# Microbial exposure across life reduces susceptibility of *Aedes aegypti* to Zika virus by enhancing blood digestion and limiting midgut cell infection

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**Short Title:** Microbes reduce Zika virus susceptibility in mosquitoes

## Abstract

The worldwide expansion of mosquito-borne pathogens necessitates improved control measures, including approaches to reduce transmission by mosquito vectors. Reducing transmission is challenging because determinants of vector competence for viruses like Zika (ZIKV) are poorly understood. Our previous work established that *Aedes (Ae.) aegypti* larvae reared in environmental water containing microbes are less susceptible to ZIKV as adults compared to cohorts reared in laboratory tap water with fewer microbial species and lower microbial abundance. Here, we identify a process by which environment-derived microbes reduce susceptibility of *Ae. aegypti* for ZIKV. Provided that the midgut represents the first barrier to mosquito infection, we hypothesized that microbial exposure modulates midgut infection by

ZIKV. Since mosquitoes live in water as larvae and pupae and then transition to air as adults, we also define the stage in the life of a mosquito when microbial exposure reduces ZIKV susceptibility. *Ae. aegypti* larvae were reared in water containing microbes and then treated with antibiotics during the pupal and adult stages, adult stage only, or provided no antibiotics at any stage. Vector competence was next evaluated in mosquitoes that ingested ZIKV-spiked bloodmeals. Antibiotic treated mosquitoes with reduced microbiota showed enhanced ZIKV infection rates in *Ae. aegypti* treated as both pupae and adults. Antibiotic treatment to disrupt microbes in pupal and adult mosquitoes also resulted in increased midgut epithelium permeability, higher numbers of ZIKV-infected midgut cells, and impaired bloodmeal digestion. Parallel control experiments with antibiotic-treated or gnotobiotic mosquitoes reared in laboratory water showed that the dysbiotic state created by antibiotic use does not influence ZIKV vector competence or midgut permeability and that more than the bacterial species in gnotobiotic mosquitoes is responsible for reducing ZIKV vector competence. *Ae. aegypti* with disrupted microbiota via antibiotic treatment as pupae and adults that ingested ZIKV in bloodmeals showed reduced expression of genes associated with bloodmeal digestion and metabolism relative to mosquitoes whose microbes were not reduced with antibiotics. Together, these data show that exposure to microbes throughout the life of *Ae. aegypti* restricts ZIKV infection by facilitating blood digestion and reducing midgut cell infection. Understanding the connections between mosquito microbiota, midgut physiology, and arbovirus susceptibility can lead to novel approaches to decrease mosquito transmission and will improve understanding of vector competence in environmental habitats containing microbes.

## Author Summary

Mosquito-transmitted viruses like Zika continue to threaten human health. Absent vaccines or treatments, controlling mosquitoes or limiting their ability to transmit viruses represents a primary

way to prevent mosquito-borne viral diseases. The role mosquito microbiota play in shaping transmission of Zika virus has been limited to association-based studies. Our prior work showed that *Aedes aegypti* mosquito larvae that develop in water containing bacteria are less susceptible to Zika virus compared to larvae reared in laboratory tap water with fewer numbers and species of bacteria. Here we identify a process that explains this association. Since mosquitoes are aquatic as larvae and pupae and terrestrial as adults, we also define the life stage when microbes need be present to reduce Zika virus susceptibility. We used antibiotics to reduce environmental water-derived microbes at pupal and adult or only adult stages and observed that microbial disruption via antibiotic treatment increases Zika virus infection and midgut permeability and impairs bloodmeal digestion. These findings advance understanding of microbiota-mosquito-virus interactions and further implicate microbes as a means to restrict virus infection of mosquitoes.

## Introduction

*Aedes (Ae.) aegypti* and *Ae. albopictus* mosquito-transmitted viruses, including dengue (DENV), chikungunya (CHIKV), and Zika (ZIKV), pose a massive burden on human health. Vector control represents the primary means to reduce the diseases they cause. Vector competence studies, which assess the capacity for mosquitoes to become infected with, develop disseminated infections, and transmit viruses in laboratory environments, are foundational to defining the species that maintain arbovirus transmission cycles. For *Ae. aegypti,* prior studies show that temperature (1,2) and geographic origin (3,4) are major determinants of ZIKV vector competence. Other factors, including the microbial environment of mosquito vectors, have been subject to much recent study (reviewed in Ferriera *et al.* (5)). The microbiota of *Aedes spp.* mosquitoes primarily derive from the environment (6–8), reside dominantly in the midgut (8,9), and can influence physiology and life history traits through nutritional supplementation (6,7,10,11) and immune stimulation (12–14). Prior studies from our group and others show that

79 microbiota influence ZIKV competence for *Ae. aegypti* (15,16), and *Ae. albopictus* (17), and that
80 these interactions are influenced by mosquito genotype (18). We discovered that when *Ae.*
81 *aegypti* larvae reared in water with microbes sourced from cemeteries were exposed orally to
82 ZIKV as adults, they became infected, developed disseminated infections, and transmitted virus
83 less efficiently than cohorts where larvae were reared in laboratory tap water with fewer
84 numbers and species of microbes (15). We also determined that the presence of microbes
85 accelerates mosquito development. Another study demonstrated that disruption of microbiota
86 via oral treatment of adult *Ae. aegypti* with antibiotics prior to exposure to ZIKV did not affect
87 vector competence (19). Together, these findings suggest that the influence of microbiota on
88 ZIKV vector competence may be specific to the mosquito developmental stage (larval, pupal,
89 adult) during which the microbes are present. To understand whether reduced ZIKV vector
90 competence after larval development in water containing microbes is conferred transstadially
91 from larvae to pupae to adults, one goal of this study was to determine whether exposure to
92 microbes throughout life is required to reduce ZIKV susceptibility in *Ae. aegypti*.
93
94 Microbes may modify vector competence of mosquitoes via multiple mechanisms, including to
95 alter immune system activation, by direct antiviral or antiparasitic activity, or by affecting
96 physiological processes, including blood digestion. Many of these processes occur in the
97 mosquito midgut, which represents the first infection barrier for most vector-borne pathogens.
98 Microbes are likely also involved in maintaining physical barriers that restrict access of an
99 ingested virus or parasite to the mosquito midgut. The commensal bacterium *Serratia*
100 *marcescens* increases susceptibility of *Ae. aegypti* for CHIKV by secreting a protein that digests
101 membrane-bound mucins coating the gut epithelium, rendering the epithelium more accessible
102 to the virus (20). The bacterium *Bacillus thuringiensis* svar. *israelensis* (Bti) expresses δ-
103 endotoxins that create pores in midgut cells of larval mosquitoes, which is lethal and has long
104 been a mainstay of larvicidal vector control (21,22). We hypothesized that disturbance in the

105 microbiota of pupal and adult *Ae. aegypti* mosquitoes would decrease blood digestion, resulting
106 in increased ZIKV susceptibility by giving the virus more time to access the midgut, since blood
107 digestion is thought to occur concomitantly with virus infection of the midgut epithelium (23). We
108 reared *Ae. aegypti* larvae in environmental water containing microbes. Half of the experimental
109 cohorts were administered antibiotics in water during the pupal stage to reduce microbes. Some
110 cohorts of adults were also administered antibiotics in sugar water prior to exposure to ZIKV-
111 spiked bloodmeals. Since antibiotics create a dysbiotic environment, may not eliminate resident
112 antibiotic-resistant microbiota (24), and can alter cellular metabolism and transcription as
113 described in mammalian cells (25–28), *Drosophila* (29), and *Anopheles* (30) in ways that could
114 also impact ZIKV infection in *Ae. aegypti*, we first verified that antibiotic treatment did not modify
115 midgut integrity or ZIKV vector competence. We also generated gnotobiotic mosquitoes by
116 colonizing larvae with an *Elizabethkingia spp.* strain; this bacterial species was not sufficient to
117 modulate vector competence, suggesting that many species are required. Next, we measured
118 physiological differences in the midgut in blood engorged mosquitoes across cohorts treated
119 with or without antibiotics at different life stages. Microscopy was used to quantify ZIKV-infected
120 midgut cells. The magnitude and rate of bloodmeal digestion was measured by quantifying
121 hemoglobin levels over time. Since the ways microbes modify vector competence are not fully
122 understood, RNA sequencing (RNA-seq) was used to identify differential gene expression in
123 cohorts of *Ae. aegypti* treated with or without antibiotics at different life stages and in response
124 to blood with or without ZIKV. We observed that *Ae. aegypti* given antibiotics as pupae and
125 adults are more susceptible to ZIKV infection compared to those not given antibiotics. Microbe
126 reduction by antibiotic treatment of pupae and adults also increased midgut permeability, the
127 number of ZIKV-infected midgut cells, and impaired bloodmeal digestion. Antibiotic treatment
128 itself did not reduce ZIKV vector competence or affect midgut permeability, ruling out toxic or
129 physiologic effects from the antibiotic cocktail used. Gene ontology analyses of ZIKV-exposed
130 mosquitoes revealed enrichment of PM-associated genes, with more genes related to blood

131 protein metabolism upregulated in mosquitoes harboring intact microbiota. Together our results
132 show that exposure to microbes throughout the life of *Ae. aegypti* facilitates blood digestion and
133 reduces ZIKV midgut cell infection.
134
135 **Materials and Methods**
136
137 **Biosafety**
138 All ZIKV infection experiments were conducted in a biosafety level 3 (BSL3) laboratory and were
139 approved by the University of California, Davis (UC Davis) Institutional Biosafety Committee
140 under Biological Use Authorization #R1863.
141
142 **Mosquito sources**
143 *Ae. aegypti* mosquitoes colonized from field collections from 2 locations in California were used.
144 Mosquitoes were field-collected in 2016 as larvae (Los Angeles) and eggs (Clovis). Mosquitoes
145 from both locations were maintained for generations numbers noted in Figures representing $F_{20}$,
146 $F_{22,}$ or $F_{26}$, at 28°C, 80% humidity, and a 12 h:12 h light : dark (L : D) cycle with 10% sucrose
147 provided to adults and periodic presentation of sheep blood to stimulate egg laying, as
148 described in our previous study (15).
149
150 **Mosquito generation and antibiotic treatments**
151 *Microbe source and preparation*. Microbes from water collected outdoors in cemeteries were
152 added to water in which *Ae. aegypti* larvae were reared, using an approach as in our previous
153 study (15). Briefly, 1 liter (L) of collected water was filtered through a 1 mm mesh to remove
154 large debris, centrifuged at 3000 g to pellet microbes, washed 3 times with sterile 1X phosphate
155 buffered saline (PBS, Thermo Fisher Scientific, Emeryville, CA), and frozen in glycerol at -80°C.

