquitoes to high temperatures, our  
270 data clearly demonstrate that rapidly fluctuating temperatures negatively influence mosquito  
271 vector competence for ZIKV compared to optimal constant temperatures.

### 272 **Temperature and vector species alter the selective environment within mosquitoes.**

273 Our data on intrahost population structure during systemic infection provides several novel  
274 insights into how temperature alters the selective environment in epidemiologically relevant  
275 arbovirus vectors. First, our data demonstrate that, in general, increasing temperature leads to  
276 increases in nucleotide diversity (Fig 2A). This may simply reflect faster virus replication at  
277 higher temperatures, with additional rounds of replication introducing additional mutations in a  
278 somewhat clocklike fashion. Alternatively, it may be that this increase in nucleotide diversity  
279 reflects the strength of positive selection at higher temperatures. The exception to this is  
280 mosquitoes that were held at 28°C. While we cannot currently explain the higher diversity in  
281 virus from these mosquitoes, it is notable that our colony has been maintained at 28°C for more  
282 than 25 generations. Mosquitoes held at diurnally fluctuating temperatures, as above, were  
283 somewhat atypical in that they tended to have higher levels of nucleotide diversity than one  
284 would expect given the distribution of diversity observed in mosquitoes held at constant  
285 temperature. As in the case of VC, diurnal temperature fluctuations are distinct in their impacts  
286 on virus-host interactions when compared to constant temperatures and thus the impact and  
287 strength of selection is also distinct.

288 Analysis of synonymous and nonsynonymous variation at the intrahost level revealed that  
289 signatures of strong purifying selection were observed only in mosquitoes held at diurnal  
290 temperatures (Fig 2B-D; Fig 3E, J). This observation provides an important counterpoint to  
291 several previous studies that have documented a relaxation of purifying selection in flaviviruses  
292 that are undergoing replication in mosquitoes [37]. Data from mosquitoes held at constant  
293 temperatures supports these prior findings:  $d_N/d_S$  was generally near or above 1. In some cases,  
294 (e.g. 28°C and 35°C) strong positive selection was detected within the E and NS1 coding  
295 regions. Positive selection observed at 35°C seems likely to be due to virus adaption to some  
296 component of the mosquito stress response such as heat shock protein [38, 39]. The  
297 mechanisms that give rise to the signal of positive selection at 28°C are not clear but may be

298 due to a bottleneck that by chance resulted in a genome with one or more nonsynonymous  
299 variants that rose in frequency due to this stochastic event rather than natural selection. The  
300 strong purifying selection observed in mosquitoes held at diurnal temperatures is detectable  
301 mainly in nonstructural coding sequences, and principally in NS5. This seems logical given the  
302 role of NS5-encoded proteins in virus replication [40] and highlights the requirement for  
303 replicase functionality across a wide temperature gradient. Indeed, our data suggest that  
304 ZIKV NS5 is adapted to temperature variation rather than to any individual temperature.

305 Analysis of consensus sequences taken from different mosquito tissues allowed us to identify  
306 the impacts of virus dissemination and physiological barriers on ZIKV sequences during  
307 systemic infection. Generally, our data demonstrate a positive correlation between constant  
308 temperature and nucleotide diversity (Fig 2E). As the virus disseminates into saliva, nucleotide  
309 diversity increases both with temperature and in a stepwise manner as new tissue  
310 compartments are infected. These findings indicate that at higher temperatures, more new  
311 consensus variants are generated. The impact of diurnal temperatures differed between two  
312 species, with most new consensus variants generated at diurnal temperatures by the less-  
313 efficient vector *Ae. albopictus*. These observations demonstrate that temperature gradients and  
314 fluctuations, and virus transmission by new, perhaps less efficient vector species, can drive the  
315 emergence of new virus variants during mosquito infection, and are supported by analyses of  
316 divergence that incorporate both intra- and interhost variation. Therefore, migration of  
317 arboviruses such as ZIKV into new environments containing unexplored vectors represents an  
318 opportunity for the emergence of new virus variants.

