

1 **Title:** Oviposition of the mosquito *Aedes aegypti* in forest and domestic habitats in Africa

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14

15 **Abstract**

16 The theory of ecological divergence provides a useful framework to understand the adaptation of many
17 species to anthropogenic ('domestic') habitats. The mosquito *Aedes aegypti*, a global vector of several
18 arboviral diseases, presents an excellent study system. *Ae. aegypti* originated in African forests, but the
19 populations that invaded other continents have specialized in domestic habitats. In its African native
20 range, the species can be found in both forest and domestic habitats like villages. A crucial behavioral
21 change between mosquitoes living in different habitats is their oviposition choices. Forest *Ae. aegypti* lay
22 eggs in natural water containers like tree holes, while their domestic counterparts heavily rely on artificial
23 containers such as plastic buckets. These habitat-specific containers likely have different environmental
24 conditions, which could drive the incipient divergent evolution of oviposition in African *Ae. aegypti*. To
25 examine this hypothesis, we conducted field research in two African locations, La Lopé, Gabon and
26 Rabai, Kenya, where *Ae. aegypti* live in both forests and nearby villages. We first characterized a series of
27 environmental conditions of natural oviposition sites, including physical characteristics, microbial
28 density, bacterial composition, and volatile profiles. Our data showed that in both locations,
29 environmental conditions of oviposition sites did differ between habitats. To examine potential behavioral
30 divergence, we then conducted field and laboratory oviposition choice experiments to compare the
31 oviposition preference of forest and village mosquitoes. The field experiment suggested that forest
32 mosquitoes readily accepted artificial containers. In laboratory oviposition assays, forest and village
33 mosquito colonies did not show a differential preference towards several conditions that featured forest
34 versus village oviposition sites. Collectively, there is little evidence from our study that environmental
35 differences lead to strong and easily measurable divergence in oviposition behavior between *Ae. aegypti*
36 that occupy nearby forest and domestic habitats within Africa, despite clear divergence between African
37 and non-African *Ae. aegypti*.

38

39 **Keywords:** *Aedes aegypti*, oviposition, forest and domestic habitat, Africa, environmental conditions,

40 choice assays

41

42 **Introduction**

43 Ecological divergence is one of the central mechanisms contributing to biodiversity (Nosil, 2012).
44 When descendants of the same ancestral population evolve in different environments, they may
45 experience divergent selection pressures leading to morphological and/or behavioral divergence (Schluter,
46 2000). Accumulation of these phenotypic changes and their underlying genetic components, along with
47 genetic drift, could further result in reproductive isolation and speciation (Nosil, 2012; Rundle & Nosil,
48 2005; Shafer & Wolf, 2013). A core step in this process is the ecologically-based divergent selection
49 (Rundle & Nosil, 2005), which can be recognized by two essential features: consistently distinct
50 environmental conditions and organisms' corresponding phenotypic adaptations. The attribution of
51 phenotypic change to ecological selection has been demonstrated in several natural populations, such as
52 Darwin's finches (Grant & Grant, 2002, 2011), stickleback fish (Hatfield & Schluter, 1999), beach mice
53 (Mullen, Vignieri, Gore, & Hoekstra, 2009), and *Timema cristinae* walking-sticks (Nosil, 2007; Nosil &
54 Crespi, 2004).

55 In addition to explaining biodiversity, the model of ecological divergence also provides a useful
56 framework for understanding the evolution of a particular group of organisms – disease vectors living
57 with humans, such as mosquitoes. Many of these vector species experienced a transition from their
58 natural habitats into anthropogenic domestic habitats (e.g., villages and urban areas) following the
59 development of human civilization (Hulme-Beaman, Dobney, Cucchi, & Searle, 2016; Otto, 2018). The
60 striking contrast between these two types of habitats suggests a potentially strong divergent selection
61 (Johnson & Munshi-South, 2017). Alternatively, some vectors species may be predisposed to using
62 domestic habitats, in which case one would expect little phenotypic changes. Few studies have examined
63 these hypotheses or demonstrated how these disease vector species react and adapt to the 'novel'
64 environmental conditions of the domestic habitats. Addressing this question will contribute to our
65 understanding of the unique evolutionary history of these epidemiologically important animals, and
66 provide valuable information on why they are so good at living around humans and transmitting diseases.

67 The mosquito *Aedes aegypti* provides an excellent model for studying ecological divergence in
68 disease vectors. The species is the main vector of yellow fever, dengue, chikungunya (World Health
69 Organization, 2014), and Zika virus (Li, Wong, Ng, & Tan, 2012; Marcondes & Ximenes, 2016). Genetic
70 data suggested that *Ae. aegypti* is native to Africa (Brown et al., 2011; Gloria-Soria et al., 2016; Powell,
71 Gloria-Soria, & Kotsakiozi, 2018). With the establishment of human settlements, they invaded human-
72 generated domestic habitats inside Africa, probably five to ten thousand years ago (Crawford et al., 2017;
73 Kotsakiozi, Evans, et al., 2018), and later spread to the rest of the world since the 15th century (Brown et
74 al., 2014; Powell et al., 2018; Powell & Tabachnick, 2013). The mosquitoes in and out of Africa showed
75 a relatively clear genetic distinction (but see exceptions in Kotsakiozi et al. 2018 and Rose et al. 2020),
76 which roughly matches the two classical subspecies: *Ae. aegypti formosus* (Aaf) and *Ae. aegypti aegypti*
77 (Aaa), respectively. Complexities exist in this subspecies definition (Powell & Tabachnick, 2013), and in
78 this paper, we refer to them simply based on their geographic range (in or out of Africa). Non-African
79 Aaa breed in human environments, e.g., live specifically in urban areas with only a few exceptions in the
80 Caribbean and Argentina (Chadee, Ward, & Novak, 1998; Mangudo, Aparicio, & Gleiser, 2015). They
81 also display a strong preference for human hosts (McBride et al., 2014; Rose et al., 2020) and use
82 artificial containers as breedings sites (Day, 2016).

83 However, relatively little is known about the initial process of colonizing domestic environments
84 inside Africa, except for a few recent studies. For example, Rose et al. (2020) found that the mosquito's
85 preference towards humans is closely associated with seasonality and human density. *Ae. aegypti*
86 throughout Africa (Aaf) can be found in both forests, the presumed ancestral habitats, and domestic
87 settings like villages. Previous studies showed that domestic Aaf in several locations inside Africa is
88 genetically similar to their local forest counterparts, suggesting a relatively recent invasion into domestic
89 habitats (Kotsakiozi, Evans, et al., 2018; Paupy et al., 2014; Sylla, Bosio, Urdaneta-Marquez, Ndiaye, &
90 Black IV, 2009). Comparing Aaf between different habitats could allow us to understand the potential

91 incipient divergence. For example, what were the original selective pressures that may have ultimately led
92 to the clear divergence between Aaf and Aaa?

93 From a behavioral perspective, one of the critical steps during the process of colonizing domestic
94 habitats and invading other tropical regions around the world is adapting to lay eggs (i.e., oviposit) in
95 domestic breeding sites. After taking a full blood meal, which is necessary for reproduction, *Ae. aegypti*
96 females lay eggs on substrates at the edge of small containers of water, i.e., oviposition sites
97 (Christophers, 1960). Aaf in African forest and domestic habitats utilize different oviposition sites: the
98 former lay eggs mainly in natural containers like water-filled tree holes and rock pools (Lounibos, 1981),
99 while the latter uses mostly artificial containers, such as plastic buckets, tires, and discarded tin cans
100 (McBride et al., 2014; Petersen, 1977; Trpis & Hausermann, 1978). This difference in oviposition site use
101 is at least partly a function of container availability in the two habitats. However, natural and artificial
102 containers likely have different characteristics (Yee, Allgood, Kneitel, & Kuehn, 2012), such as bacterial
103 profiles (Dickson et al., 2017), that could also drive genetically based divergence in container preference.
104 Such divergence likely exists between Aaa and Aaf, as shown in studies comparing Aaf and a once
105 existed Aaa introduced to coastal Kenya from non-African populations (Leahy, VandeHey, & Booth,
106 1978; Petersen, 1977). Whether a similar divergence also exists within Aaf remains mostly unclear.

107 Conversely, ovipositional modifications could have a significant effect on the evolution of the
108 mosquitoes. If forest and domestic Aaf actively prefer natural and artificial containers, respectively, it
109 could facilitate the isolation between them: selective oviposition could keep forest populations in the
110 forest and domestic populations close to humans, which reduces gene flow between them and promotes
111 other adaptations (Servedio, Van Doorn, Kopp, Frame, & Nosil, 2011). Therefore, the evolution of
112 oviposition behaviors could be a key factor in understanding how *Ae. aegypti* became domesticated
113 (Powell et al., 2018; Rose et al., 2020). This process is of particular interest in the initial colonization of
114 domestic habitat within Africa. How different are the oviposition sites in the forest versus domestic

115 habitats? Was Aaf an ovipositional generalist, pre-adapted to jump into human environments? Or are
116 there genetically-based differences between populations breeding in wild and human environments?

117 *Ae. aegypti* choose oviposition sites based on the interactions between their innate preference and
118 external oviposition cues. Various abiotic and biotic factors have been shown to influence oviposition
119 choices of *Ae. aegypti* (Day, 2016), including container size (Bond & Fay, 1969; Burkot et al., 2007;
120 Harrington, Ponlawat, Edman, Scott, & Vermeylen, 2008), shading (Barrera, Amador, & Clark, 2006;
121 Prado, Maciel, Leite, & Souza, 2017), water salinity (Matthews, Younger, & Vosshall, 2019), color and
122 texture of the sites (Bentley & Day, 1989; Fay & Perry, 1965), presence of conspecific eggs, larvae, and
123 pupae (Zahiri, Rau, & Lewis, 1997, 1997), predators (Albeny-Simoes et al., 2014; Pamplona Lde,
124 Alencar, Lima, & Heukelbach, 2009), bacterial density and community composition (Arbaoui & Chua,
125 2014; Hazard, Mayer, & Savage, 1967; Ponnusamy, Schal, Wesson, Arellano, & Apperson, 2015), and
126 chemical components (Afify & Galizia, 2015; Melo et al., 2019). However, most of the existing studies
127 were conducted in laboratory settings with artificial oviposition choices. Although these studies provided
128 rich knowledge on the sensory mechanisms of oviposition (Matthews et al., 2019; Ponnusamy et al.,
129 2015), the conditions examined in these studies may not necessarily reflect the characteristics of breeding
130 sites in the field. These studies are also heavily biased to Aaa, while detailed examination of oviposition
131 preference in Aaf is mostly missing, let alone comparisons between forest and domestic Aaf. As a result,
132 it is still unclear how oviposition behaviors evolved during the domestication of *Ae. aegypti*.

133 As a first step to address this question, we examined oviposition of *Ae. aegypti* living in forest
134 and domestic habitats in two locations in Africa, La Lopé in Gabon and Rabai in Kenya. Mosquitoes in
135 both locations are Aaf, but can be found in forest and villages in close proximity. They also showed little
136 genetic differentiation between habitats (Xia et al., submitted), which suggested gene flow between forest
137 and domestic populations. We first characterized the environmental conditions of natural oviposition
138 sites, including physical characteristics, competition and predation, bacterial profiles, and chemical
139 volatiles, in natural sites (tree holes) and artificial containers. To examine whether environmental

140 differences may translate into behavioral differences, we then investigated the oviposition preference of
141 forest and domestic Aaf through field oviposition experiments and laboratory oviposition assays. The
142 results could also provide useful information on identifying the critical environmental variables that
143 potentially drove the divergent evolution of oviposition, if such behavioral divergence exists.