156 Microbe aliquots were thawed at room temperature and placed in laboratory tap water prior to
157 addition of mosquito eggs.
158 *Larval rearing.* Mosquito eggs were hatched in 1 L deionized (di$H_2O$) water spiked with 1 mL of
159 microbes from a glycerol stock aliquot. Approximately 1500 larvae were reared in three pans at
160 a density of ~500 larvae/pan. Each pan contained an agarose plug that was prepared by mixing
161 1% agarose (Sigma-Aldrich, St. Louis, MO) with pulverized fish food (final concentration of 100
162 g/L, or 10% [Tetra, Melle, Germany]) and rodent chow (final concentration of 80 g/L, or 8%
163 [Teklad Global 18% Protein Rodent Diet, Envigo, Indianapolis, IN]), which was autoclave-
164 sterilized before casting into 12-well plates. One to 2 plugs were added to larval pans every
165 other day until day 15 post-hatch. Larval trays were kept in environmental growth chambers
166 (Binder, Bohemia, NY) at 28°C, 80% humidity, and a 12 h:12 h L:D cycle.
167 *Antibiotic treatments to pupae and adults*. Pupae or adults were divided into three treatments: 1)
168 mosquitoes exposed to antibiotics (Abx) both as **p**upae and **a**dults (AbxPA), 2) mosquitoes
169 exposed to antibiotics as **a**dults (AbxA) only, and 3) mosquitoes not exposed to antibiotics (No
170 Abx), where the latter represents the control group. First, pupae were picked and randomly
171 divided into 2 batches each day. The AbxPA batch of pupae was washed in di$H_2O$ containing an
172 antibiotic cocktail consisting of 50 U/mL each of penicillin, streptomycin, and kanamycin (Sigma-
173 Aldrich, St. Louis, MO) for 10 minutes before transfer to another water vessel containing fresh
174 antibiotic cocktail. This concentration is in the middle of the range of doses used previously in
175 similar studies with *Ae. aegypti* (20) and *An. stephensi* (31). The other batch of pupae, from
176 which the AbxA and No Abx groups were derived, was transferred to a separate cage without
177 washing or antibiotics. Adults in the AbxPA group were maintained on the antibiotic cocktail
178 dissolved in 10% sucrose (Thermo Fisher Scientific, Emeryville, CA) *ad libitum*. Adults in the
179 other batch were initially maintained on filter-sterilized 10% sucrose without antibiotics *ad libitum*
180 until eclosion was complete. Once adults reached a density of ~500, females were aspirated out
181 and transferred in cohorts of 80-120 mosquitoes to 32-ounce plastic containers (Amazon,

182 Seattle, WA) with mesh lids. At this point, the second non-antibiotic treated batch was further
183 divided into two treatments: AbxA was provided 10% sucrose *ad libitum* with the antibiotic
184 cocktail, and No Abx was fed 10% sucrose *ad libitum* without the antibiotic cocktail. As a control
185 for toxicity or physiological effects stemming from antibiotic treatment, we also reared
186 mosquitoes in laboratory tap water (conventional) with or without antibiotics, which were
187 provided as described above, to pupal and adult mosquitoes (conventional + Abx). Adult
188 mosquitoes were maintained at 28°C, 80% humidity, and 12 hour : 12 hour L : D cycle
189 throughout the experiments. All mosquitoes were housed in the same incubator. Antibiotic
190 treatment experiments with conventional mosquitoes and experiments with mosquitoes provided
191 environmental microbes each performed once.
192
193 **Gnotobiotic mosquito generation**
194 An *Elizabethkingia spp.* strain isolated from colonized *Ae. aegypti* from Los Angeles was
195 cultured in LB broth at 37°C for 24 hours prior to inoculation into larval flasks. *Ae. aegypti* eggs
196 attached to their egg papers cut into strips were surface sterilized by submersion in 1% bleach
197 for 10 minutes. After 10 minutes, bleach was removed by vacuum filtration of eggs through 1000
198 mL of di$H_2O$ and a 0.22 μm filter (Genesee Scientific, San Diego, CA). Eggs were then washed
199 twice in 70% ethanol and allowed to dry. To prepare larval flasks, 50 mL of di$H_2O$ and one larval
200 nutrient plug were added to four T75 tissue culture flasks. Paper strips containing ~200 eggs
201 were placed into each tissue culture flask and spiked with 1 mL of 1.4 x $10^8$ CFU/mL
202 *Elizabethkingia* culture before sealing the lid and incubating at 28°C with 80% humidity and 12
203 h:12 h L:D cycle for 5 days. When pupae developed, lids of the tissue culture flasks were
204 removed, and a sterilized fabric sleeve feeding into a makeshift plastic bucket with a mesh lid
205 was taped around the opening of the flask to allow adults to emerge into the new holding
206 container. Individual mosquitoes of each life stage were sampled, homogenized, and cultured

207 on LB plates to confirm *Elizabethkingia* colonization and the absence of other contaminating
208 bacteria, which was verified by colony morphology.
209
210 **Bacterial quantification by 16S qPCR**
211 We measured the efficacy of antibiotic treatment for removing bacteria from AbxA and AbxPA
212 groups and to verify dominance of *Elizabethkingia* in gnotobiotic mosquitoes by performing 16S
213 qPCR. AbxA and AbxPA larvae, pupae, and adults 3-5 days post-eclosion (dpe) were
214 evaluated. L4 larvae and pupae before antibiotic treatment were also included. Whole adult
215 mosquitoes in the gnotobiotic group were evaluated and conventional and conventional+ Abx
216 mosquitoes were included as comparators. DNA from 5 L4 larvae, 5 pre-treatment pupae, 5 No
217 Abx, 10 AbxA, 10 AbxPA, 8 gnotobiotic, 10 conventional, and 10 conventional + Abx individuals
218 was extracted with the Quick-DNA Tissue/Insect Microprep Kit (Zymo Research, Irvine, CA,
219 USA), according to the manufacturer instructions and eluted in 30-40 µL elution buffer.
220 Extracted DNA was PCR-amplified in the V3-V4 (32) hypervariable region of the 16S rRNA
221 gene, as previously described (15,32). FASTQ files were processed using a standard DADA2
222 pipeline(33) (package version 1.22.0) into phyloseq (package version 1.38.0)(34). Sequences
223 were assigned to ASVs, and samples were filtered by prevalence (ASVs at > 0.1% abundance),
224 known taxonomy (filtered unknown taxa), and presence in the negative control (ASVs also
225 present in the negative control were excluded).Quantification of bacteria was performed by
226 SYBR Green Real-Time PCR (Thermo Fisher Scientific, Emeryville, CA) to amplify the 16S
227 rRNA gene. No Abx, AbxA, and AbxPA samples were assayed in technical duplicates;
228 gnotobiotic, conventional, and conventional + Abx were assayed once. 16S quantities were
229 normalized to an *Ae. aegypti* reference ribosomal protein S17 gene (RPS17) and reported as a
230 16S:RPS17 ratio (35). The rationale for reporting the ratio instead of absolute values was to
231 enable comparison of relative measurements across mosquito treatment groups to verify that
232 antibiotic treatment reduces bacteria in mosquitoes.

233 **Hemolytic activity**

234 Hematophagous bacteria can directly lyse blood cells and confound hemoglobin digestion
235 measurements. Bacterial isolates from adult female *Ae. aegypti* were screened for hemolytic
236 activity via culturing on agar plates. Individual mosquitoes were homogenized (TissueLyser,
237 Retsch, Haan, Germany) in 500 µL PBS and 40 µL of supernatant was plated on either R2A
238 agar (Sigma-Aldrich, St. Louis, MO) or sheep blood agar plates (Biological Media Services, UC
239 Davis) for 3 days at 37°C. Up to 10 colonies from each mosquito were streaked on media plates
240 2-3 additional times to generate axenic cultures. Resulting colonies were streaked on sheep
241 blood agar at both 30°C and 37°C and screened for clearing zones around colonies over the
242 course of 3 days. The viability of this assay was tested in-house with hemolytic *Streptococcus*
243 *spp.* isolates from the UC Davis School of Veterinary Medicine.

244

245 **Virus source and titrations**

246 We used ZIKV strain BR15 (Brazil 2015, SPH2015, GenBank Accession # KU321639), the
247 same strain we used previously for mosquito infection experiments (15). This virus strain was
248 originally isolated from human serum from a patient in Brazil in 2015 and passaged 3 times in
249 Vero cells (CCL-81, ATCC, Manassas, VA) before freezing in stocks. Stocks were titrated on
250 Vero cells prior to bloodmeal presentation to approximate stock titers, and ZIKV-spiked sheep
251 blood was also titrated after bloodfeeding to confirm the dose presented in each bloodmeal. For
252 titrations, serial 10-fold dilutions of cell culture supernatant containing virus stock or ZIKV-spiked
253 blood were inoculated into multi-well plates containing confluent Vero cells at one dilution per
254 well and incubated for 1 hour at 37°C in 5% $CO_2$ with rocking every 10 minutes. After
255 incubation, each well was filled with an agar overlay (0.5% agarose [Thermo Fisher Scientific,
256 Emeryville, CA] in Dulbecco's modified Eagle's medium [DMEM, Thermo Fisher Scientific,
257 Emeryville, CA], + 2% fetal bovine serum [FBS, Genesee Scientific, San Diego, CA] +
258 penicillin/streptomycin [Thermo Fisher Scientific, Emeryville, CA]). The cells were incubated for