### 319 **Increased extrinsic incubation temperatures drive viral variant fixation in mosquitoes**

320 We identified 8 consensus mutations (5 non-synonymous and 3 synonymous) in multiple  
321 mosquitoes during the course of this study. These all were present in the input population at low  
322 frequencies (0.01- 0.35, Table 1) and rose in frequency during mosquito infection. Four

323 consensus changes were found in both *Aedes* species, and in mosquitoes held under most  
324 temperature regimes. L330V E (Fig S1A) is within domain III of the envelope protein, which  
325 plays a role in host cell receptor binding and entry [41]. W98G NS1 (Fig S1B) is a surface  
326 exposed aromatic to aliphatic amino acid change on the wing section of NS1, which contributes  
327 to cellular membrane association [42]. M220T NS1 (Fig S1C) replaces a sulfur containing side  
328 group with a hydroxylic side group and is located on the loop surface the NS1 172-352  
329 homodimer [43]. G83 NS5 (Fig S1D) is a synonymous mutation found in the middle of the  
330 coding sequence for the NS5 methyltransferase domain active site. Moreover most of these  
331 substitutions were not particularly conservative and may be of functional significance.  
332 While none of these mutations increased significantly in frequency at 25°C, mosquito exposure  
333 to 35°C or diurnal temperatures cause some of these mutations to rise in frequency,  
334 sometimes in a species-dependent manner, again highlighting the temperature-dependence  
335 of variant frequencies in our studies.  
336 To assess the fitness implications of these and other mutations that were repeatedly detected in  
337 mosquitoes, we engineered individual mutations into a ZIKV infectious clone and conducted *in*  
338 *vivo* competition studies at 25° and 35°C. The most notable finding from these studies is that  
339 higher temperatures tended to favor frequently detected mutations, which is consistent with our  
340 data on variant frequencies, consensus level changes and the strength of purifying selection.  
341 Accordingly, we conclude that the variants we examined are more likely to reach high frequency  
342 at higher temperature.  
343 The work presented here was designed to address the hypothesis that temperature determines  
344 not only the efficiency with which mosquitoes transmit arboviruses (which has been well  
345 established for decades) but that it also influences virus evolutionary dynamics. The lack of  
346 quantitative data on how temperature effects arbovirus mutational diversity and selective forces  
347 within mosquitoes is a critical shortcoming in the literature. Data presented here provides some  
348 novel insights into this. The most significant findings reported here are related to how increases

349 in temperature increase the rate of fixation of novel variants in virus populations, both at the  
350 population and consensus sequence levels. This suggests that as global temperatures rise, new  
351 virus variants may emerge more rapidly. This observation requires validation using other virus-  
352 vector pairs, but the implications are ominous and require further attention.

353 A second key finding is that diurnal temperatures impose heretofore undetected purifying  
354 selection on virus populations as they pass through mosquitoes. This finding was not predicted,  
355 but was consistent in both mosquito species examined. We suspect that this finding is related to  
356 both (a) increased constraint imposed by the requirement that the virus replicase act efficiently  
357 across a ten degree Celsius temperature range and (b) the inability of potentially temperature-  
358 specific adaptive mutations to rise in frequency. Our data on individual mutations in various  
359 mosquito tissues supports this. Moreover, this work collectively highlights the significance of  
360 temperature changes on the evolutionary biology of the mosquito-virus interaction. It also  
361 indicates that studies of arbovirus-vector interactions conducted using multiple temperatures,  
362 including diurnal temperature cycles, may capture subtle yet significant evolutionary forces that  
363 act on viruses during mosquito infection.

## 364 **Methods**

### 365 **Cells and Virus**

366 African Green Monkey kidney cells (Vero; ATCC CCL-81) were maintained at 37°C and 5% CO<sub>2</sub>  
367 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum  
368 (FBS) and 1% penicillin-streptomycin (Pen-Strep). Zika virus strain PRVABC59 (ZIKV-  
369 PRVABC59; GenBank # KU501215) obtained from the Center for Disease Control and  
370 Prevention branch in Fort Collins, CO was originally isolated from the sera of a patient returning  
371 from travel to Puerto Rico in December 2015. The virus was isolated on Vero cells and a 4<sup>th</sup>  
372 passage frozen at -80 was used for all *in vivo* and *in vitro* experiments. ZIKV-PRVABC59  
373 infectious clone (ZIKV-PR-IC) served as a backbone for the reverse genetic platform developed

374 by our lab [44] to introduce all point mutations. ZIKV-REF was designed using the  
375 aforementioned reverse genetic platform, incorporating 5 synonymous mutations amino acid  
376 108-arginine and 109-serine of the prM protein coding sequence. The ZIKV-PR-IC sequence  
377 nucleotides were changed from ZIKV-PR-IC 795-CGG TCG-800 to ZIKV-REF 795-AGA AGT-  
378 200.