144 We hypothesized that natural and artificial containers represent very different environmental
145 characteristics, and that both forest and domestic Aaf will prefer conditions that are more alike the
146 oviposition sites from their own habitats, as would be predicted under a model of ecological divergence
147 and local adaptation. By examining the two main elements of ecological divergence, environmental
148 variation and behavioral differences, this study provides valuable information on how oviposition
149 behaviors in *Ae. aegypti* evolved during the domestication history of the mosquito.

150

151 **Materials and methods**

152 *Field study*

153 We conducted field studies in La Lopé, Gabon in Central Africa from November to December
154 2016, and in Rabai, Kenya in East Africa from April to May 2017. La Lopé has an extensive continuous
155 tropical rainforest surrounding La Lopé village (Figure 1a). The forest in Rabai, on the other hand, is
156 more fragmented, with several villages scattered around the forest patch (Figure 1b). In each location, we
157 searched for water-holding containers as potential mosquito oviposition sites in both the forests and
158 nearby villages. A potential oviposition site was defined as one that holds at least one mosquito larva (not
159 necessarily *Ae. aegypti*) at the time of sampling, which suggested that the site had been present long
160 enough for a mosquito to lay eggs. We categorized oviposition sites into three habitat groups: forest,
161 peridomestic (outdoor containers in a village area), and domestic (indoor containers) (Table 1). We
162 separated indoor and outdoor containers as classical studies from the 1970s reported that, at least in
163 Rabai, Kenya, the mosquitoes living indoor and outdoor showed distinct behavioral and genetic

164 difference (Leahy et al., 1978; McBride et al., 2014; Petersen, 1977; Tabachnick, Munstermann, &
165 Powell, 1979; Trpis & Hausermann, 1975). Genetic analysis showed that these indoor mosquitoes in
166 Rabai were likely descendent of non-African Aaa, which is a unique case in the evolutionary history of
167 *Ae. aegypti* (Brown et al., 2011; Gloria-Soria et al., 2016). However, this previously described Aaa-like
168 indoor form was no longer found at the time of sampling, which is also supported by genetic data (Xia et
169 al., submitted).

170 In La Lopé, we visited 60 oviposition sites in seven forest locations, and 38 sites in six village
171 locations. The sampling sites separate by 5-17 km. Forest oviposition sites were predominantly rock pools
172 around streams and tree holes that accumulated water. In the village, mosquito larvae were found in a
173 variety of artificial containers, including construction bricks, tires, metal cans, and plastic containers.
174 Because residents in the village rarely store water indoor, all village oviposition sites were 'peridomestic.'
175 In Rabai, Kenya, we sampled 31 oviposition sites consisting of mainly plastic buckets, earthenware pots,
176 and metal barrels in four villages. They were mostly indoor (i.e., domestic) containers. The 37 oviposition
177 sites in Rabai forest were all tree holes holding rainwater (Figure 1b). We recorded the GPS coordinates
178 of each sampling location (which may consist of more than one oviposition sites) in La Lopé, and of each
179 oviposition site in Rabai, Kenya (Figure 1).

180 Upon identifying a potential oviposition site in any habitat, we measured 11-16 physical
181 variables. We also collected water samples for further analysis of bacterial and chemical volatile profiles.
182 Method details are described in the following sections and the Appendix. In addition, we collected all
183 mosquito larvae using pipets and reared them to adults in field stations, keeping larvae and pupae from
184 different oviposition sites separate. Upon eclosion, the adults were identified to species or genus based on
185 taxonomic keys using a dissection microscope in the field. We kept *Ae. aegypti* adults alive to establish
186 lab colonies for future behavioral tests. We categorized each site as '*Ae. aegypti* present' or '*Ae. aegypti*
187 absent' based on whether it held any *Ae. aegypti* larvae or pupae (Table 1). It is worth noting that the
188 absence of *Ae. aegypti* may not necessarily suggest an avoidance. Some sites may be suitable for

189 oviposition but not yet colonized by *Ae. aegypti* at the time of collection. The combinations of habitats
190 and *Ae. aegypti* presence will be referred to as ‘oviposition site groups’ in the rest of the paper for the
191 purpose of communication. We summarized the sample sizes for analyses of different environmental
192 variables in Table 1. Almost all peridomestic and domestic habitats in Rabai were present with *Ae.*
193 *aegypti*. This mainly results from the fact that there were rarely other species present in these
194 environments, but we required at least one mosquito larvae to include the site in the dataset, so there are
195 effectively no ‘*Ae aegypti* absent’ sites. Because the peridomestic *Ae. aegypti* absent group contained only
196 one sample, it is excluded from group-level analyses, but retained in comparisons between habitats or
197 between *Ae. aegypti* present vs. absent sites.

198 The fieldwork in La Lopé was approved by the CENAREST with the authorization
199 AR0013/16/MESRS/CENAREST/CG/CST/CSAR, and by the La Lopé National Parks with the
200 authorization AE16008/PR/ANPN/SE/CS/AEPN. The fieldwork in Rabai was approved by the Kenya
201 Medical Research Institute Scientific and Ethical Review Unit with the authorization
202 KEMRI/SERU/3433.

203 *Characterizing oviposition sites: physical variables*

204 We measured 11 physical variables for each oviposition site in La Lopé, Gabon, and five
205 additional variables in Rabai, Kenya (Table S1 in Appendix). The variables were selected partially based
206 on previous laboratory studies of mosquito oviposition (Harrington et al., 2008; Madeira, Macharelli, &
207 Carvalho, 2002; Petersen, 1977; Reiskind & Zarrabi, 2012; Wong, Stoddard, Astete, Morrison, & Scott,
208 2011), as well as the availability of equipment and resources in the field. These variables include the size
209 of the oviposition sites (e.g., diameters, circumference, surface area, volume, container depth, water
210 depth, etc.), ambient environments (temperature, relative humidity, and canopy coverage), and water
211 characteristics (pH, conductivity, salinity, water temperature, and total dissolved solids). Methodological
212 details can be found in Table S1 in the Appendix.

213 After removing eight oviposition sites with excessive missing data, we first compared each
214 variable individually across oviposition site groups. Because our data do not follow a normal distribution,
215 we used the Kruskal–Wallis test and post hoc pairwise Wilcoxon rank sum test in R v3.5.0 (R
216 development core team, 2018) with *Holm* correction for multiple comparisons. We then tested the
217 difference between habitats or between *Ae. aegypti* present and absent sites separately, regardless of the
218 other grouping factors. We also performed a principal component analysis (PCA) to summarize all
219 physical variables. The multivariate differences between oviposition site groups, habitats, and *Ae. aegypti*
220 presence status were tested by multiple response permutation procedure (MRPP) with 999 permutations.
221 The p values for multiple comparisons were adjusted using the *Holm* method. Lastly, we attempted to
222 identify the variables that are most differentiated in each comparison by ranking variable importance
223 using a random forest algorithm in R package *randomForest v4.6-14* (Liaw & Wiener, 2002). Random
224 forest is a decision-tree based classification algorithm that works well with small sample size and
225 correlated variables (Qi, 2012).

226 *Characterizing oviposition sites: competition and predation*

227 Competition and predation could influence larval development and female oviposition choice
228 (Pamplona Lde et al., 2009; Soman & Reuben, 1970; Vonesh & Blaustein, 2010; Zahiri & Rau, 1998). To
229 consider their effects, we counted the number of individual mosquitoes (*Ae. aegypti* as well as other
230 mosquito species) present in each oviposition site. We also noted the presence of predatory larvae,
231 predominately *Toxorhynchites* mosquitoes, and removed them immediately if found. We first compared
232 the number and density of *Ae. aegypti* between habitats, using only the oviposition sites where *Ae. aegypti*
233 was present. We also carried out an additional analysis that used mosquitoes of all species (including *Ae.*
234 *aegypti*) to include possible interspecific competition effects, and included oviposition sites without *Ae.*
235 *aegypti*. In La Lopé, records of other mosquito species were only available for the forest, so we only
236 compared forest sites present versus absent of *Ae. aegypti*. We used negative-binomial models to compare
237 mosquito numbers with habitat as the predictors, and used Kruskal–Wallis tests and *post hoc* pairwise

238 Wilcoxon rank sum tests to compare mosquito density. In addition, we analyzed the frequency of finding
239 predators in different oviposition site groups or habitats with chi-squared tests.

240 *Characterizing oviposition sites: microbial density*

241 We examined the microbial profile in a subset of oviposition sites, inspired by previous studies
242 showing that the microbiome, particularly bacteria, affect *Ae. aegypti* oviposition (Arbaoui & Chua, 2014;
243 Ponnusamy et al., 2015). We collected 15 mL (in La Lopé) or 50 mL (in Rabai) water samples from each
244 field oviposition site using sterile pipets and conical tubes (Thermo Scientific, USA). This procedure was
245 performed before measuring physical characteristics to avoid contamination. We kept the water samples
246 in a cooler with ice packs in the field until returning to the field station. To measure microbial density, we
247 added an aliquot of each water sample to formaldehyde solution (Millipore Sigma, USA) with a final
248 concentration of 1% - 3% formaldehyde and kept it in 4 °C. After returning to Yale University, we
249 stained the formaldehyde preserves with DAPI (4',6-diamidino-2-phenylindole, final concentration 5
250 ug/mL, Thermo Scientific, USA), and counted the microbial cells using hemocytometers (DHC-N01,
251 INCYTO, Korea) under a widefield fluorescence microscope (Leica DMi8, Leica, German) Densities
252 were log-transformed before statistical analysis. We then compared the microbial density among
253 oviposition site groups in La Lopé with the Kruskal–Wallis test and post hoc pairwise Wilcoxon rank sum
254 tests. The distribution of data in Rabai samples did not violate parametric test assumptions, so we
255 performed the comparisons using analysis of variance (ANOVA) and *post hoc* Tukey tests.

256 *Characterizing oviposition sites: bacterial community composition*

257 In addition to the overall density, we performed 16s-rRNA gene amplicon sequencing to explore
258 the bacterial community composition in most oviposition sites (Table 1), inspired by previous studies
259 suggested different bacteria between habitats (Dickson et al., 2017). The details of sample processing and
260 sequencing library preparation are described in the Appendix. In short, we collected cells from the water
261 samples by centrifuge or filtering, extracted DNA, and amplified the 16s-rRNA gene V4 region using

262 primers reported in Kozich et al. (2013) . The primers label each sample with a unique combination of
263 index sequences. The PCR products were cleaned and mixed with equal quantity and sequenced on
264 Illumina MiSeq (Illumina, USA) at the Yale Center for Genome Analysis. We also included commercial
265 mock communities of bacteria in our library. The composition of these mock communities are known,
266 which allows validation of the sequencing accuracy. Amplicon sequencing for La Lopé and Rabai were
267 conducted separately.

268 We demultiplexed the sequencing reads using USEARCH v10.0.240 (Edgar, 2010) and followed
269 the pipeline of DADA2 (v1.8.0) (Callahan et al., 2016) to determine the bacterial community
270 composition. DADA2 estimates sequencing errors and infers the exact sequence variants (i.e., amplicon
271 sequence variants, or ASVs), which are analog to the conventional operational taxonomic unit (OTU). We
272 summarized the frequency of each ASV in every water sample, and blasted the ASVs to the Ribosomal
273 Database Project (RDP) 16s-rRNA gene reference database (RDP trainset 16 and RDP species
274 assignment 16) (Cole et al., 2014) for taxonomic assignment. We then agglomerated ASVs into higher
275 taxonomic levels for further analysis.