259 1 week at 37°C in 5% $CO_2$, then fixed with 4% formalin (Thermo Fisher Scientific, Emeryville,
260 CA). Fixed cells were stained with 0.025% crystal violet (Thermo Fisher Scientific, Emeryville,
261 CA) in 20% ethanol and visualized on a light table to count plaques. ZIKV bloodmeal titers are
262 represented as plaque-forming units (PFU) per mL of blood.
263
264 **ZIKV vector competence experiments**
265 Stock ZIKV in DMEM, or DMEM with no virus as a control, were diluted in 10-fold increments
266 with fresh heparinized sheep blood (HemoStat Laboratories, Dixon, CA) to achieve ZIKV titers
267 of $10^6$ PFU/mL. Bloodmeals were presented to female *Ae. aegypti* at 3-5 days post-eclosion in
268 cohorts of 80-120 per container, at 2-3 containers per treatment. Mosquitoes were sucrose-
269 starved 18-24 hours before the presentation of bloodmeals. Mosquitoes were offered
270 bloodmeals for 60 minutes through a collagen membrane rubbed with an artificial human scent
271 (BG-Sweetscent mosquito attractant, Biogents USA) and heated to 37°C in a membrane feeder
272 (Hemotek Limited, Blackburn, United Kingdom). Bloodmeals were presented to mosquitoes
273 inside a plexiglass glove box containing an open bottle of boiling water to maximize humidity.
274 After feeding, mosquitoes were immobilized with $CO_2$ for 5 seconds and then immobilized on a
275 chill table (Bioquip (R.I.P.), Compton, CA, USA) to assess their bloodfeeding status using a
276 microscope at 10X magnification. Fully engorged females (18-45 per treatment for each
277 experiment) were sorted into fresh plastic containers at a density of 10-20 mosquitoes per
278 container and held at 28°C with 80% humidity and 12 h:12 h L:D cycle for 14 days. No Abx
279 cohorts were provided 10% sucrose *ad libitum,* while AbxPA, AbxA, and conventional + Abx
280 cohorts were maintained on 10% sucrose with the antibiotic cocktail *ad libitum*.
281
282 At 14 days post-bloodfeed (dpf), surviving mosquitoes were $CO_2$-anesthetized and immobilized
283 on a chill table. Bodies, legs/wings, and saliva were collected to assess infection, dissemination,

284 and transmission rates, respectively. Legs/wings were removed before collection of expectorate
285 into capillary tubes containing PBS by forced salivation, using an established approach (36). To
286 minimize cross-contamination, surgical tools were washed once in CaviCide® (Metrex, Orange,
287 CA) and twice in 70% ethanol between dissections. Each capillary tube was placed in a 1.5-mL
288 tube containing 250 µL PBS and pulse-centrifuged at 8,000 g to recover saliva. Bodies and
289 legs/wings were placed into 2-mL tubes containing 500 µL PBS and a 5-mm glass bead
290 (Thermo Fisher Scientific, Emeryville, CA), then homogenized at 30 hertz for 5 minutes in a
291 TissueLyser (Retsch, Haan, Germany). Viral RNA was extracted using a MagMax Viral RNA
292 Extraction Kit (Thermo Fisher Scientific, Emeryville, CA), into 60 µL elution buffer following the
293 manufacturer's recommendations.
294
295 Detection and quantification of viral RNA in mosquito tissues and saliva were performed by
296 quantitative reverse transcription polymerase chain reaction (RT-qPCR). The rationale for using
297 RT-qPCR was to enable rapid and high throughput analyses, because the limit of detection
298 (LOD) is lower than for titrations of volume limited samples, and to reproducibly enable
299 comparison of relative measurements across mosquito treatment groups. Furthermore, our prior
300 studies with ZIKV strain BR15 in Vero cells showed high reproducibility and a ZIKV RNA:PFU
301 ratio of 100-2000 (37). We used a TaqMan Fast Virus 1-Step Master Mix and a ZIKV-specific
302 primer set (ZIKV 1086F/1162c, probe: ZIKV 1107-FAM) using established methodologies
303 (36,37). Cycle threshold (Ct) values from the RT-qPCR were converted to RNA genome copies
304 using standard curves established with ZIKV RNA of known concentration. Each sample was
305 assayed in technical duplicates and converted to copies per mosquito before calculating the
306 average value. The LOD was calculated from the standard curve linear regression line where
307 the Ct value = 40. Samples without a detectable Ct of less than 40 were reported at the LOD.
308 Infection rates are reported as the number of ZIKV-positive individuals divided by the number
309 that ingested ZIKV-spiked blood. Dissemination rates are reported as the number of ZIKV-

310 positive legs and wings divided by the number of ZIKV-infected individuals. Transmission rates
311 are reported as the number of ZIKV-positive saliva samples divided by the number individuals
312 with disseminated ZIKV in legs and wings.
313
314 **Midgut permeability assessments**
315 A low molecular weight dextran-FITC (MW 4000, Sigma-Aldrich, St. Louis, MO) was co-spiked
316 into ZIKV-bloodmeals and used to measure midgut permeability in No Abx, AbxA, AbxPA,
317 conventional, conventional + Abx, and gnotobiotic groups that were evaluated within 1 hour
318 after bloodfeeding. Permeability was assessed by measuring the fluorescent intensity of
319 dextran-FITC inside versus outside at three locations in the midgut for each mosquito for a total
320 of 5 mosquitoes per treatment. A stock solution of 20 mg/mL dextran-FITC dissolved in PBS
321 was spiked into sheep blood at a 1:20 dilution to achieve 1 mg/mL, the concentration used in a
322 previous study (38). Dextran-spiked blood mixed with $10^5$ PFU/ml ZIKV and presented to
323 mosquitoes as described above for vector competence assessments. Blood-engorged
324 mosquitoes were visualized by fluorescence microscopy to detect FITC in tissues. Samples
325 were covered in foil for all post-dissection steps to minimize photobleaching. Quantification of
326 FITC fluorescence was then performed on Fiji (ImageJ) (39) by measuring intensity relative to
327 background by subtracting fluorescence of an equal-sized region outside the sample area.
328 Fluorescence intensity ratios between the gut lumen and hemocoel were calculated at three
329 randomly drawn areas of equal size around the gut perimeter, then averaged together. Each
330 average was also conducted across three slices per sample to minimize focal plane variation in
331 fluorescence. At least 4 mosquitoes per group were included in each permeability assessment.
332
333 **Fluorescence imaging of mosquito midguts**
334 Mosquitoes in No Abx, AbxA, and AbxPA groups that ingested ZIKV-spiked bloodmeals were
335 imaged using immunofluorescence microscopy to examine midgut structure and to quantify the

336 number of ZIKV-infected midgut epithelial cells. Legless and wingless mosquito bodies or
337 dissected midguts from mosquitoes at 2 or 3 dpf were placed in 10% formalin in PBS for 24
338 hours, then prepared for various image-based assessments as described below. Mosquitoes
339 were imaged at the UC Davis Advanced Imaging Facility using a Leica TCS SP8 STED 3X
340 inverted microscope.
341 *Midgut structure*: Mosquito bodies were washed once in PBS, submerged in PBS with 30%
342 sucrose overnight, and then molded and frozen at -80°C in Tissue Plus OCT medium (Thermo
343 Fisher Scientific, Emeryville, CA). Tissue molds were cut into 10 μm slices at the median sagittal
344 plane (at a depth where the blood bolus reaches maximum diameter, determined with test
345 samples) using a Leica CM1860 Cryostat Microtome (Leica Biosystems, Deer Park, IL, USA)
346 and embedded onto charged microscope slides. A single drop of ProLong Gold Antifade
347 Mountant with DAPI (Thermo Fisher Scientific, Emeryville, CA) was used to mount each slide.
348 *ZIKV infection of midguts:* Dissected midguts in 10% formalin were washed twice in PBS.
349 Tissues were submerged in a blocking solution consisting of 1X PBS, 0.2% Triton X-100
350 (PBS+T) (Bio-Rad Laboratories, Hercules, CA), 1% bovine serum albumin (BSA, Sigma-Aldrich,
351 St. Louis, MO), and 10% normal goat serum (Lampire Biological Laboratories, Pipersville, PA)
352 for 10 minutes before staining with a primary monoclonal antibody that reacts with multiple
353 flaviviruses (Flavivirus treatment antigen antibody D1-4G2-4-15, Novus Biologicals, Littleton,
354 CO) at a 1:1000 dilution in blocking solution at 4°C overnight. Midguts were washed five times
355 via direct tube transfer in PBS+T before staining with the secondary antibody (Goat anti-mouse
356 Alexa Fluor 594, Abcam, Cambridge, United Kingdom) at a 1:1000 dilution in blocking solution
357 for 1 hour at room temperature. Midguts were washed 5X in PBS+T before mounting onto
358 microscope slides with ProLong Gold Antifade Mountant with DAPI. ZIKV detection and
359 quantification were done by manually counting distinct red patches, using blue DAPI as a
360 reference for individual cells across three slices of a single midgut, and averaging the values.

361 FITC, DAPI, and Alexa Fluor 594 were each visualized (and FITC was quantified) in their
362 respective channels before merging.
363
364 **Histologic analyses**
365 Histological images at 20X and 40X magnification were used to visualize mosquito anatomy and
366 to validate that sample preparation for immunofluorescence microscopy did not mechanically
367 disrupt the midgut, which could affect interpretations of midgut integrity. Blood-engorged
368 mosquitoes with their legs and wings removed were placed in 10% formalin in PBS for 24 h,
369 then encased within tissue cassettes (Thermo Fisher Scientific, Emeryville, CA) submerged in
370 70% ethanol. Samples were submitted to the UC Davis School of Veterinary Medicine Anatomic
371 Pathology Service, where whole mosquitoes were paraffin-embedded, cut into 5 μm slices, and
372 stained with hematoxylin and eosin (H&E). Slides were visualized at 20X and 40X magnification
373 under a Zeiss Axio Vert A1 inverted light microscope connected to an Axiocam 208 color
374 camera (Carl Zeiss, Jena, Germany). Although we evaluated sections from 5 individual
375 mosquitoes each for No Abx, AbxA, and AbxPA, groups, we used histology readouts as a
376 qualitative approach to evaluate gross midgut anatomy. Anatomical structures were identified by
377 an expert in mosquito anatomy. Representative images for each of the three groups are shown.
378
379 **Blood digestion analyses**
380 Quantification of hemoglobin in bloodfed mosquitoes with a hemoglobin colorimetric detection
381 kit (Thermo Fisher Scientific, Emeryville, CA) was used to measure blood digestion. The blood
382 only and ZIKV-spiked bloodmeals each included 3 ml of blood. An additional 150 ul ZIKV was
383 added to the ZIKV-spiked bloodmeal, such that the volume of virus was 5% of the total
384 bloodmeal volume. At 0, 1, 2, or 3 dpf, 10-12 individual mosquitoes each in No Abx, AbxA, and
385 AbxPA, groups exposed to blood only or ZIKV-spiked blood with their legs/wings removed were
386 homogenized in 300 μL hemoglobin diluent, 100 μL of which was used for the assay, per