## 379 **Mosquitoes**

380 *Ae. aegypti* colonies for this study were established from individuals collected in Poza Rica,  
381 Mexico [45] and used at F13-F18 generation. A lab adapted colony (greater than 50  
382 generations) of *Ae. albopictus* were established from individuals collected in Florida, USA; the  
383 colony was provided by the Centers for Diseases Control and Prevention (CDC-Fort Collins,  
384 CO, USA) in 2010. Mosquitoes were reared and maintained at 27-28°C and 70-80% relative  
385 humidity with a 12:12 L:D photoperiod. Water and sucrose were provided ad libitum.

## 386 **Infection of *Aedes* mosquitoes and sample collection**

387 Adult mosquitoes used for experiments were 3-7 days post-eclosion. Mosquitoes were provided  
388 a bloodmeal containing calf blood mixed 1:1 with ZIKV-PRVABC59 (1E7 PFU/mL) using a water  
389 jacketed glass membrane feeder. Engorged female mosquitoes were sorted into cartons and  
390 housed at 25°C, 28°C, 32°C, 35°C at constant temperatures or alternating between 25°C -35°C  
391 to simulate diurnal condition, with 70-80% relative humidity and 12:12 L:D photoperiod.  
392 Mosquitoes were cold anesthetized in preparation for dissociations. Mosquito midguts,  
393 legs/wings, and saliva from the first batch of mosquitoes were collected after 7- and 14-days  
394 post-feed for NGS processing. Mosquito carcass, legs/wings and saliva from the second batch  
395 of mosquitoes were collected at 3, 5, 7, 10, and 14 days post-feed for assessing systemic  
396 infecting dynamics. Tissues represent infection (midgut), dissemination (legs), and transmission  
397 (saliva). Tissues were removed using forceps cleaned with 70% ethanol between samples and

398 were homogenized in 200  $\mu$ l of mosquito diluent with a stainless-steel ball bearing using a  
399 Retsch Mixer Mill 400 at 24 Hz for 45 seconds, as previously described [46]. Saliva was  
400 collected by inserting mosquito mouthparts into capillary tubes containing mineral oil for 30 to 45  
401 minutes. Saliva in oil was removed from the capillary tube by centrifugation into 100  $\mu$ l of  
402 mosquito diluent for 5 minutes at  $>20,000 \times g$ . All samples were stored at  $-80^{\circ}\text{C}$  until  
403 manipulation.

#### 404 **Plaque assay**

405 ZIKV stocks and infectious bloodmeal were quantified by plaque assay on Vero cell cultures  
406 seeded in 12-well plates. Duplicate wells were infected with 0.2 ml aliquots from serial 10-fold  
407 dilutions of virus stocks and infectious blood meals in media (DMEM supplemented with 1%  
408 FBS and 1% penicillin/streptomycin), and virus was adsorbed for one hour by incubating at  
409  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . Following incubation, the inoculum was removed, and monolayers were  
410 overlaid with tragacanth-EMEM overlay containing 1x EMEM, 5x L-glutamine, sodium  
411 bicarbonate 3.75%, tragacanth 1.2%, gentamicin (25mg/ml), and Amphotericin B 40mL/L. Cells  
412 were incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  for four days for plaque development. Cell monolayers then  
413 were stained with 1 mL of overlay containing a 20% ethanol and 0.1% crystal violet. Cells were  
414 incubated at room temperature for 30-60 minutes and then gently washed and plaques were  
415 counted. Plaque assays for 3,5,7,10 and 14 days post infection (dpi) mosquitoes were  
416 performed similar to above with the following changes, 50  $\mu$ l of homogenized midgut and leg  
417 tissues or 30  $\mu$ l of saliva samples were added to Vero cultures in 24-well plates (final volume of  
418 200  $\mu$ l), and plaques were observed post processing.

#### 419 **Viral RNA isolation**

420 Viral RNA was extracted from 50  $\mu$ l of either cell culture supernatant, homogenized mosquito  
421 tissues, or saliva-containing solution using the Mag-Bind<sup>®</sup> Viral DNA/RNA 96 kit (Omega Bio-



422 Tek) on the KingFisher Flex Magnetic Particle processor (Thermo Fisher Scientific). Nucleic  
423 acid extraction was performed as directed by the manufacturer and eluted in 50 µl nuclease-free  
424 water. Viral RNA was then quantified by qRT-PCR using the iTaq™ Universal Probes One-Step  
425 Kit (BIO-RAD) according to manufacturer's protocol using a forward primer (5'-  
426 CCGCTGCCCAACACAAG-3'), reverse primer (5'- CCACTAACGTTCTTTTGCAGACAT-3'), and  
427 FAM probe (5'- AGCCTACCTTGACAAGCAGTCAGACACTCAA-3') sequences [47].