276 Using the DADA2 outputs and the R package *phyloseq* (McMurdie & Holmes, 2013), we first
277 calculated the alpha diversity of the bacteria community in each oviposition site indicated by the Shannon
278 index (Shannon, 1948), using the raw read counts of all samples. We then compared the index across
279 oviposition site groups, habitats, and between *Ae. aegypti* present and *Ae. aegypti* absent sites. The
280 community compositions were summarized by non-metric multidimensional scaling (NMDS) with the
281 Bray-Curtis distance matrix. Similar to PCA, NMDS analysis summarizes multivariate data (each
282 bacterial taxa as one variable), but is more appropriate for bacterial composition data (Ramette, 2007).
283 Before NMDS analysis, we first removed samples with fewer than 5000 reads to avoid low-quality
284 samples, and we thinned each sample proportionally to the lowest read depth of all samples to remove the
285 impact of uneven sequencing depth between samples. Bacterial communities may show different
286 assembly patterns at different taxonomic levels (Goldford et al., 2018). Therefore, we calculated the

287 Shannon index and performed NMDS at four taxonomic levels: ASV, Species, Genus, and Family. To
288 provide more information on the detailed compositions of the bacterial communities, we also
289 demonstrated the major bacterial groups at the Family level. Lastly, we used R package *DESeq2* to
290 identify families that are most differentiated between habitats (Love, Huber, & Anders, 2014).

291 To estimate the temporal stability of the bacterial communities, for five oviposition sites in each
292 habitat, we collected water samples more than once. The average number of days between two
293 consecutive collections ranges from 3 to 21, with an average of 8.4 days in La Lopé and 17 days in Rabai.
294 All temporal samples were sequenced, but only the first-day samples were included in analyses described
295 above. We performed a separate NMDS analysis to examine variation between temporal samples.

296 *Characterizing oviposition sites: chemical volatiles in Rabai, Kenya*

297 Chemical volatiles released from an oviposition site could act as olfactory cues for mosquitoes
298 during oviposition site selection (Afify & Galizia, 2015), yet the volatile profiles of natural oviposition
299 sites have rarely been examined. We attempted to describe the volatile profile in oviposition sites in
300 Rabai, Kenya (we did not collect chemical data in La Lopé due to financial constraints). In brief, we
301 collected water samples from a subset of oviposition sites (Table 1) and extracted the volatiles into an
302 absorbent with a steady airflow. The captured volatiles were examined by Gas Chromatography–Mass
303 Spectrometry (GC-MS) at Yale West Campus Analytical Core. We then identified and quantified each
304 compound using the GC-MS results. The technical details of volatile extraction and GC-MS were
305 described in the Appendix. Due to the sparsity of many compounds in the final dataset, we did not
306 perform statistical analysis across oviposition site types or habitats, but instead summarized the
307 compound concentrations using a heatmap.

308 *Field oviposition choice experiments*

309 We conducted field oviposition experiments in both La Lopé and Rabai. We placed artificial and
310 natural containers at forest sites and village sites and left them for use by wild mosquitoes. Bamboo

311 segments were used for the natural containers since they are similar to tree holes in size and shape and
312 have been commonly used by African researchers to collect forest mosquitoes (Kemp & Jupp, 1991). The
313 artificial containers used in La Lopé included tires, plastic bottles, plastic bags, bricks, and metal cans
314 (see the insets in Figure 7 for a representation of these experimental containers). These containers are
315 frequently found in the villages. We paired five bamboo with the five artificial containers to form a group
316 of ten containers. We then placed these groups in four forest locations and four peridomestic locations.
317 All containers were set up empty and filled by rainwater naturally. We retrieved all containers after
318 roughly two weeks, collected larvae and pupae from them, and reared all mosquitoes to adults to count
319 the number of *Ae. aegypti*. Because of the low yield in these experimental containers, within each
320 habitats, we combined mosquitoes from all bamboos or all artificial containers, respectively. This resulted
321 in a single count of *Ae. aegypti* from each types of container in habitat. We used a chi-squared test to
322 examine whether habitat influences the distribution of *Ae. aegypti* in the bamboo versus artificial
323 containers.

324 In Rabai, we followed similar procedures but used plastic buckets and earthenware pots as the
325 artificial containers. Each container group thus consisted of two artificial containers and two bamboo
326 frgments. Another difference is that instead of placing the container group in peridomestic as in La Lopé,
327 we left them in domestic habitats (indoor), after receiving verbal permission from homeowners. We set up
328 ten container groups in the Kaya Bomu forest and ten in Bengo village (Figure 1B). Tap water was added
329 to the containers on the first day, as rains were not frequent enough and could not reach indoor containers.
330 The experiment lasted for 7-10 days. In the end, containers were flooded to hatch all eggs, and we reared
331 larvae and pupae in the field. We then counted the number of *Ae. aegypti* present in the bamboo or either
332 type of artificial container. Instead of combining mosquito counts as described above for La Lopé
333 experiments, we kept data from the ten containers groups (i.e. ten replicates) within each habitat separate.
334 Each of the ten container groups thus represents a replicate in the choice experiment. After removing
335 groups that produced no *Ae. aegypti*, we applied a beta-binomial model to address the effect of habitat on

336 the distribution of eggs between bamboo and artificial containers. The beta-binomial model was
337 implemented in the R package *glmmTMB* (Brooks et al., 2017).

338 Although we chose containers as similar as possible to natural oviposition sites in both habitats, it
339 is critical to examine how the environmental conditions of these experimental containers reflect the
340 natural conditions. Therefore, we collected water samples from them at the end of the experiments and
341 applied the 16s-rRNA gene amplicon sequencing and downstream NMDS analysis to examine the
342 bacterial community in these experimental containers.

343 *Laboratory oviposition assays*

344 In an attempt to disentangle the effects of different environmental variables on oviposition choice,
345 we performed laboratory oviposition assays in a common-garden setup. The goal was to examine whether
346 forest and village *Ae. aegypti* have different oviposition preferences towards a subset of environmental
347 variables that differed between forest and village oviposition sites.

348 We established a forest colony and a peridomestic colony from La Lopé using *Ae. aegypti*
349 collected from natural breeding sites, supplemented with oviposition traps and human landing capture
350 (approved by the National Research Ethics Committee of Gabon under the protocol 0031/2014/SG/CNE).
351 In Rabai, we created six independent village colonies from the four villages (four domestic colonies and
352 two peridomestic colonies) and four forest colonies from the the Kaya Bomu forest. We blood-fed the
353 mosquitoes in the field and brought the eggs back (i.e., the second generation) to our lab at Yale
354 University and the McBride lab at Princeton University. The detailed information of the mosquito
355 colonies and the protocol for rearing these colonies are in the Appendix. The two peridomestic colonies
356 correspond to K63 and K65 in Rose et al. (2020) and the Rabai forest colonies correspond to K66 and
357 K67. All laboratory oviposition assays were performed in the insectary at Yale University. We used the
358 fourth to the sixth generation of mosquitoes in these assays. For simplicity, we will refer to the
359 peridomestic colony in La Lopé and the domestic colonies in Rabai as village colonies.

360 In the first series of assays, we used two-choice tests. In each cage, five gravid females were
361 allowed to choose from two oviposition cups with different conditions (see Appendix). Using this assay,
362 we compared the oviposition preference of forest versus village colonies from La Lopé and Rabai towards
363 several environmental variables.

364 We first tested a pair of Rabai forest and domestic colony (K66 vs. Kwa Bendegwa village) for
365 their preference for water samples directly collected from forest and domestic oviposition sites in Rabai.
366 The rest of the oviposition assays focused on specific environmental variables. We determined the target
367 variables from the list of variables that showed a significant difference between forest and village
368 oviposition sites in the field. The decision was also constrained by space, resources, and our experimental
369 setup. For example, we were unable to test variables related to container size and height as well as
370 ambient temperature and humidity, as they require much larger space than the capacity of our insectary.
371 As a result, we tested pH, shading, larval density, and a combined effect of pH, conductivity, and shading
372 in the K66 versus Kwa Bendegwa colony pair. Additionally, we conducted oviposition assays examining
373 bacterial community composition in all La Lopé and Rabai colonies (Table S2 in the Appendix).

374 Conditions we examined in the assays replicated the median conditions of forest and village
375 oviposition in nature (details of each assays are described in the Appendix). Specifically, in the
376 experiments testing the preference for bacterial compositions, we create forest and village type of
377 bacterial community by inoculating water samples collected from natural forest and village oviposition
378 sites in nutritionally rich Lysogeny broth (LB). After growing the two bacterial cultures overnight, they
379 were diluted to the same cell density and used as the two choices in the behavioral assays. Although the
380 bacterial communities in these LB cultures likely vary from the actual bacterial communities in natural
381 oviposition sites, they should still contain some representative bacterial taxa from each habitat.

382 We counted the number of eggs in oviposition cups at the end of each assay. Cages with fewer
383 than ten eggs in total were removed from further analysis. We first calculated the oviposition activity
384 index (OAI) (Kramer & Mulla, 1979) for each cage:

385
$$OAI = \frac{N_1 - N_2}{N_1 + N_2}$$

386 where N_1 and N_2 are the number of eggs deposited in the two cups, respectively. OAI ranges from -1 to 1,
387 which represents a complete preference for the second choice to a complete preference for the first
388 choice. We performed beta-binomial models in the R package *glmmTMB* (Brooks et al., 2017) to examine
389 whether colonies differ in their oviposition preference, using the two egg counts in each cage as the
390 dependent variable (Rose et al., 2020). We added the batch/trial IDs as random effects if the experiments
391 testing a condition spanned more than one trial. The statistical significance of colony or habitat effects
392 were determined by comparing the full model with a null model that excludes colony or habitat
393 information (Table S9). We then extracted mean OAI with a 95% confidence interval from the model
394 using the R package *emmeans* (Lenth, Singmann, & Love, 2018; Rose et al., 2020).

395 In addition to the above two-choice assays, in the last series of laboratory assays, we tested the
396 oviposition preference of all mosquito colonies to five bacterial densities. This is inspired by the large
397 variation in bacterial density among field oviposition sites (more than two orders of magnitude) and that
398 previous laboratory experiments with *Ae. aegypti* found density-dependent ovipositional responses to
399 bacteria (Ponnusamy et al., 2015; Ponnusamy, Wesson, Arellano, Schal, & Apperson, 2010). We used a
400 similar experimental design as the two-choice assays described above but provided each cage of gravid
401 female mosquitoes five cups instead of two. The cups contained bacterial cultures at densities ranging
402 from zero to nearly the maximal bacterial density in field oviposition sites. The bacterial culture was
403 generated from an even mixture of forest and domestic water samples (Table S2 in the Appendix). We
404 counted the numbers of eggs laid in the five cups and fitted a negative-binomial model using the R
405 package *lme4* (Bates, Mächler, Bolker, & Walker, 2014) to detect significant differences between
406 colonies and between the habitat type of the colonies. A full model with an interactive term of
407 colony/habitat with bacterial densities was compared to a null model excluding this interactive term
408 (Table S9). Because the number of eggs in the five cups from the same cage were not independent from

409 each other, we added the ID of cages as a random effect to control for this data structure. Lastly, we used
410 the *emmeans* package to estimate the expected number of eggs in each bacterial density with 95%
411 confidence intervals.