387 manufacturer's instructions. Absorbance at 570 nm was measured from each sample once in
388 96-well plates using a SpectraMax iD5 Multimode Microplate Reader (Molecular Devices, San
389 Jose, CA). Absorbance values were converted to hemoglobin concentrations via standard
390 curves generated from hemoglobin standards and serial dilutions of fresh sheep blood used for
391 feeding. Output absorbance values were normalized to homogenized, non-bloodfed mosquito
392 negative controls, and units were converted to µg per mosquito body. Before conducting the
393 assay, we validated and optimized this kit for bloodfed mosquitoes, including ensuring that heat
394 incubation of mosquitoes at 60°C for 10 minutes to destroy ZIKV infectivity did not affect
395 hemoglobin quantification (**Figure S1**).**RNA sequencing and bioinformatics**
396 Mosquito messenger RNA was sequenced to determine differences in gene expression
397 associated with antibiotic treatments after ZIKV exposure. Total RNA was extracted from adult
398 female mosquitoes in triplicate pools of 5 individuals each for No Abx, AbxA, and AbxPA groups
399 using the Quick-RNA Tissue/Insect Microprep kit (Zymo Research, Irvine, CA, USA). RNA
400 extracts were eluted into 20 µL elution buffer and quantified using a Qubit fluorometer RNA
401 high-sensitivity (HS) assay (Thermo Fisher Scientific, Emeryville, CA). Approximately 1 µg of
402 total RNA per sample was submitted to Genewiz Inc. from Azenta Life Sciences (Chelmsford,
403 MA, USA), where poly-A selection, library preparation, and paired-end Illumina HiSeq 2x150 bp
404 were performed. Raw FASTQ data files were received and treated by filtering and trimming
405 reads with *cutadapt v3.2* (40) using default parameters (Quality cutoff >= 30, minimum length of
406 100 bp). Next, *FastQC* (41) was used to visualize quality scores. Processed reads were then
407 aligned to version AaegL5.2 of an *Ae. aegypti* transcriptome obtained from VectorBase (42)
408 using *bowtie2 v2.4.3* (43). Aligned reads were imported into R and analyzed with the package
409 Differential Expression Analysis using *DESeq2 v1.28.1* (44). Default parameters were used to
410 determine the differential abundance of each gene using an input experimental design of
411 [antibiotic treatment + bloodmeal status + interaction]. Genes with a significance (adjusted P

412 value) below 0.05 are reported. Significant genes were mapped to biological processes using
413 the Gene Ontology program *topGO* (45).
414
415 **Statistical analyses**
416 The 16S:RPS17 ratios to verify antibiotic efficacy from qPCR were compared using a Kruskal-
417 Wallis test with multiple comparisons for adult mosquito treatments and across life stages.
418 Differences in ZIKV infection, dissemination, and transmission rates between groups were
419 determined using Fisher's exact tests. Differences in ZIKV RNA levels in bodies, legs/wings,
420 and saliva across groups were assessed by Kolmogorov-Smirnov tests. Differences in ASV and
421 Evenness were evaluated using unpaired t tests. Differences in hemoglobin levels across
422 groups were evaluated using Kruskal-Wallis tests with repeated measures. Relative
423 fluorescence values and numbers of nuclei were averaged in triplicate and tested for
424 significance by one-way ANOVA tests. Differences in numbers of ZIKV-infected midgut
425 epithelial cells were assessed using Fisher's exact tests. For RNA-Seq, statistical tests built into
426 the *DESeq2* package were used. In short, each sample was normalized by library size, and the
427 geometric mean for each gene was calculated. Negative binomial generalized linear models
428 (glm) were used for each gene to determine differential expression across antibiotic-treated
429 groups. The Wald test was used for statistical significance, with correction for the false
430 discovery rate (FDR) set to 0.05. For principal components analyses (PCA), variance stabilized
431 transformation was implemented before plotting. All statistical analyses, except RNA-Seq, were
432 performed using GraphPad PRISM 9.0.2 (GraphPad Software, San Diego, CA). RNA-Seq
433 analyses were conducted in R *v3.6* (46). P-values of less than 0.05 were considered statistically
434 significant. For gene ontology (GO), Fisher's exact tests were used on GO terms and corrected
435 for the false discovery rate.
436

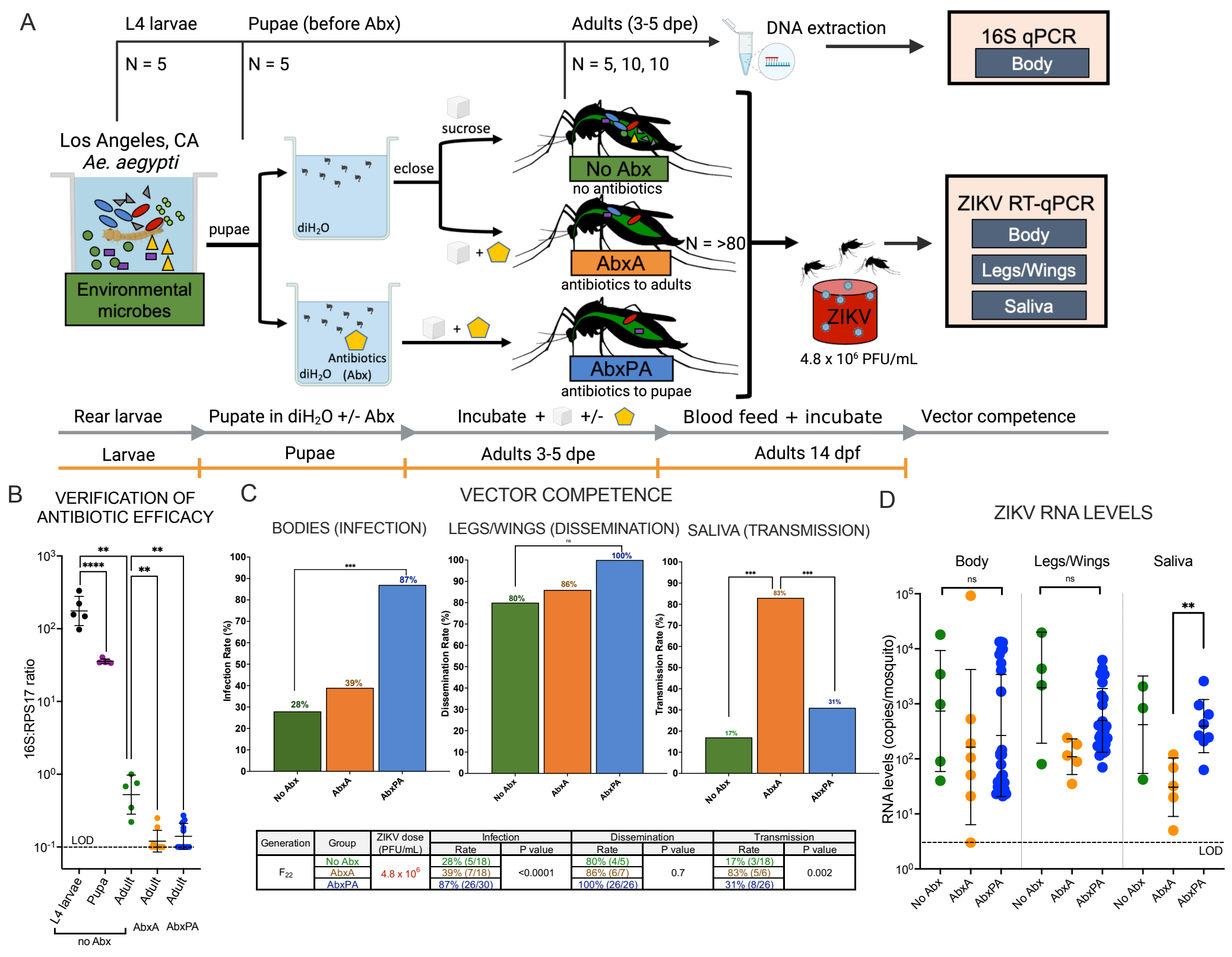
437 **Data availability.** Raw sequencing data are available from the NCBI Sequence Read Archive
438 under BioProject entry PRJNA818687. Scripts for the RNA-Seq analysis are available on Github
439 (DOI: 10.5281/zenodo.7259822).
440

441 **Results**

442 **Reduced *Ae. aegypti* susceptibility to ZIKV conferred by microbial exposure is life stage-**
443 **dependent**

444 In our previous study, we observed that adult *Ae. aegypti* reared in environmental water as
445 larvae were less competent ZIKV vectors, assessed as lower infection and transmission rates,
446 compared to *Ae. aegypti* reared in laboratory tap water (15). In this study, to understand
447 whether reduced ZIKV vector competence is conferred transstadially from larvae to pupae to
448 adults, we first determined whether exposure to microbes throughout life (i.e. at larval, pupal,
449 and adult stages) is required to reduce ZIKV susceptibility. *Ae. aegypti* larvae were reared in
450 laboratory water spiked with microbes that were collected from water in cemeteries that we
451 characterized in a previous study (15) which contain >1000 bacterial species from the phyla
452 *Bacteroides*, *Firmicutes*, and *Proteobacteria*. Control mosquitoes were not provided antibiotics
453 (No Abx) at any life stage. To assess the effects of microbe modulation at pupal and adult life
454 stages, cohorts of **p**upae and the **a**dults they developed into were administered an antibiotic
455 (Abx) cocktail (AbxPA) that was provided to pupae in water and orally in sucrose to adults from
456 eclosion to 3 days of age (**Figure 1A**). To study the effects of microbe modulation on adult
457 mosquitoes that were not antibiotic treated at earlier life stages, we also included cohorts where
458 **a**dults were provided antibiotics (AbxA) orally for 3-5 days post-eclosion. Antibiotics significantly
459 reduced the number of prokaryotes in Abx treated adults as assessed by 16S:RPS17 ratios, our
460 proxy for prokaryote levels (Kruskal-Wallis [K-W] with multiple comparisons, P = 0.002 between
461 No Abx and AbxA [adj. P = 0.002] and No Abx and AbxPA [adj. P = 0.006]) (**Figure 1B**).
462 Significantly higher 16S:RPS17 ratios were also observed in No Abx L4 larvae and pupae