#### 428 **Generation of ZIKV mutant clones**

429 An infectious clone for ZIKV-PRVABC59 was used to generate mutants [44]. To engineer the  
430 point mutations (Table 1) into the ZIKV genome, the corresponding single nucleic acid  
431 substitution was introduced into the ZIKV-PR-IC using *in vivo* assembly cloning methods [48].  
432 The infectious clone plasmids were linearized by restriction endonuclease digestion, PCR  
433 purified, and ligated with T4 DNA ligase. From the assembled fragments, capped T7 RNA  
434 transcripts were generated, and the resulting RNA was electroporated into Vero cells. Infectious  
435 virus was harvested when 50-75% cytopathic effects were observed (5-8 days post  
436 transfection). Viral supernatant was clarified by centrifugation and supplemented to a final  
437 concentration of 20% fetal bovine serum and 10 mM HEPES prior to freezing and storage as  
438 single use aliquots. Titer was measured by plaque assay on Vero cells. All stocks (both wildtype  
439 and infectious clone-derived viruses) were sequenced via sanger sequencing to verify complete  
440 genome sequence.

#### 441 **Competition studies**

442 Competitive fitness was determined largely as described in previous studies [49, 50].  
443 Competitions were conducted with orally infected *Ae. aegypti* (Poza Rica) mosquitoes. Three to  
444 seven day old mosquitoes were offered a bloodmeal containing the 1:1 mixture of viruses  
445 (ZIKV-REF and ZIKV-clone of interest) at a concentration of 1 million PFU/mL and bodies were

446 collected 14 days post blood feed. RNA was extracted as above, and amplicons were generated  
447 via qRT-PCR using iTaq™ Universal SYBR® Green One-Step Kit (BIO-RAD) according to  
448 manufacture protocol. A locked nucleic acid (LNA) forward primer was used to ensure amplicon  
449 specificity. The forward LNA primer 5'-A+CTTGGGTTGTGTACGG-3' and reverse primer 5'-  
450 GTTCCAAGACAACATCAACCCA-3' were used to generate amplicons for Quantitative Sanger  
451 sequencing. Genotype fitness was analyzed using polySNP software [51] to measure the  
452 proportion of the five synonymous variants present in the ZIKV-REF sequence allowing us to  
453 compare the proportion of ZIKV-REF virus to competitor virus.

#### 454 **Library preparation for next-generation sequencing**

455 Positive controls were generated in triplicate, each generated with 1 million genome equivalents  
456 of a 100% ZIKV PRVABC59 viral stock, a mixture of 90% ZIKV PRVABC59 and 10% ZIKV  
457 PA259359 (GenBank # KX156774.2), and a mixture of 99% ZIKV PRVABC59 and 1% ZIKV  
458 PA259359. The negative control was water (no template control, or NTC). Controls and sample  
459 RNA (10ul) was prepared for NGS using the Trio RNA-Seq Library Preparation Kit (NUGEN) per  
460 manufacturer standard protocol. Final libraries were pooled by tissue type and analyzed for size  
461 distribution using the Agilent High Sensitivity D1000 Screen Tape on the Agilent TapeStation  
462 2200, final quantification was performed using the NEBNext® Library Quant Kit for Illumina®  
463 (NEB) according to manufacturer's protocol. 150 nt paired-end reads were generated using the  
464 Illumina HiSeq4000 at Genewiz.

#### 465 **NGS processing and data analysis**

466 NGS data were analyzed using a workflow termed "RPG (RNA virus Population Genetics)  
467 Workflow"; this workflow was generated using Snakemake [52] and workflow and related  
468 documentation can be found at <https://bitbucket.org/murrieta/snakemake/src>. Briefly, Read 1  
469 and Read 2 .fastq files from paired-end Illumina HiSeq 4000 data were trimmed for Illumina

470 adaptors and quality trimming of phred scores < 30 from the 3' and 5' read ends using cutadapt  
471 [53]. The reads were then mapped to the ZIKV-PRVABC59 reference sequence (GenBank #  
472 KU501215) using MOSAIK [54], similar to that previously described [55]. Picard [56], Genome  
473 Analysis Toolkit (GATK) [57], and SAMtools [58] were used for variant calling preprocessing.  
474 SNV's and inserts and deletions (INDELS) we called using LoFreq [59] with the --call-indels  
475 command; otherwise, all settings were default. Consensus sequences were generated using the  
476 .vcf files generated above and VCFtools [60]. NTC had less than 0.02% of reads mapping to  
477 ZIKV and an average of < 8x coverage across the genome indicating little to no contamination,  
478 sequencing bleed through, or index hopping (S1 Table). Only variants in the coding sequence  
479 (nt position 108-10379), with 100x coverage or greater and a cut off of 0.01 frequency were used  
480 for analysis to account for low coverage (reads per genome position) in the 3' and 5'  
481 untranslated regions (S1 Table, Fig S2).