412

413 Results

414 *Characterizing oviposition sites: physical characteristics*

415 PCA analysis summarizing the 11 physical variables in La Lopé showed that the four oviposition
416 site groups (two habitats \times *Ae. aegypti* present/absent) overlap extensively in the space described by the
417 first two principal components, which together account for 38% of the total variance (Figure 2a).

418 However, forest and peridomestic village sites appeared to differ slightly. In support of that, MRPP tests
419 found a significant multivariate difference among the four groups ($p = 0.019$) and between habitats when
420 including both *Ae. aegypti* present and absent sites ($p = 0.001$). Sites with *Ae. aegypti* did not differ
421 significantly between habitats ($p = 0.316$), possibly due to the small sample size (only five samples in the
422 forest *Ae. aegypti* present category). Sites with and without *Ae. aegypti* did not differ significantly (all
423 sites regardless of habitats: $p = 0.311$, only forest sites: $p = 1$, only peridomestic sites: $p = 1$).

424 Examining the rotations of the original physical variables onto the first two principal component
425 axes suggested two main groups of variables. The first one includes ambient temperature and humidity,
426 shading (i.e., canopy coverage), container opening height, and water pH, which axis roughly corresponds
427 to differentiation between forest and peridomestic oviposition sites. For these variables, we found a
428 significant difference between habitats and between the four oviposition site groups (Figure S2, Table S3
429 and S4). They also ranks highly in their variable importance measures (Figure S4a). These observations
430 support the idea that variables in this group differentiate habitats. The second group mainly represents
431 container size and water volume, with the latter differing significantly between forest and peridomestic
432 oviposition sites (Table S3). *Ae. aegypti* present and absent sites have similar conditions for all variables

433 except the height of the container opening (Figure S2, Table S3 and S4). Container height is the main
434 factor differentiating *Ae. aegypti* present vs. absent sites, especially within the peridomestic sites (Figure
435 S3a).

436 In Rabai, similarly as in La Lopé, forest and peridomestic sites was modestly but significantly
437 different in their physical characteristics (Figure 2b, PCA summarizing 16 physical variables). In addition
438 to these two habitat types, we also measured indoor ‘domestic’ sites. The PCA results suggest a strong
439 differentiation between forest and domestic oviposition sites (Figure 2b). Sites with and without *Ae.*
440 *aegypti* in the forest did not show much difference. Consistent with PCA, MRPP found significant
441 multivariate differences in most comparisons, except between forest sites with *Ae. aegypti* present vs.
442 absent ($p = 0.157$) and between domestic vs. peridomestic sites ($p = 0.192$).

443 Forest and domestic oviposition sites were separated primarily along the first PC, which is
444 explained by container size (e.g., diameter, circumference, etc.), water volume, and water pH (Figure 2b).
445 Single variable comparisons confirmed that these variables are indeed different between forest and
446 domestic/peridomestic oviposition sites (Figure S4, Table S5 and S6). On the other hand, comparisons of
447 forest *Ae. aegypti* present vs. absent sites as well as between domestic and peridomestic sites found very
448 few significant differences (Table S5 and S6). Canopy coverage, a measure of shading, also showed a
449 strong difference across oviposition site groups and between habitats (Figure S4, Table S5 and S6). We
450 expected this difference as domestic oviposition sites are always under roof, and peridomestic containers
451 are mostly exposed, while forest tree holes are partially shaded by the canopy. Lastly, variables with
452 significant differences between oviposition site groups or between habitats also generally have high ranks
453 in the output of the Random Forests analysis of the corresponding comparisons (Figure S3b).

454 *Characterizing oviposition sites: competition and predation*

455 The density of *Ae. aegypti* was similar between forest and peridomestic oviposition sites in La
456 Lopé (Figure 3a, Table S4). Many oviposition sites in both habitats produced only one *Ae. aegypti* (Figure

457 S5a). Although peridomestic sites contained significantly more *Ae. aegypti* than forest sites (Figure S5a),
458 they also had larger volumes (Figure S2f). Mosquitoes other than *Ae. aegypti* were recorded only in the
459 La Lopé forest. Oviposition sites with and without *Ae. aegypti* in the forest did not show a significant
460 difference in the total number or density of all mosquitoes (Figure S5b and S5c, Table S4). Analysis of
461 predation found that the presence of predatory *Toxorhynchites* larvae did not differ among oviposition site
462 groups, between habitats, or between *Ae. aegypti* present and absent sites ($p > 0.05$ in all chi-squared
463 tests).

464 In Rabai, *Ae. aegypti* density was significantly lower in domestic oviposition sites (Figure 3b,
465 Table S6) in comparison with the other two habitats. The density difference between forest and domestic
466 containers was mainly driven by the difference in water volume (Figure S4e). In contrast, the difference
467 between peridomestic and domestic sites are due to the higher number of mosquitoes found in
468 peridomestic sites (Figure S6a). When including other mosquito species, comparisons of mosquito
469 numbers and densities between oviposition sites groups reached the same conclusion (Figure S6b and
470 S6c, Table S5 and S6).

471 *Characterizing oviposition sites: microbial density*

472 Microbial densities do not show significant differences between oviposition site groups, habitats,
473 or *Ae. aegypti* present and absent sites in La Lopé (Figure 4a, Table S3 and S4). In Rabai, we found
474 significantly lower microbial density in domestic oviposition sites than forest or peridomestic oviposition
475 sites (Figure 4b, Table S5 and S6). Microbial densities were similar in forest and peridomestic oviposition
476 sites. Lastly, *Ae. aegypti* present and absent sites have comparable levels of microbial density (Figure 4b,
477 Table S5 and S6).

478 *Characterizing oviposition sites: bacterial community composition*

479 The median depth of the amplicon sequencing was 17,420 reads per La Lopé samples and 56,478
480 reads per Rabai samples. Negative controls yielded 0 - 976 reads (median: 33) and 0 - 11 ASVs (median:

481 4) per sample, which suggested minimal contamination from the sampling and library preparation
482 procedures. We also reconstructed the mock communities relatively well using the sequencing results: we
483 found 18-23 ASVs from mock communities containing 20 bacterial taxa and nine ASVs from another two
484 mock communities that contain eight bacterial colonies (see the Appendix for more information on the
485 mock communities).

486 In La Lopé, alpha diversity of the bacterial communities varies considerably. The Shannon index
487 differs significantly across the four oviposition site groups at the Species and Genus level (Table S3). The
488 pairwise comparison did not find any significant difference between any pairs of oviposition site groups
489 at any taxonomic level (Table S4). Still, visually the peridomestic groups have higher Shannon indexes
490 (Figure S7). This observation was reflected in the comparisons between habitats regardless of *Ae. aegypti*
491 presence, as it suggested a significantly higher alpha diversity in peridomestic oviposition sites at the
492 species, genus, and family level (Table S3). *Ae. aegypti* present and absent sites have similar alpha
493 diversity at all taxonomic levels (Table S3). In Rabai samples, we did not find significant differences in
494 the Shannon index across oviposition site groups or between habitats (Figure S8, Table S5 and S6). *Ae.*
495 *aegypti* present sites have lower diversity than *Ae. aegypti* absent sites, but only when we included
496 oviposition sites from all habitats (Table S5).

497 NMDS analysis suggested that forest and village (including peridomestic and domestic)
498 oviposition sites had a very different bacterial community in both La Lopé and Rabai at the ASV level.
499 Peridomestic sites in Rabai clustered with domestic sites (Figure 5). The forest-village divergence was
500 less evident at higher taxonomic levels for the La Lopé oviposition sites, especially at the Family level
501 (Figure S9). Rabai samples, on the other hand, retained the substantial difference between forest and
502 village oviposition sites at all four taxonomic levels (Figure S10). In all NMDS analysis, oviposition sites
503 present and absent with *Ae. aegypti* within each habitat always overlap extensively, which suggested that
504 they likely have similar bacterial community composition (Figure 5, S9, and S10).

505 When examining the most abundant bacterial families across different oviposition site groups, we
506 observed considerable variation among samples (Figure S11). Most oviposition sites contained
507 representatives of multiple families with no clear dominance. Among the top ten families in La Lopé
508 samples, *Microbacteriaceae*, *Flavobacteriaceae*, and *Burkholderiaceae* showed higher abundance in
509 forest oviposition sites, while *Oxalobacteraceae* and *Sphingobacteriaceae* are more abundant in
510 peridomestic sites (Figure S11a, Table S7). In Rabai oviposition sites, *Moraxellaceae* has an apparent
511 dominance in domestic oviposition sites, but its abundance is not significantly different between habitats.
512 *DESeq2* found a significantly higher abundance of *Enterobacteriaceae*, *Xanthomonadaceae*,
513 *Pseudomonadaceae*, and *Planococcaceae* in forest oviposition sites than domestic and peridomestic sites
514 (Figure S11b, Table S8). A full list of bacterial families that showed differential abundance between
515 habitats are in Table S7 and S8 in the Appendix.

516 Lastly, NMDS analysis at the ASV level found that temporal samples collected from the same
517 oviposition site do vary in their bacterial community, but remain in the same cluster defined by habitats
518 (Figure S12). That is, temporal samples from forest cluster with the rest of forest oviposition sites instead
519 of sites from other habitats and vice versa. This result suggests that the strong divergence in bacterial
520 communities between habitat are likely temporally stable.

521 *Characterizing oviposition sites: chemical volatiles in Rabai, Kenya*

522 The volatile profiles of a subset of oviposition sites in Rabai were summarized in Figure 6. After
523 filtering, 31 oviposition sites remained in the final dataset. There was substantial variation in the chemical
524 composition of samples, both within habitats and across habitats. GC-MS analysis identified a total of 29
525 chemical compounds. The majority of them were shared across different habitats, but we found a few
526 chemicals that were unique to either forest or domestic habitat.

527 *Field oviposition choice experiments*

528 Experimental containers in the forest and the village produced in total 61 and 95 *Ae. aegypti*,
 529 respectively, in La Lopé. The majority of the *Ae. aegypti* were from bamboo in the forest and artificial
 530 containers in the village (Figure 7a). This habitat-associated bias in *Ae. aegypti* production between the
 531 two types of containers was statistically significant (chi-square test: $\chi^2 = 52.1$, df = 1, p < 0.001). In
 532 Rabai, we collected all but one *Ae. aegypti* from artificial containers in the village (Figure 7b). *Ae. aegypti*
 533 were also more abundant in artificial containers than in bamboo in the forest, which is the opposite of the
 534 finding in La Lopé. However, the beta-binomial model still found a significant effect of habitat (Figure
 535 7c, AIC of full model: 54.3, AIC of null model: 48.9, model comparison: $\chi^2 = 7.38$, df = 1, p = 0.007).

When examining the bacterial community composition of these experimental containers, NMDS analysis found that regardless of the container type and the habitats where they were located, all experimental containers clustered with natural village (peridomestic and domestic) oviposition sites (Figure S13).

540 *Laboratory oviposition assays*

The results of the laboratory oviposition assays are shown in Figure 8 and Figure S14. Based on the OAI confidence interval estimated by the beta-binomial models, we found three significant preferences among all experiments: Rabai Kwa Bendegwa village colony preferred forest water samples over village water samples, and forest mosquito larval density over village larval density; La Lopé forest colony preferred the bacterial culture started with peridomestic water samples over that started with forest water samples. However, there is significant within-colony variation in most experiments. When comparing between colonies or between the habitat types of the colonies, the beta-binomial models did not find any significant difference in any assays (Table S9). Lastly, we applied a negative-binomial model to analyze the results of oviposition assays testing bacterial densities (Figure 9). Neither colonies nor the habitats of the colonies have a significant effect on the mosquito's preference for the five bacterial densities (Table S9). La Lopé village colonies showed a weak preference for lower bacterial densities, but

552 the trend was not statistically significant (ANOVA for the effect of oviposition choices: $F = 1.56$, $df = 4$,
553 $p = 0.200$).