463 relative to adult mosquitoes (K-W multiple comparisons, P = <0.0001 between No Abx and larva
464 [adj. P = 0.0012] and No Abx and pupa [adj. P = 0.0079]). This is consistent with ours (15) and
465 others' (7,13) observations that microbial abundance decreases during mosquito development.
466 Mosquitoes were orally presented with ZIKV-spiked bloodmeals for vector competence
467 assessments. AbxPA showed significantly increased ZIKV infection rates compared to No Abx
468 (Fisher's exact test, P < 0.0001) while AbxA and No Abx infection rates were not different
469 (Fisher's exact test: P = >0.05). Dissemination rates were not significantly different across
470 treatments (Fisher's exact test: P = >0.05). Significantly more AbxA mosquitoes transmitted
471 compared to No Abx or AbxPA (Fisher's exact test: P < 0.002) (**Figure 1C**). Levels of ZIKV RNA
472 in saliva were higher in AbxPA compared to AbxA (Kolmogorov-Smirnov (KS) test, P = 0.009),
473 but not higher than No Abx (KS test, P = 0.71) (**Figure 1D**). Collectively, these results show that
474 microbe disruption across *Ae. aegypti* life stages increases rates of ZIKV infection (AbxPA),
475 transmission (AbxA) and the magnitude of ZIKV transmitted in saliva (AbxPA).
476
477 **Figure 1. Microbial disruption using antibiotic treatment of *Ae. aegypti* pupae and adults from**
478 **Los Angeles, California, increases ZIKV susceptibility. A) Experimental design**. Microbes from
479 cemetery headstones were added to tap water during rearing of *Ae. aegypti* larvae from Los Angeles
480 (LA), CA. Pupae in the antibiotic (Abx) groups were treated by adding an antibiotic cocktail to pupal
481 water and orally in sugar to adults (AbxPA). Antibiotics were delivered orally in sugar to adult Abx
482 (AbxA) groups. No antibiotics (No Abx) groups received no antibiotics at any life stage. Mosquitoes
483 from each group and some life stages were sequenced to verify the efficacy of antibiotic treatment.
484 Adult mosquitoes 3-5 days of age were presented with ZIKV spiked into bloodmeals, and ZIKV
485 vector competence was assessed by measuring ZIKV RNA levels in bodies to determine infection,
486 legs/wings of individuals with infected bodies to assess dissemination, and in saliva of individuals
487 with infected legs/wing to measure transmission, which are presented as rates in cohorts and mean
488 levels from individuals. **B) Antibiotic treatment reduces bacterial DNA in mosquitoes.** Bacteria



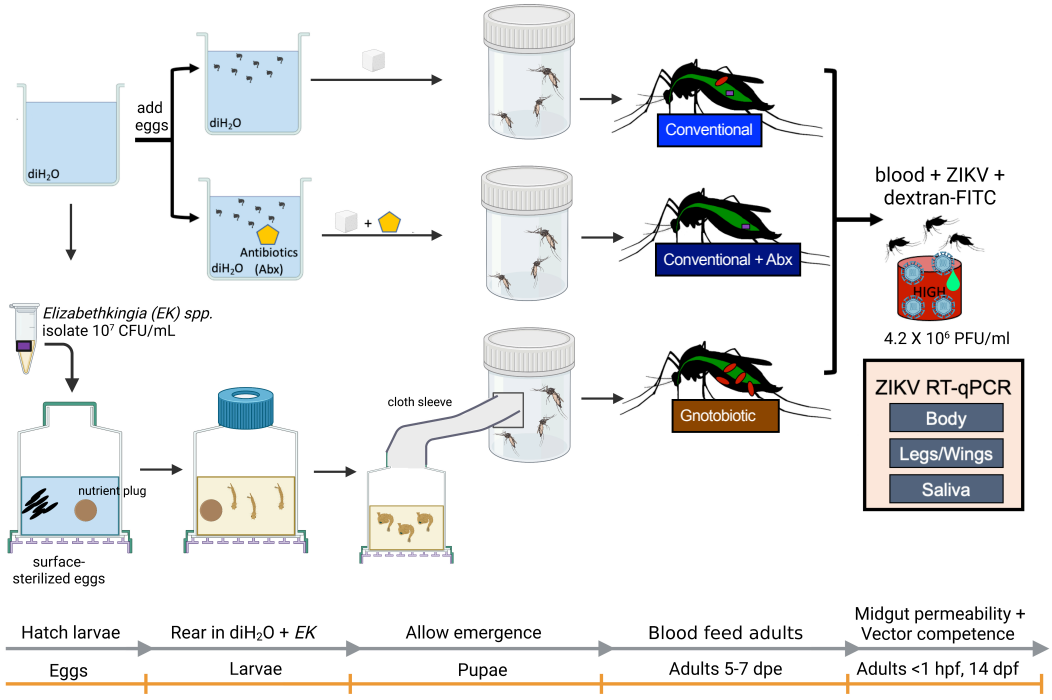

| Generation | Group | ZIKV dose (PFU/mL) | Infection | | Dissemination | | Transmission | |
|---|---|---|---|---|---|---|---|---|
| | | | Rate | P value | Rate | P value | Rate | P value |
| $F_{22}$ | No Abx | $4.8 \times 10^6$ | 28% (5/18) | <0.0001 | 80% (4/5) | 0.7 | 17% (3/18) | 0.002 |
| | AbxA | | 39% (7/18) | | 86% (6/7) | | 83% (5/6) | |
| | AbxPA | | 87% (26/30) | | 100% (26/26) | | 31% (8/26) | |

489 was quantified in mosquitoes by 16S qPCR. Each point represents a single mosquito (N larvae,
490 pupae, No Abx = 5; N AbxA, AbxPA = 10). 16S copies were normalized to the *Ae. aegypti* RPS17
491 gene. The limit of detection (LOD) was a 0.1 16S:RPS17 ratio; below this value, 16S was not
492 detected. Statistical significance across all groups was determined by a Kruskal-Wallis test with
493 multiple comparisons. **C) ZIKV vector competence in microbe-modulated mosquitoes.** Bar
494 graphs show ZIKV infection, dissemination, and transmission rates from cohorts as determined by
495 ZIKV RNA RT-qPCR. The summary table shows the same data in the bar graphs. Rates are
496 calculated as the number of bodies, legs/wings, or expectorates that yielded at detectable ZIKV
497 RNA, divided by the total number of individuals that ingested blood within a cohort, were infected, or
498 developed disseminated infections, respectively. Statistical significance across all treatments was
499 determined by Fisher's exact tests. Vector competence experiments were conducted once where
500 mosquitoes in different cohorts were presented the same bloodmeal. **D) ZIKV RNA levels in**
501 **mosquito tissues after ingestion of ZIKV-spiked bloodmeals**. Each dot shows the mean value
502 for an individual mosquito based on triplicate measurements. The average LOD across all
503 experiments was 3 copies per mosquito and is represented as the dotted line. Mosquitoes without
504 detectable ZIKV RNA are not shown. Kolmogorov-Smirnov tests were used to compare mean RNA
505 levels. For B and D geometric means are shown and error bars show standard deviations. *Denotes
506 $P < 0.05$ **denotes $P < 0.01$, *** denotes $P < 0.001$, ns is not significant at $P<0.05$.
507

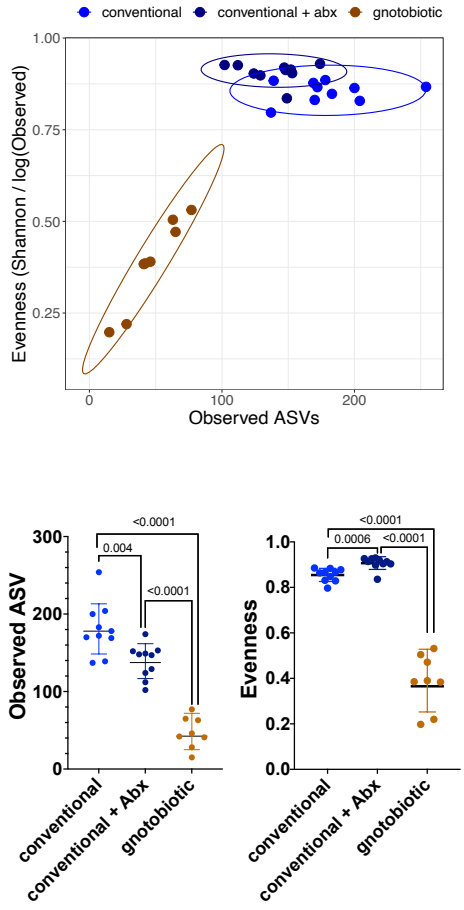
508 **Antibiotics do not directly impact midgut permeability or ZIKV vector competence**

509 Next, we performed experiments to understand whether increased ZIKV vector competence in
510 *Ae. aegypti* is due to direct effects of antibiotics on mosquito physiology or the dysbiotic
511 microbial environment. We compared ZIKV vector competence and midgut integrity in
512 mosquitoes treated with antibiotics (Conventional + Abx) or not antibiotic treated (Conventional),
513 as well as in gnotobiotic mosquitoes (Gnotobiotic) (**Figure 2A**). For the antibiotic treated group,
514 antibiotics were administered to both larval water and adults in sugar water using the same