482 Data analysis was performed using custom Python and R code integrated into the RPG  
483 Workflow. Using .vcf files generated by LoFreq and .depth files generated by GATK  
484 DepthOfCoverage command, the workflow generates .csv files that provides sequencing  
485 coverage across the CDS, Shannon entropy, richness, nucleotide diversity,  $d_N/d_S$ , and  $F_{ST}$   
486 (compared to input population) of a specified locus. Additionally, amino acid changes,  
487 synonymous (S) and non-synonymous (NS) changes, and Shannon entropy are reported by  
488 variant positions. The same scripts are called manually outside of the RPG Workflow to perform  
489 the above analysis on specific protein coding regions or to compare divergence of populations  
490 other than the input.

#### 491 **Genetic diversity**

492 All genetic diversity calculations were incorporated into Python and R code located at  
493 <https://bitbucket.org/murrieta/snakemake/src/master/scripts/>. In short, richness was calculated

494 by the sum of the intrahost SNV (iSNV) sites detected in the CDS in each population. Diversity  
495 was calculated by the sum of the iSNV frequencies per coding sequence. Complexity was  
496 calculated using Shannon entropy ( $S$ ) which was calculated for each intrahost population ( $i$ )  
497 using the iSNV frequency ( $p$ ) at each nucleotide position ( $s$ ):

$$498 \quad (1) \quad S_{i,s} = -(p_s(\log_2 p_s) + (1 - p_s)\log_2(1 - p_s))$$

499 The mean  $S$  from all sites  $s$  is used to estimate the mutant spectra complexity. Divergence was  
500 calculated using  $F_{ST}$  to estimate genetic divergence between two viral populations as described  
501 previously [55]

## 502 **Selection**

503 Intrahost selection was estimated by the ratio of nonsynonymous ( $d_N$ ) to synonymous ( $d_S$ ) SNVs  
504 per site ( $d_N/d_S$ ) using the Jukes-Cantor formula as previously described [55], and incorporated  
505 into custom Python code found at <https://bitbucket.org/murrieta/snake/branch/master/scripts/>.  
506 DnaSP software [61] was used to determine the number of nonsynonymous (7822.83) and  
507 synonymous (2446.17) sites from the ancestral input ZIKV consensus sequence. When no  
508 synonymous SNVs sites were present in replicates,  $d_N/d_S$  was set to 1, and no nonsynonymous  
509 SNV's  $d_N/d_S$  was set to 0.

## 510 **Statistical analysis**

511 All analyses were performed using GraphPad Prism (version 7.04) and R. Fisher's exact test  
512 were used to determine significant difference in virus titers and viral loads. All other tests were  
513 done using Kruskal-Wallis with Dunn's correction unless otherwise noted.

514 To evaluate the relationship between external factors and the infection dynamics of ZIKV, we  
515 examined the data with generalized linear models (Supplemental material). The predictors we

516 used include days post infection (days), temperature (scaled), species, and tissue type. We  
517 evaluated the impact of these variables on consensus changes, vector competence, complexity,  
518 nucleotide diversity and richness. We assumed that consensus changes and richness follow a  
519 quasi-Poisson distribution, complexity and nucleotide diversity follow a linear distribution, and  
520 assumed that dissemination efficiency and vector competence follow a binomial distribution. Our  
521 original models follow the base structure

$$522 \quad (2) \quad \text{response} \sim \exp [\beta_1(\text{days}) * \beta_2(\text{temp})^2 * \beta_3(\text{species}) * \beta_4(\text{tissue})]$$

523 Each model was reduced to a best fit structure using AIC values and/or a chi-square goodness  
524 of fit test. The polynomial on temperature allows us to differentiate between the linear and  
525 quadratic effect of temperature. Vector competence was evaluated with the following base  
526 structure for each tissue response

$$527 \quad (3) \quad \text{response} \sim \exp [\beta_1(\text{days}) * \beta_2(\text{temp})^2 * \beta_3(\text{species})]$$

## 528 **Data Availability**

529 Zika virus sequence data have been deposited in the NCBI Sequence Read Archive  
530 (PRJNA659260). All other data supporting the findings given are available within the article and  
531 supplementary information files, or from corresponding author upon request.

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