554 **Discussion**

555 In this study, we found that oviposition sites in different habitats tend to have different physical
556 properties and bacterial community composition, in both La Lopé and Gabon (Figure 2 and 5). Outdoor
557 peridomestic sites have moderately different physical characteristics from forest sites in both locations,
558 while the forest-domestic comparison unique to Rabai reveals a even stronger differentiation (Figure 2).
559 The bacterial composition in forest oviposition sites is consistently very distinct from the other two
560 village habitats (Figure 5). Unique to the Rabai system, we also found significantly lower larval and
561 bacterial densities in domestic oviposition sites (Figure 3b and 4b), as well as some differences in the
562 chemical profiles between forest and domestic oviposition sites (Figure 6). These results support our
563 hypothesis that *Ae. aegypti* living in their ancestral forest habitats and invaded anthropogenic habitats are
564 using oviposition sites with different average properties.

565 Within each habitat, oviposition sites with *Ae. aegypti* present or absent at the time of collection
566 share similar environmental conditions. We found some significant differences between *Ae. aegypti*
567 present and absent sites, but only when we combined sites across all habitats. These results can be
568 partially explained by the uneven distribution of *Ae. aegypti* present vs. absent sites across habitats. For
569 example, in Rabai, all but one *Ae. aegypti* absent sites were in the forest, so the comparison between all
570 *Ae. aegypti* absent and present sites were largely confounded by the contrast between forest and domestic
571 sites. Another caveat for this comparison between *Ae. aegypti* present vs. absent sites lies in the difficulty
572 of confirming that the absence of *Ae. aegypti* in any site was due to active avoidance. However, if *Ae.*
573 *aegypti* actively choose only a subset of oviposition sites with specific environments, we would expect to
574 see a tighter clustering of sites with *Ae. aegypti* present than absent, which is not the case in our data
575 (Figure 2 and 5). Therefore, it is likely that wild *Ae. aegypti* do not have a strong preference when
576 choosing oviposition sites within their native habitats and could use most available sites.

577 Many environmental characteristics of natural oviposition sites, such as the bacterial
578 communities, predation risk, and competition are likely dynamic and vary depending on weather, season,
579 and stochastic events (e.g., leaf litter falling into a site). In our data, we did find variation in bacterial
580 communities in temporal samples from a few oviposition sites. However, these temporal differences did
581 not exceed the scale of within-habitat variation (Figure S12), which suggested that the main difference we
582 found between forest and peridomestic/domestic oviposition sites were likely stable over time. We expect
583 a similar or even higher level of temporal stability in most physical characteristics of oviposition sites as
584 they are more intrinsic to the containers (e.g., container size) or their locations (e.g., shading).
585 Unfortunately, all our sampling was conducted during the rainy season, and the largest interval between
586 two temporal samples was 21 days, which is not enough to evaluate seasonal variabilities. In dry seasons,
587 it is generally easier to collect *Ae. aegypti* by oviptraps in the field (personal observations), which may
588 suggest that either the mosquitoes are less selective when there are fewer natural oviposition sites
589 available, or the mosquitoes have an altered preference in dry seasons. Recently studies suggested that
590 seasonality may play an important role in driving the domestication of *Ae. aegypti* (Powell & Tabachnick,
591 2013; Rose et al., 2020). Therefore, future studies are needed to examine the seasonal change of
592 oviposition site conditions, in order to provide a full picture of the ecological backdrop for mosquito
593 oviposition.

594 Only a few studies so far has characterized the environmental conditions of *Ae. aegypti* natural
595 oviposition sites. Dickson et al. (2017) described the bacterial community composition in field
596 oviposition sites in La Lopé, Gabon and found a strong differentiation between habitats, which is echoed
597 in our study. Yet that study did not examined other environmental conditions such as the physical
598 variables. Another study compared several environmental conditions between tree holes and tires in
599 Hattiesburg, MS, USA, and found consistent differences between them (Yee et al., 2012). However, *Ae.*
600 *aegypti* were not present in most containers in that study. Therefore, the environmental data reported in
601 the current study added useful information to our understanding of the ecology of *Ae. aegypti* oviposition.

602 Admittedly, our data may not cover the full temporal variations in the field, and the complex field
603 conditions limited the accuracy of some measures. For instance, accurate volume and surface area
604 estimates were challenging for some irregularly shaped sites. However, we hope this initial quantification
605 of natural oviposition sites could provide useful information for generating hypotheses regarding the
606 evolution of *Ae. aegypti* oviposition.

607 One hypothesis we wanted to test in this study is that environmental differentiation between
608 forest and village (peridomestic and domestic) oviposition sites leads to divergent oviposition preference
609 in the mosquitoes. The results of the field oviposition assay did suggest some behavioral differences
610 between forest and village *Ae. aegypti* in both La Lopé and Rabai (Figure 7). However, these results need
611 to be interpreted with caution. Because we counted *Ae. aegypti* after they developed into adults instead of
612 at the egg stage, the number in each experimental container could be affected by factors other than
613 oviposition preference, such as egg hatching rate and larval survival etc. In addition, although bamboo
614 segments are similar to tree holes in size and shape, they possessed bacterial communities that resembled
615 the artificial containers (Figure S13). Therefore, results of the field oviposition experiments might reflect
616 a behavioral difference, if it truly exists, that does not fully correspond to the between-habitat difference
617 of natural oviposition sites. It is unclear what are the exact mechanisms of this differential production of
618 *Ae. aegypti* from bamboo and artificial containers in different habitats, which could be of interest for
619 future studies. An interesting possibility is that choice of bamboo versus artificial containers was affected
620 by their apprenency in each habitat (Harrington et al., 2008; Strauss, Cacho, Schwartz, Schwartz, & Burns,
621 2015). For example, domestic habitats may present less visual obstacles and make the aritifical container
622 stand out more. This potential visual effect may be of interest for future studies.

623 Nevertheless, the results of field experiments provided strong evidence that *Ae. aegypti* in the
624 forest habitat readily accept artificial containers. They might even prefer these containers, as we collected
625 more *Ae. aegypti* from these artificial containers placed in the forest (22 in La Lopé and 645 in Rabai)
626 than from tree holes or rock pools (9 in La Lopé and 156 in Rabai). These results imply that *Ae. aegypti*

627 may be predisposed to use artificial containers for oviposition. Oviposition choices have been suggested
628 to have a strong impact on the movement of *Ae. aegypti* (Reiter, 2007). Previous studies also proposed
629 that females turning to human stored water for oviposition during dry seasons may be a key driver for the
630 human specialization of *Ae. aegypti* inside Africa (Brown et al., 2014; Powell et al., 2018; Powell &
631 Tabachnick, 2013; Rose et al., 2020). As suggested by our field experiment results, this crucial
632 ovipositional transition might happen relatively easily and frequently. Consistent with this hypothesis, a
633 population genetic study using *Ae. aegypti* collected in La Lopé and Rabai found very little evidence of
634 genetic differentiation between habitats, which indicates that mosquitoes could move between habitats
635 freely (Xia et al. submitted). On the other hand, this extensive connectivity in the local scale between
636 habitats may hinder any phenotypic divergence from evolving, consistent with the lack of oviposition
637 differences in the lab (Figure 8). In a more regional scale where gene flow is less frequent, there may be
638 differences between mosquitoes from different habitats, as found for host odor preference by Rose et al.
639 (2020).

640 In line with this “predisposal” hypothesis, it is possible that *Ae. aegypti* from La Lopé and Rabai
641 are not very selective in their oviposition choices in general. We found considerable OAI variation within
642 each colony despite the well-controlled rearing and experimental procedures. Only a few trials found any
643 significant preference for any choices. Yet in these assays, the direction of preference was opposite our
644 prediction (e.g., one domestic colony from Rabai showed a preference for forest water samples over
645 domestic water samples; Figure 8a). Lastly, we did not find a significant difference between forest and
646 village mosquito colonies in any assays (Figure 8 and 9). However, these results of the laboratory
647 oviposition assays need to be interpreted with caution. For example, we cannot rule out the possibility
648 that we simply lacked the power to detect the preference. Yet, our sample sizes are comparable to
649 many previous studies that used a similar experimental design and found significant oviposition
650 preference (Afify, Horlacher, Roller, & Galizia, 2014; Allan & Kline, 1995; Ganesan, Mendki,
651 Suryanarayana, Prakash, & Malhotra, 2006; Melo et al., 2020). It is also possible that the choices we

652 tested are not of a magnitude detectable by female *Ae. aegypti*. However, these choices were informed by
653 the characteristics of natural oviposition sites, and therefore should be ecologically relevant for the
654 mosquitoes. We are currently testing some more extreme conditions (e.g., complete shading vs. complete
655 exposure) using the same groups of La Lopé and Rabai colonies, which will be summarized in a future
656 report.

657 Another strong possibility is that mosquitoes use multiple cues simultaneously in choosing
658 oviposition sites, as previous studies found a broad spectrum of factors influencing *Ae. aegypti*
659 oviposition (Afify & Galizia, 2015; Arbaoui & Chua, 2014; Day, 2016; Harrington et al., 2008; Leahy et
660 al., 1978; Wong, Stoddard, Astete, Morrison, & Scott, 2011). Because most of our choice assays focused
661 on a single variable, it is premature to reach a definitive conclusion of no behavioral difference. Future
662 experiments testing more combinations of environmental factors are needed to gain a deeper
663 understanding of the potential synergistic effects of the environments on driving oviposition evolution in
664 *Ae. aegypti formosus*.

665 In summary, this study confirmed a strong environmental difference between forest and village
666 oviposition sites in both Gabon (La Lopé) and Kenya (Rabai). Our ecological divergence hypothesis
667 suggested that *Ae. aegypti* in different habitats may evolve divergent oviposition preferences
668 corresponding to these environmental differences. However, direct behavioral data from this study was
669 insufficient to support this hypothesis. The similar environmental conditions between *Ae. aegypti* present
670 vs. absent sites in the field also suggested no strong selectivity within habitats. Considering all the
671 findings, it is possible that *Ae. aegypti* in La Lopé and Rabai behave as generalists when choosing
672 oviposition sites. If this is the case, the initial transition between habitats may not require significant
673 changes in oviposition behavior. After occupying different habitats, mosquitoes may start to evolve some
674 minor behavioral differences, but likely not strong enough to discriminate against oviposition sites from
675 the other habitats and impede gene flow at this small geographic scale. This speculation is consistent with
676 the documentation of multiple independent invasions of domestic habitats in Africa in recent years

677 (Kotsakiozi, Evans, et al., 2018; Powell & Tabachnick, 2013), including the latest cases of La Lopé and
678 Rabai (Xia et al., submitted). Being an ovipositional generalist benefits *Ae. aegypti* as they are capable of
679 utilizing a large variety of containers (Petersen, 1977; Simard, Nchoutpouen, Toto, & Fontenille, 2005),
680 and thus quickly respond to environmental changes such as the drying of tree holes as well as within-
681 container competition. In the forest, most oviposition sites we surveyed contained multiple species. A
682 previous study in Kenya found a positive association between *Ae. aegypti* and a few other *Aedes* species
683 in tree holes (Lounibos, 1981), which could lead to resource competition. It is possible that this
684 competition and possibly predation, in combination with the flexibility of *Ae. aegypti* oviposition choices,
685 drove the mosquito to exploit artificial containers. This raises the question of why most other mosquito
686 species do not exploit domestic habitats. What makes *Ae. aegypti* so special?