# A STUDY DESIGN



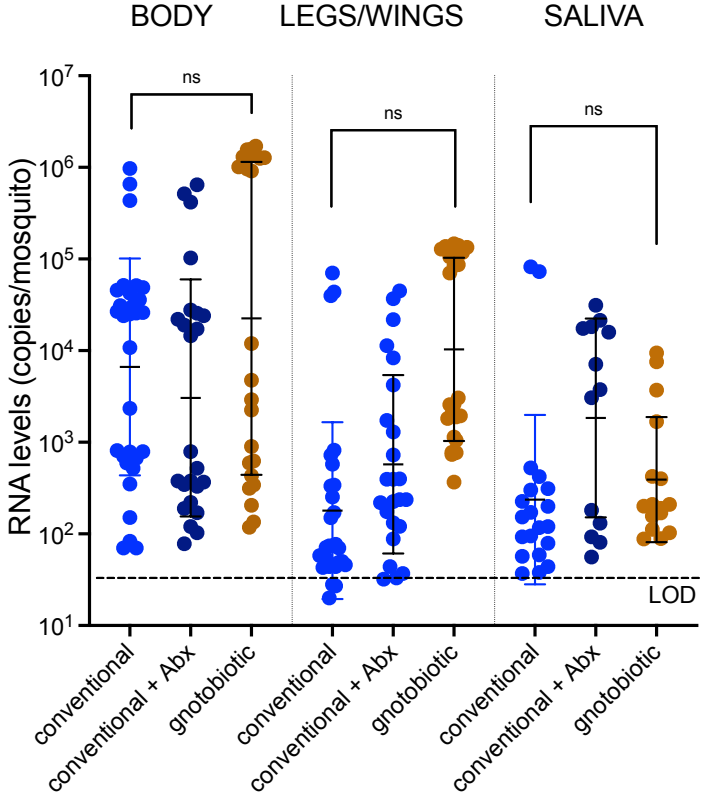

# B VERIFICATION OF TREATMENT STATUS



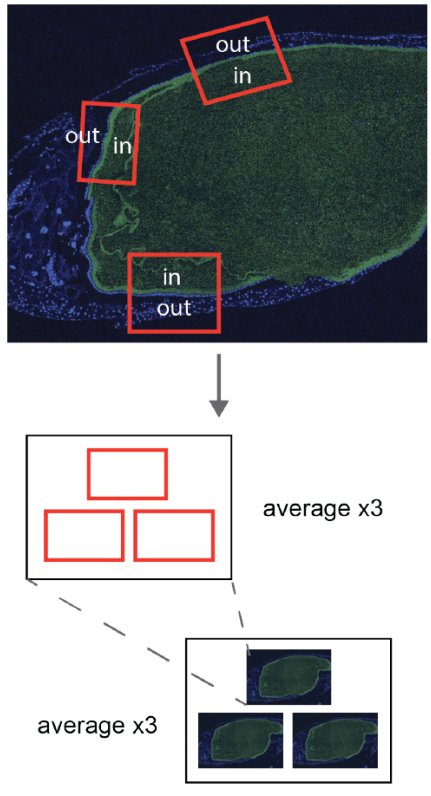

# C VECTOR COMPETENCE

| Generation | Group | ZIKV dose (PFU/mL) | Infection | | Dissemination | | Transmission | |
|---|---|---|---|---|---|---|---|---|
| | | | Rate | P value | Rate | P value | Rate | P value |
| $F_{26}$ | conv. | 4.2 x 10$^6$ | 94% (31/33) | 0.87 | 90% (28/31) | 0.09 | 71% (20/28) | 0.41 |
| | conv. + Abx | | 92% (24/26) | | 100% (24/24) | | 54% (13/24) | |
| | gnotobiotic | | 100% (24/24) | | 100% (24/24) | | 67% (16/24) | |

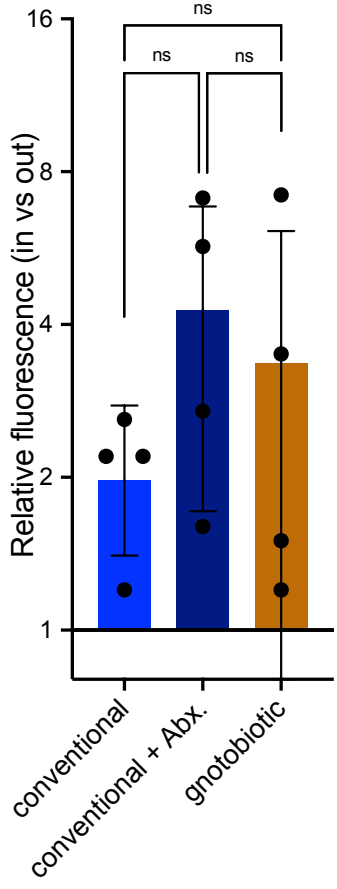
# D MAGNITUDE OF ZIKV INFECTION




# E DEXTRAN QUANTIFICATION



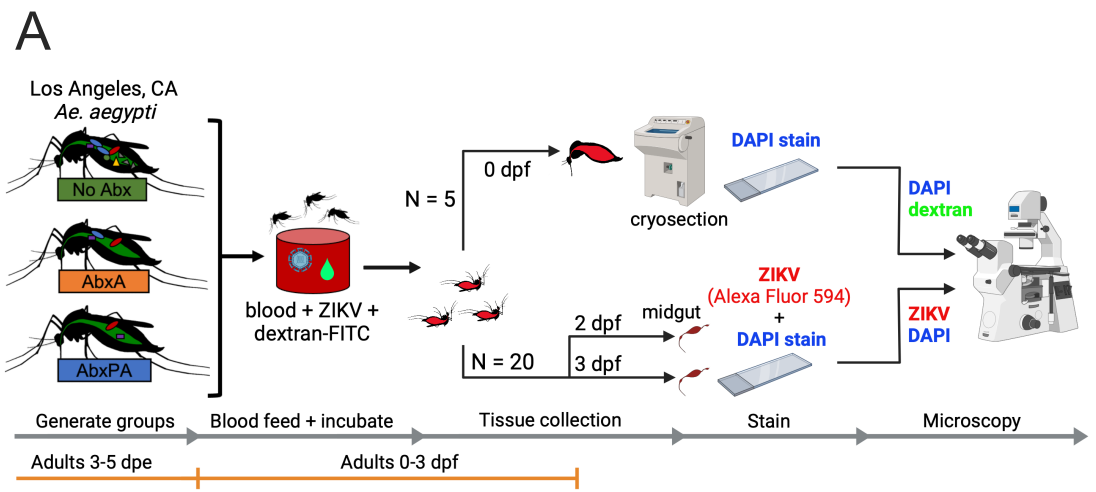

# F DEXTRAN IN VS OUT OF GUT LUMEN

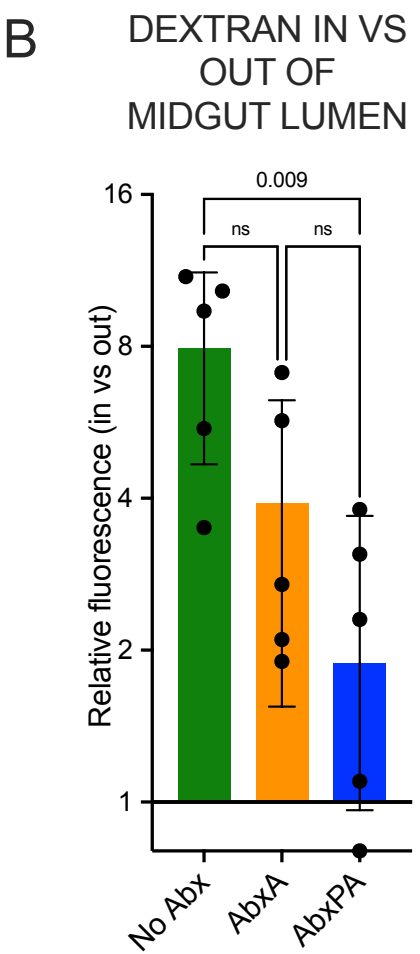


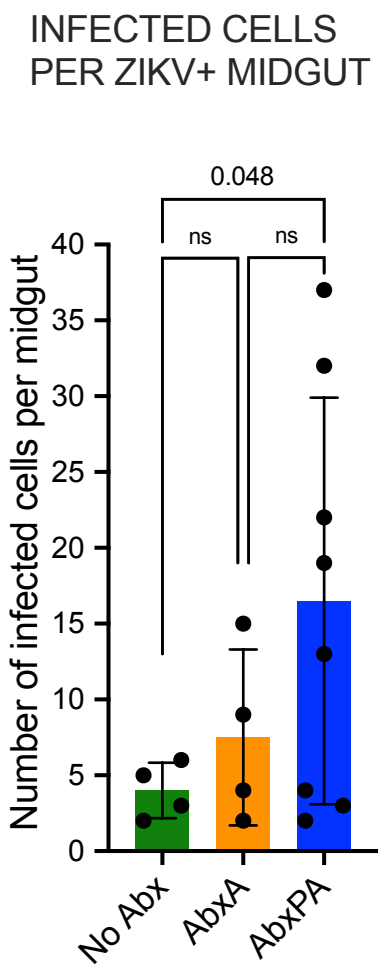
515 design as for the AbxPA cohorts. Gnotobiotic mosquitoes have a known microbiome compared
516 to mosquitoes reared in environmental water, and, unlike axenic mosquitoes that do not
517 possess any microbes, do not suffer from the lack of microbiome-derived essential nutrients that
518 can impact fitness (47). Gnotobiotic *Ae. aegypti* were generated by inoculating larval water with
519 an *Elizabethkingia spp.* culture isolated from colonized *Ae. aegypti* from LA. *Elizabethkingia*
520 *spp*. was selected since it is ubiquitous in the environment (47) and represents a dominant
521 bacterial sequence in our larval and adult *Ae. aegypti* colonies (15). We verified the gnotobiotic
522 status by sequencing (**Figure 2B**). Gnotobiotic mosquitoes showed significantly lower mean
523 observed ASV and evenness (where lower evenness indicates dominance by fewer species)
524 compared to Conventional or Conventional+Abx ($p<0.05$, unpaired t test). A mean of 180 ASVs
525 were detected in Conventional mosquitoes, Conventional +Abx had 139, and Gnotobiotic had
526 47. Conventional mosquitoes reared with or without antibiotics had *Chryseobacteria* as the
527 dominant ASV while in gnotobiotic mosquitoes, *Elizabethkingia* and *Asaia* comprised >95% of
528 all observed ASVs (**Figure S2**). The co-dominance of our intended *Elizabethkingia* inoculum
529 and *Asaia* spp., a commonly found bacterium in our mosquito colony that may be vertically
530 transmitted (15), supports a successful gnotobiotic state. The three groups were presented with
531 high dose ZIKV-spiked blood with and without fluorescent dextran (FITC-dextran). A subset of
532 mosquitoes were evaluated within 1 hour of bloodfeeding for midgut permeability assays and
533 the remaining bloodfed individuals were assessed for vector competence at 14 days. The
534 purpose of adding FITC-dextran, which is a small molecule that can traverse cell membranes,
535 was to evaluate whether antibiotics or a gnotobiotic environment influence midgut permeability.
536 Infection, dissemination, and transmission rates were not statistically different across any group
537 (Fisher's exact test: $P >0.09$) (**Figure 2C**). Mean ZIKV RNA levels in ZIKV-positive bodies,
538 legs/wings, and saliva were also not statistically different across groups (KS test, $P >0.05$)
539 (**Figure 2D**). We visualized and quantified the relative fluorescent intensity of FITC-dextran
540 inside versus outside the midgut (**Figure 2E**), where higher fluorescence inside versus outside