687 *Ae. aegypti* are known to spread risks during oviposition by a conservative bet-hedging strategy
688 (Starrfelt & Kokko, 2012), namely ‘skip oviposition’: A gravid female distributes her eggs across
689 multiple containers to prevent losing all eggs due to the destruction of any single oviposition site (Colton,
690 Chadee, & Severson, 2003; Swan, Lounibos, & Nishimura, 2018). If *Ae. aegypti* can accept a large
691 variety of oviposition choices, they could further spread the risk. It would be interesting to examine
692 whether the large inter-individual variation we observed in oviposition choice assays are heritable and
693 consistent across the lifetime of individual mosquitoes.

694 This study examined *Ae. aegypti* in forests and rural villages in Africa, where the domestication
695 of this epidemiologically important species likely first occurred (Powell et al., 2018; Powell &
696 Tabachnick, 2013). Outside of Africa, *Ae. aegypti* are closely associated with human communities and
697 use almost exclusively artificial containers for oviposition, except in the Caribbean and Argentina
698 (Chadee et al., 1998; Mangudo et al., 2015). Studies from the 1970s and continuing through 2014 found a
699 human-specialized strain of *Ae. aegypti* reintroduced to Rabai from America or Asia (Brown et al., 2011;
700 McBride et al., 2013; Tabachnick et al., 1979; Tabachnick & Powell, 1978; Trpis & Hausermann, 1978),
701 which have likely gone extinct before our study in Rabai in 2017 (Xia et al. submitted). Comparing this

702 re-introduced strain with the local sylvatic *Ae. aegypti* back then revealed significant behavioral
703 differences, including their oviposition preference (Leahy et al., 1978; Petersen, 1977; Trpis &
704 Hausermann, 1975). These pieces of evidence suggested that *Ae. aegypti* outside of Africa have
705 behaviorally specialized to the domestic oviposition sites. When did the ovipositional adaptation happen,
706 if *Ae. aegypti* remain largely generalists during the initial invasion inside Africa? A few recent studies
707 suggested that human specialization may happen somewhere in West Africa, such as Sahel or Angola
708 (Crawford et al., 2017; Powell et al., 2018; Rose et al., 2020). This specialization may not always
709 accompany the use of domestic habitats inside Africa, but may play a key role for the spread of this
710 species to the rest of the world. More studies examining the intial domestication process inside Africa and
711 the later human specialization are necessary for providing a more comprehensive understanding of the
712 evolutionary history of *Ae. aegypti*.

713

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953

954 **Tables**

955 **Table 1.** Number of oviposition sites measured for different environmental variables

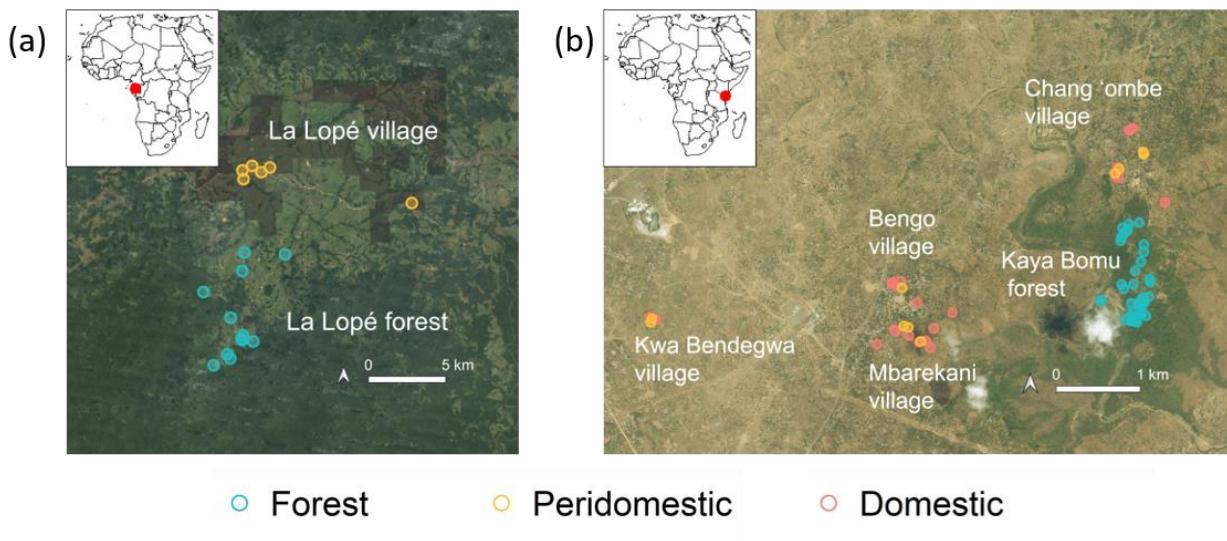
Field site	Habitat	<i>Aedes</i>	Physical characteristics	Larval density	Microbial density	Bacteria composition	Volatile profile
	location	<i>aegypti</i>					
	Forest	Present	5	5	5	5	na*
		Absent	48	55	10	33	na*
La Lopé, Gabon	Peridomestic (Village)	Present	13	13	10	10	na*
		Absent	24	25	12	23	na*
	Total		90	98	37	71	na*
	Forest	Present	15	15	15	15	7
		Absent	22	22	11	22	12
Rabai, Kenya	Peridomestic (Village)	Present	8	8	8	8	5
		Absent	1	1	1	1	1
	Domestic (Village)	Present	22	22	22	22	17
	Total		68	68	57	68	42

956 * Headspace volatiles were not collected in La Lopé, Gabon.

957

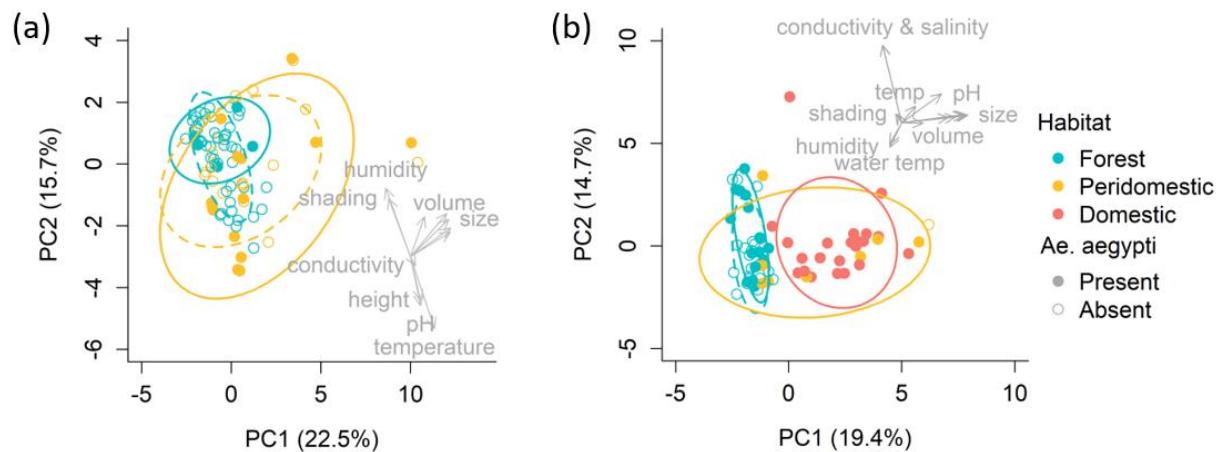
958

959 **Figures**



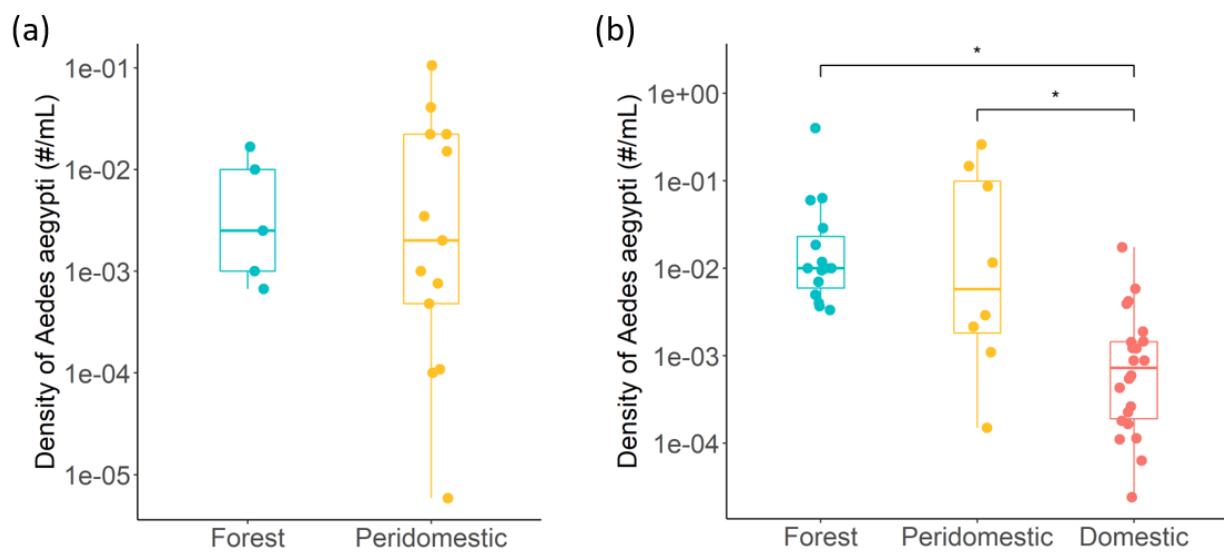
960

961 **Figure 1.** Sampling locations in (a) La Lopé, Gabon, and (b) Rabai, Kenya. The inset in each graph
962 shows the location of the field site in continental Africa. In (a), each point represents a sampling site
963 where one to multiple oviposition sites were found. In (b), each point represents a single oviposition site.
964 The color of the point indicates the habitat category: red points are domestic (village indoor) sites, yellow
965 points are peridomestic sites (village outdoor), and green points are forest sites. The satellite image were
966 from (a) Google Satellite and (b) Bing Satellite in QGIS, respectively.

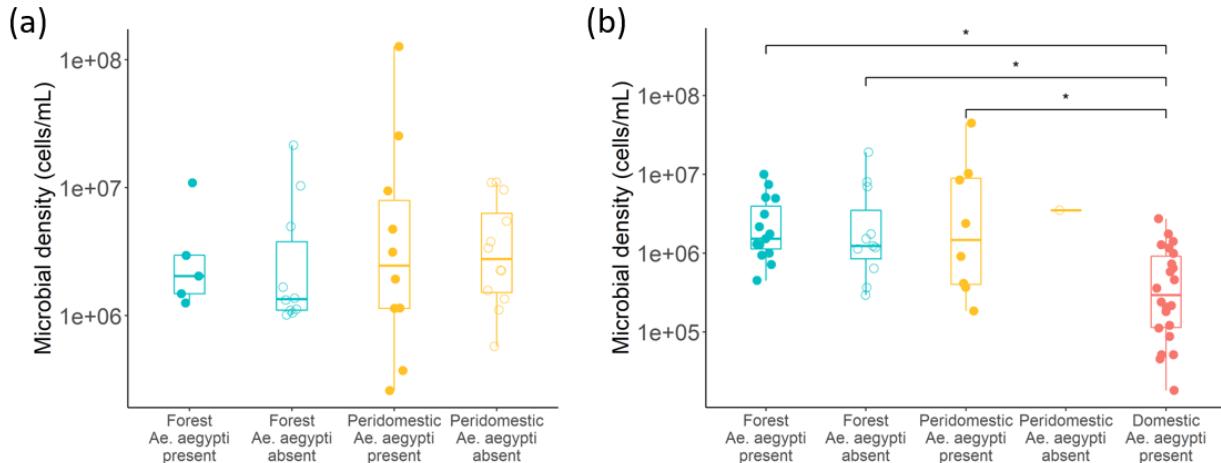


967

968 **Figure 2.** Principal component analysis (PCA) of all physical variables in (a) La Lopé and (b) Rabai. The
969 first two PCs are shown, and the variance explained by each PC was indicated in the axis label. Each
970 point represents a single oviposition site. Colors and point shapes indicate habitat and whether *Ae. aegypti*
971 were found in the sites, respectively. An ellipse was drawn for each oviposition site group with a 75%
972 confidence level. The colors of the ellipses represent habitat types and match the colors of the points. The
973 solid and dashed ellipses correspond to *Ae. aegypti* present and absent sites. The original variables were
974 overlaid on the PC1-PC2 plate with major variables labeled.



976 **Figure 3.** Comparison of *Ae. aegypti* density between habitats in (a) La Lopé and (b) Rabai. Only
977 oviposition sites present with *Ae. aegypti* were included. Each point represents a single oviposition site.
978 The color and shape are as in Figure 2. The boxplots show the minimum, 25% quartile, median, 75%
979 quartile, and maximum. Differences between habitats were tested using pairwise Wilcoxon rank sum test
980 with Holm multiple comparison corrections (*: p < 0.05, Table S4-S6).



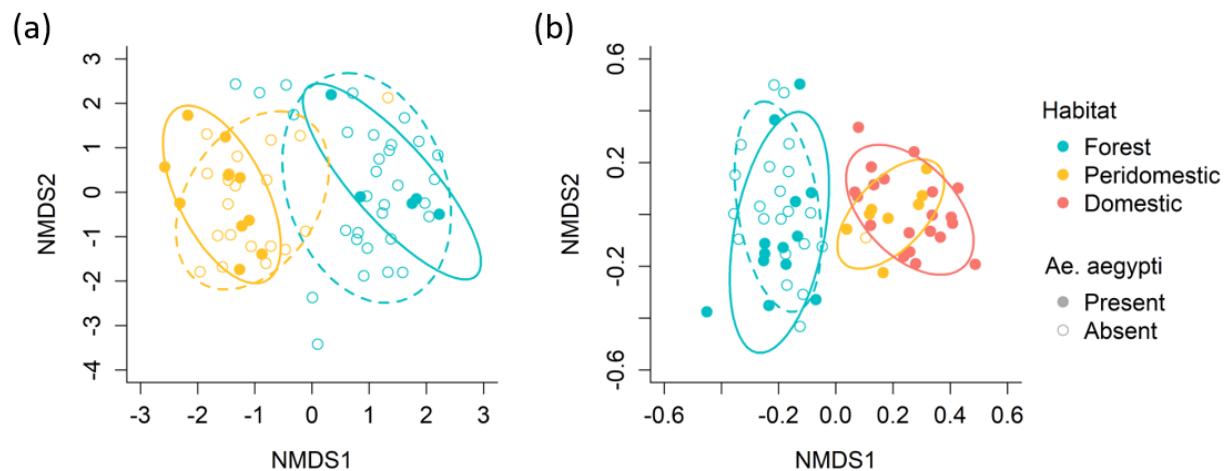
981

982 **Figure 4.** Comparison of microbial density between oviposition site groups in (a) La Lopé and (b) Rabai.

983 Each point represents an oviposition site. The color and shape are as in Figure 2. Differences between

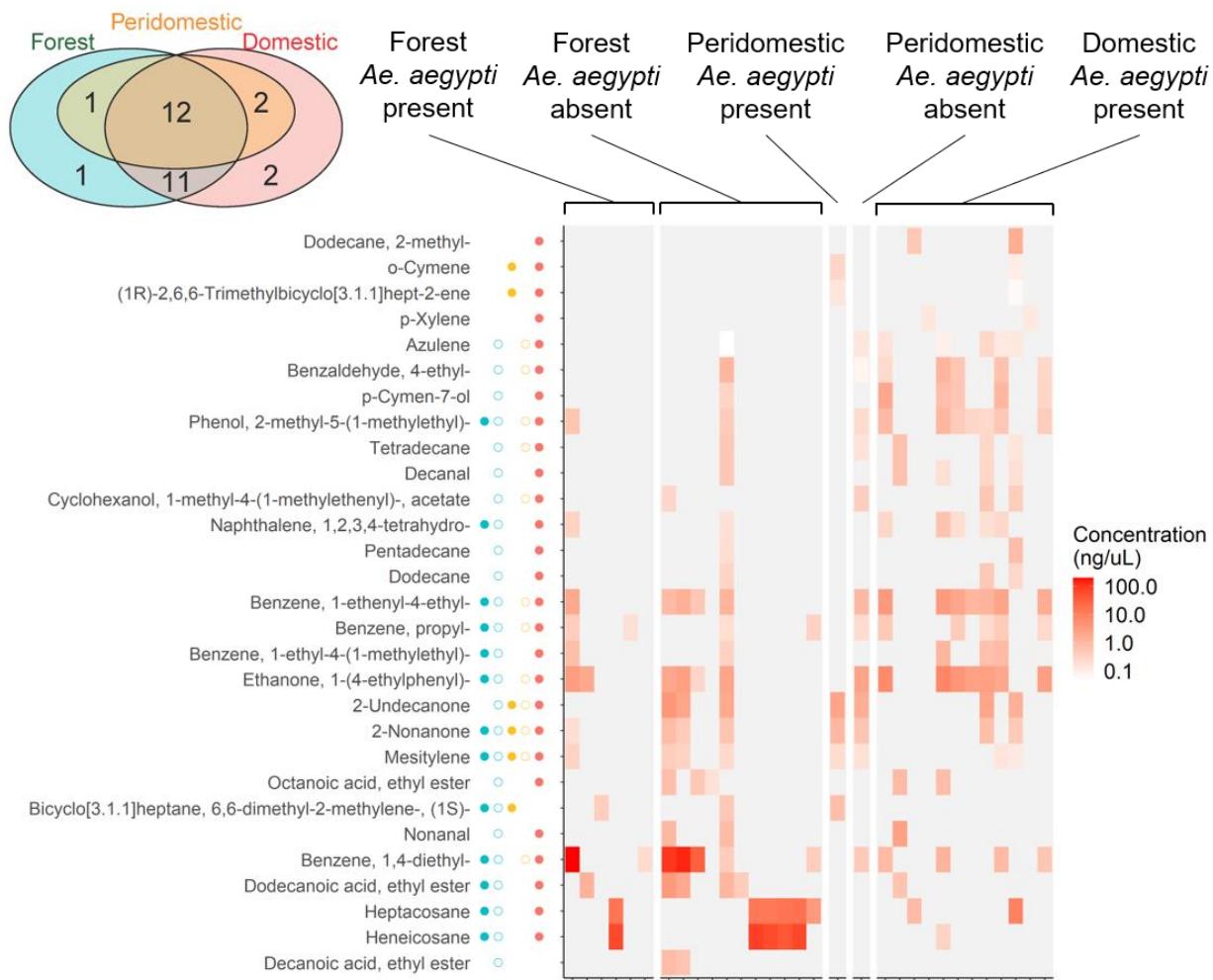
984 groups were tested using pairwise Wilcoxon rank sum test with *Holm* multiple comparison correction (*: $p < 0.05$, Table S3-S6).

985



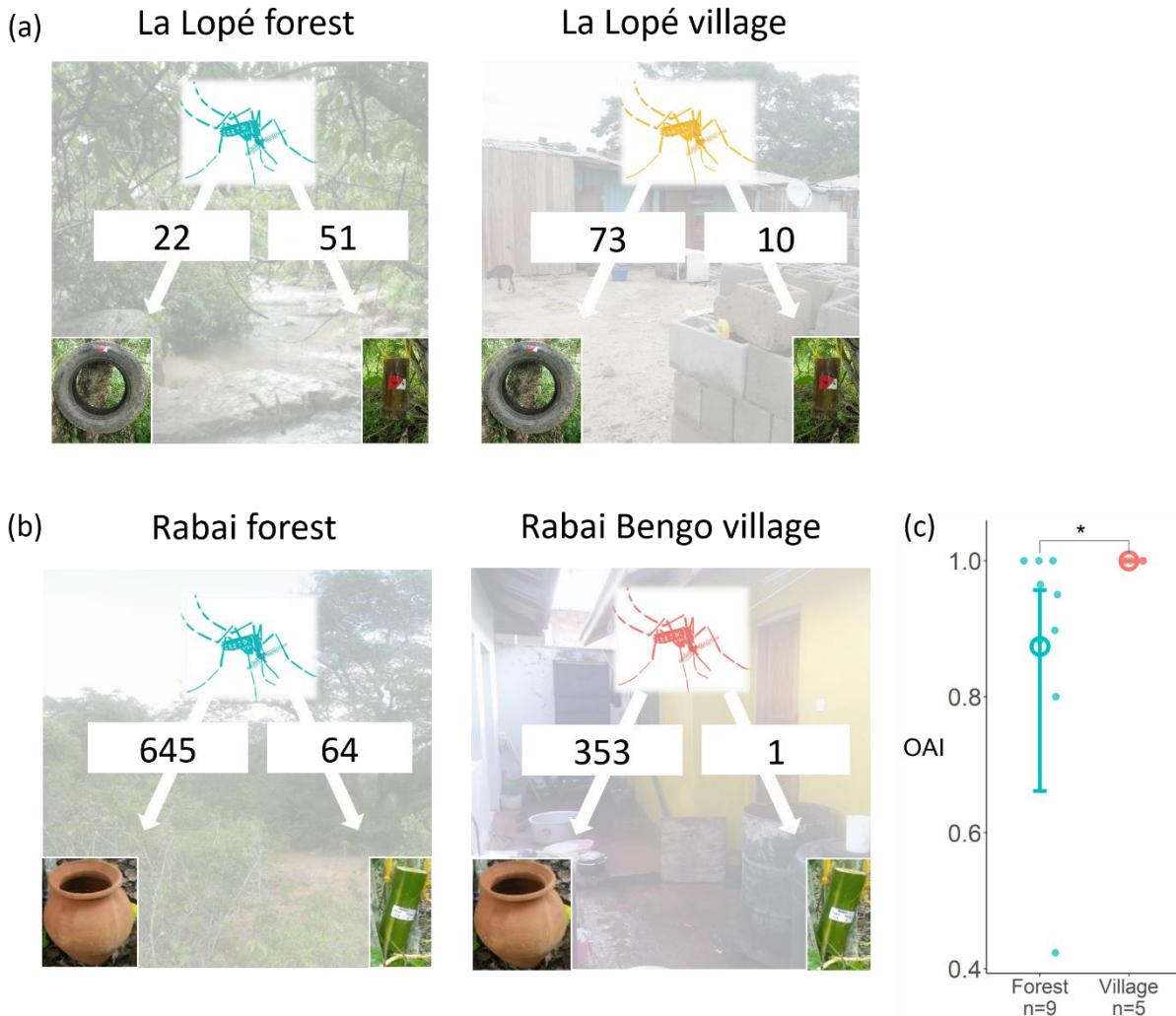
986

987 **Figure 5.** NMDS analysis of bacterial community compositions in oviposition sites in (a) La Lopé and (b)
988 Rabai. The analysis was performed with the amplicon sequencing results at the sequencing variants
989 (ASVs) level. Each point represents an oviposition site. The color and shape of points, as well as the
990 ellipses, are the same as in Figure 2.