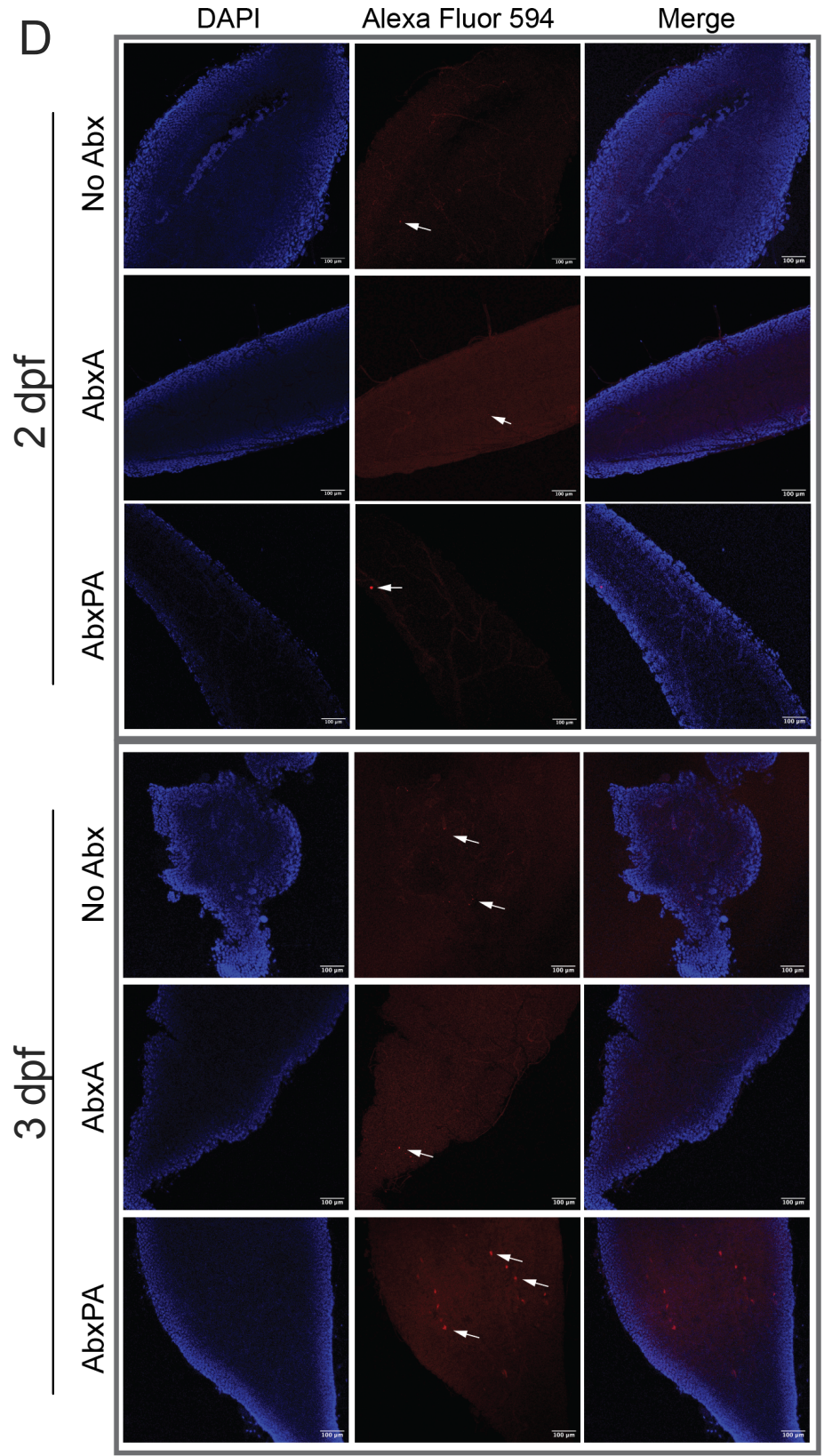
541 is indicative of greater midgut integrity. Dextran permeability measurements showed no
542 significant difference across groups in mean fluorescence inside versus outside of the midgut
543 (KS test, P >0.05) (**Figure 2F**). These data indicate that antibiotics themselves or a gnotobiotic
544 state do not perturb midgut permeability or affect ZIKV vector competence and rule out
545 confounding effects of these treatments on the structural integrity of the mosquito midgut
546 epithelium.
547
548 **Figure 2: A) Experimental design.** *Aedes aegypti* from LA, CA, were reared in laboratory tap water
549 without any antibiotics (Conventional), with antibiotics (Conventional + Abx), where antibiotics were
550 also provided orally to adults in sugar, or with *Elizabethkingia spp*. (Gnotobiotic) bacteria isolated
551 from our *Ae. aegypti* colony. Mosquito eggs in the Gnotobiotic group were surface sterilized before
552 hatching and reared in sterile flasks. Adult mosquitoes 3-5 days of age were presented with ZIKV
553 spiked into bloodmeals, and ZIKV vector competence was assessed by measuring ZIKV RNA levels
554 in bodies to determine infection, legs/wings of individuals with infected bodies to assess
555 dissemination, and in saliva of individuals with infected legs/wing to measure transmission, which
556 are presented as rates in cohorts and mean levels from individuals. The bloodmeals also contained
557 1 µg dextran-FITC (green drop) for midgut permeability assessments. **B) Verification of treatment**
558 **status using 16S sequencing from adult mosquitoes in each treatment group.** The scatterplot
559 shows the relationship between observed ASVs and bacterial species evenness. ASVs with
560 unassigned taxonomy were included. Evenness is calculated by dividing Shannon diversity by the
561 natural log of observed ASVs. Ellipses encompass the 95% confidence interval for samples within
562 the group. Each dot on Observed ASV and Evenness graphs represents an individual mosquito
563 (N=10 Conventional, N=10 Conventional + Abx, N=8 Gnotobiotic). P values reported were
564 determined using unpaired t tests. **C) ZIKV vector competence** data comparing infection,
565 dissemination, or transmission rates in Conventional, Conventional + Abx, and Gnotobiotic *Ae.*
566 *aegypti* groups**.** Vector competence experiments were conducted once where mosquitoes in
567 different cohorts ingested the same bloodmeal. Statistical evaluation across all treatments used

568 Fisher's exact tests. **D) ZIKV RNA levels in mosquito tissues after ingestion of ZIKV-spiked**
569 **bloodmeals**. Each dot shows the mean value for an individual mosquito based on triplicate
570 measurements. The average LOD across all experiments was 62 copies per mosquito and is
571 represented as the dotted line. Mosquitoes without detectable ZIKV RNA are not shown. **E) Dextran**
572 **fluorescence quantification approach** that was used to generate data in panel F. Fluorescence
573 ratios were calculated in triplicate from randomly selected areas in a single midgut slice. Dextran
574 permeation was calculated as the ratio of fluorescence intensity inside the gut lumen to fluorescence
575 intensity outside the gut lumen for randomly selected regions of the midgut (red rectangles). **F)**
576 **Mosquito midgut permeability assessed by relative dextran inside versus outside the midgut**
577 **lumen**. Each symbol represents 9 measurements from one mosquito, N=4 mosquitoes per
578 treatment. For C and E geometric means are shown, and error bars show standard deviations.
579 Kolmogorov-Smirnov tests were used to compare mean RNA or fluorescence levels. ns is not
580 significant at $P<0.05$.
581
582 **Microbe disruption increases *Aedes aegypti* midgut permeability**
583 To understand how microbial exposure of larvae, pupae, and adults reduces susceptibility of *Ae.*
584 *aegypti* to ZIKV, we applied the midgut permeability assays (**Figure 2E**) to evaluate ZIKV-
585 exposed mosquitoes that were microbe-exposed or microbe-disrupted. Our rationale for
586 focusing on the midgut is that it represents the first barrier to infection of *Ae. aegypti* by ZIKV
587 after oral exposure. We asked whether higher infection rates in AbxPA compared to No Abx are
588 explained by reduced midgut integrity resulting from microbe disruption. We compared
589 fluorescent intensity across antibiotic-treated groups immediately after ingestion of a bloodmeal
590 with ZIKV (**Figure 3A**). All mosquitoes had only bloodfed once, eliminating potential
591 confounding effects of repeated bloodfeeding on midgut integrity (48) and were fully engorged;
592 hemoglobin measurements (see below) also revealed no statistically significant differences in
593 mean ingested bloodmeal volumes across groups. The relative dextran-FITC fluorescence


A
Los Angeles, CA
Ae. aegypti
No Abx
AbxA
AbxPA
blood + ZIKV +
dextran-FITC
N = 5
0 dpf
cryosection
DAPI stain
DAPI
dextran
N = 20
2 dpf
3 dpf
midgut
ZIKV
(Alexa Fluor 594)
+
DAPI stain
ZIKV
DAPI
Generate groups
Blood feed + incubate
Tissue collection
Stain
Microscopy
Adults 3-5 dpe
Adults 0-3 dpf


B
DEXTRAN IN VS
OUT OF
MIDGUT LUMEN
0.009
ns
ns
Relative fluorescence (in vs out)
16
8
4
2
1
No Abx
AbxA
AbxPA


C
INFECTED CELLS
PER ZIKV+ MIDGUT
0.048
ns
ns
Number of infected cells per midgut
40
35
30
25
20
15
10
5
0
No Abx
AbxA
AbxPA