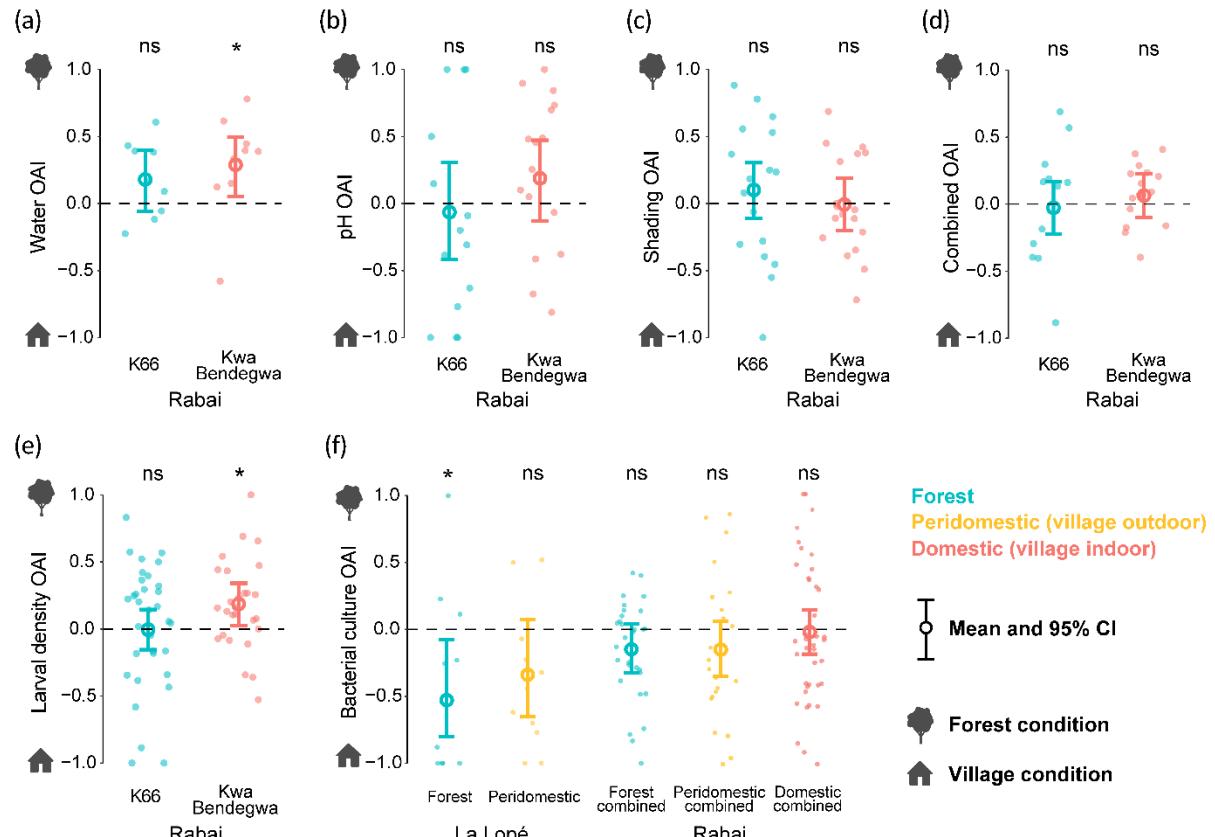
991

992 **Figure 6.** Chemical profile of the volatile samples collected from Rabai oviposition sites. Each row
 993 represents a compound, and each column represents an oviposition site. The five columns of points
 994 between the compound names and the heatmap summarize whether the compounds were present in each
 995 of the five oviposition site groups. The color and shape of points are the same as in Figure 2. The color of
 996 each cell in the heatmap quantifies the concentration on a log scale. Gray cells indicate that the compound
 997 was not found in the oviposition sites according to the GC-MS results. The inset Venn diagram shows the
 998 total numbers of compounds unique in each habitat or shared between different habitats.



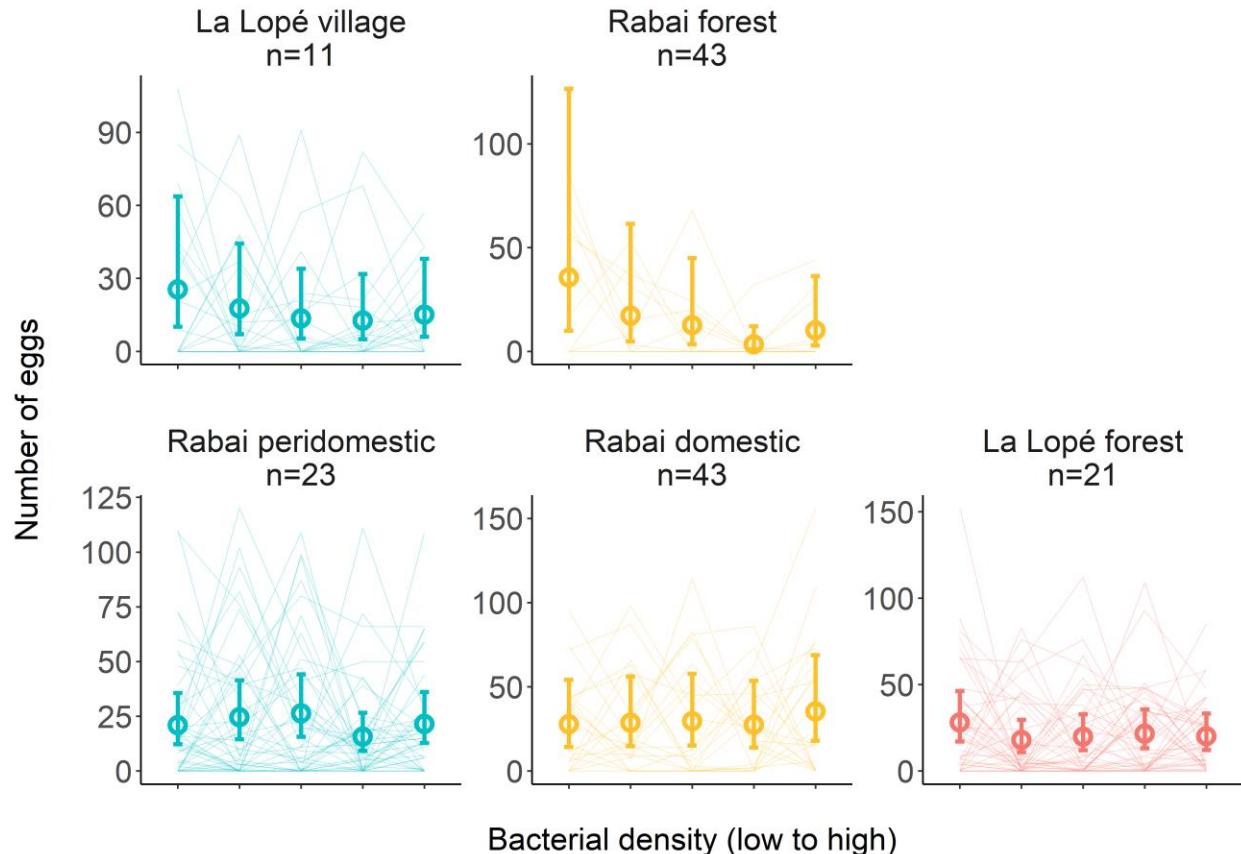
999

1000 **Figure 7.** Field oviposition choice experiments in (a) La Lopé and (b) Rabai. Artificial containers and
1001 bamboo segments (inset photos as examples) were placed in both the forest and the villages. The numbers
1002 in (a) and (b) are the total numbers of *Ae. aegypti* produced by the two types of containers in the two
1003 habitats. In Rabai, the ten replicates of container pairs in each habitat were examined separately. An OAI
1004 was calculated for each container group that had no fewer than ten *Ae. aegypti*, as shown by points in (c).
1005 Larger OAI implies more *Ae. aegypti* from artificial containers. The hollow circles and error bars show
1006 the mean OAI and a 95% confidence interval estimated by a beta-binomial model with habitats as the
1007 predictor. The model was significantly better than a null model, which suggested a significant difference
1008 between habitats (*: $p < 0.05$).



1009

1010 **Figure 8.** Two-choice laboratory oviposition assays testing preference for field-collected waters, pH,
 1011 shading, a combination of water pH, salinity and shading, *Ae. aegypti* larval density, and bacterial culture.
 1012 Colony-wise results are shown in Figure S14 in the Appendix. The details of the two choices in each
 1013 assay were described in Table S2 in the Appendix. Higher OIA implies a preference for the forest
 1014 condition. Each point represents the OIA of one cage with five gravid females. The mean and 95%
 1015 confidence interval (CI) were estimated by beta-binomial models. The asterisks and 'ns' above each
 1016 colony indicates whether the 95% CI excludes zero. No significant differences were found between habitats
 1017 or between colonies in any experiments.



1018

1019 **Figure 9.** Five-choice laboratory oviposition assays testing preference for bacterial density. Five cups
1020 were provided in each cage with increasing bacterial density at 0, 2×10^5 , 1×10^6 , 5×10^6 , 2.5×10^7 cells/mL
1021 (details in Table S2), which correspond to the five columns (left to right) in each panel. Each line
1022 connects the five egg counts in one cage. Colors represent the habitats from where the colonies came.
1023 Multiple colonies from the same habitat in Rabai were combined in this figure. Colony-wise results are
1024 shown in Figure S15 in the Appendix. A negative-binomial model was used to fit the results of each
1025 oviposition assay. The model estimates the mean number of eggs in each bacterial density and a 95%
1026 confidence interval, which are shown by the open circles and the error bars, respectively.

1027

1028 **Data Accessibility Statement**

1029 The datasets that describe the basic information, physical characteristics, larval density, predator
1030 presence, microbial density, and chemical profile of oviposition sites in La Lopé and Rabai are archived
1031 in Dryad: doi:10.5061/dryad.7m0cfxprg (La Lopé) and doi:10.5061/dryad.3tx95x6cz (Rabai), respectively.
1032 The 16s-rRNA gene amplicon sequencing data was deposited in the NCBI SRA database with ID
1033 SUB7716639 (La Lopé samples) and SUB7719551 (Rabai samples).

1034

1035 **Competing Interests Statement**

1036 The authors declare that they have no competing interests.

1037

1038 **Author contributions**

1039 SX and JRP designed and conceptualized the study. SX, DA, RS, JL, CSM, NHR, and JRP coordinated
1040 the fieldwork. SX, DA, and JL conducted field sampling of oviposition sites and field oviposition
1041 experiments. SX and HD designed the volatile collection in Rabai and performed the GC-MS analysis.
1042 SX performed the lab work to generate the data on microbial density and bacterial community
1043 composition. SX, CSM, and NHR established mosquito colonie, and SX performed the laboratory
1044 oviposition assays. SX wrote the first draft of the manuscript. JRP provided funding, coordinated the
1045 entire study, and interpreted results with SX. All authors provided critical feedback on the manuscript.

1046

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