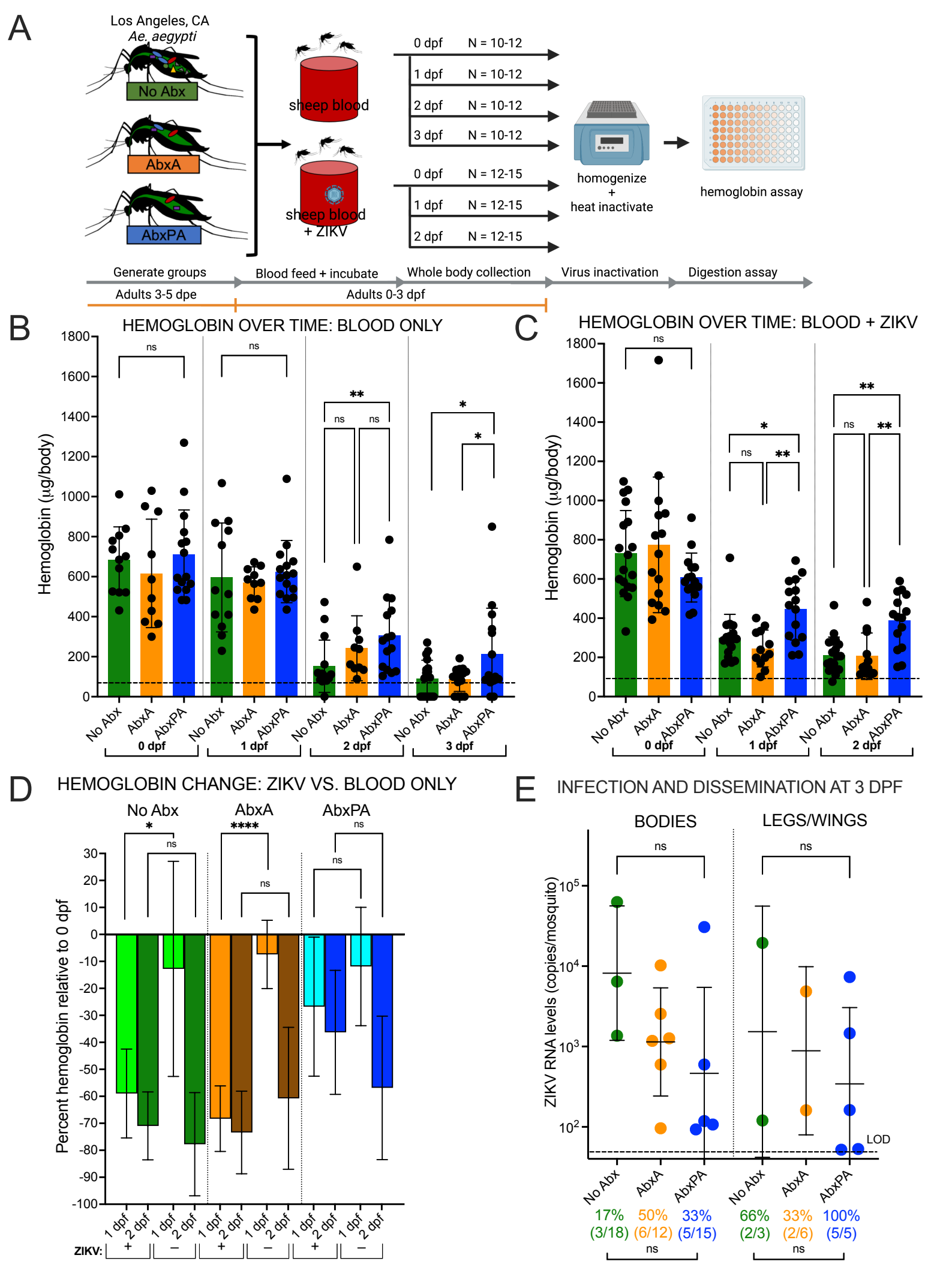

D
DAPI
Alexa Fluor 594
Merge
2 dpf
3 dpf
No Abx
AbxA
AbxPA

594 inside versus outside of the midgut lumen for the AbxPA group was significantly lower than the
595 No Abx group (P = 0.009), and the AbxA group trended lower (although not significantly) than
596 the No Abx group (**Figure 3B**). To rule out physical rupture or other disruptions to the midgut
597 during sample preparation that may have reduced structural integrity, we treated and stained
598 midgut sections from a separate cohort of similarly treated mosquitoes with DAPI or H&E. At
599 10X, 20X, and 40X magnification, midgut epithelia across treatments did not appear structurally
600 different (**Figures 3D**, **S1**). Together these data support a process where disruption of microbial
601 colonization at pupal and adult *Ae. aegypti* life stages increase midgut cell permeability in adult
602 mosquitoes after bloodmeal ingestion.
603
604 **Figure 3. Microbial disruption increases *Aedes aegypti* midgut permeability. A) Schematic of**
605 **bloodfeeding experiment and sample preparation for immunofluorescence microscopy.**
606 *Aedes aegypti* from LA, CA were used to generate three groups of mosquitoes: no antibiotics (No
607 Abx), antibiotics as adults (AbxA), and antibiotics as larvae, pupae, and adults (AbxPA). Adult
608 mosquitoes aged 3-5 days were presented with a bloodmeal containing 1 mg/mL dextran-FITC
609 (green) and 3.3 x $10^6$ PFU/mL ZIKV. Whole mosquitoes were harvested within 1 hour of feeding at 0
610 days post-feed (dpf), cryosectioned, DAPI stained, and examined using fluorescence microscopy.
611 Midguts from the same cohorts were harvested at 2 and 3 dpf, and ZIKV antigen and DAPI were
612 stained before fluorescence microscopy. **B) Microbe disruption of pupae and adults increases**
613 **midgut permeability, evidenced by reduced relative dextran inside versus outside the gut**
614 **lumen in AbxPA compared to No Abx mosquitoes**. Dextran permeation was calculated as the
615 ratio of fluorescence intensity inside the gut lumen to fluorescence intensity outside the gut lumen
616 using the same approach as in Figure 2E. Each symbol represents 9 measurements from one
617 mosquito. **C) Mosquitoes that were antibiotic treated as pupae and adults have more ZIKV-**
618 **infected midgut epithelial cells**. The number of ZIKV-infected cells, as determined by ZIKV antigen
619 staining, were counted for each ZIKV-positive midgut on 2 and 3 dpf. Data from both days is shown.

620 Each symbol represents one mosquito. **D) Antibiotic treated ZIKV-positive midguts at 2 and 3**
621 **dpf.** Midguts examined under immunofluorescence at 10X magnification, at 2 and 3 dpf. Cell nuclei
622 (blue) and ZIKV antigen (red) are shown with the combined (merged) channels. Images reflect
623 representative observations for each antibiotic treatment. Arrows point to ZIKV antigen positive cells.
624 P values in B and C use ANOVA with multiple comparisons and error bars show standard deviations.
625 ns is not significant.
626
627 **Microbe disruption via antibiotic treatment increases ZIKV infection of the midgut**
628 **epithelium**
629 Next, we asked microbe disruption leads to increased midgut infection success, measured as
630 higher numbers of ZIKV-infected midgut epithelial cells. We detected ZIKV in midguts by
631 staining sections with a flavivirus binding antibody 2 or 3 dpf and counting the total number of
632 cells in each midgut that were stained with fluorescent anti-ZIKV antibody (**Figure 3A**). The
633 number of ZIKV-positive cells in midguts was significantly higher in the AbxPA group compared
634 to the No Abx group (Brown-Forsythe ANOVA test with Dunnett's multiple comparisons test, P =
635 0.048) (**Figure 3C**). These data support the role of microbes in larvae and pupae in maintaining
636 the integrity of the mosquito midgut which associates with reduced midgut epithelium infection
637 by ZIKV.
638
639 **Microbial disruption via antibiotic treatment impairs blood digestion**
640 Since midgut integrity can affect physiology of blood digestion in addition to ZIKV susceptibility,
641 we next assessed whether elevated midgut permeability stemming from microbe reduction by
642 antibiotic treatment affects blood digestion. We employed a commercially available hemoglobin
643 colorimetric assay as a proxy for blood protein digestion by quantifying the reduction of
644 hemoglobin levels in blood-engorged mosquitoes on 1 and 2 dpf compared to immediately after
645 feeding on 0 dpf. Before performing the assay, which is typically used for human blood, we first

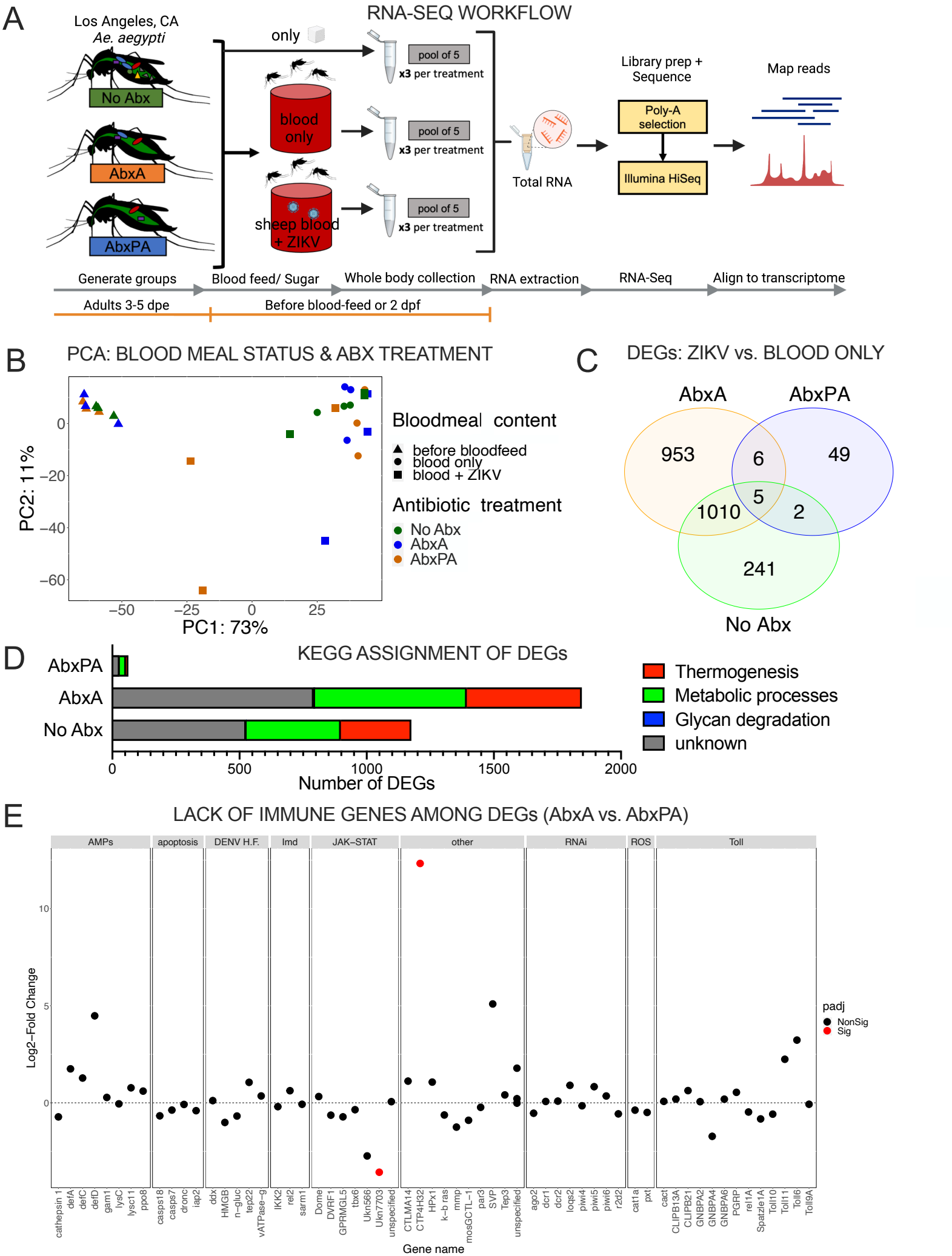
646 validated and optimized the approach for use with homogenized mosquitoes that ingested
647 sheep bloodmeals. We observed that sheep blood absorbance varies linearly as a function of
648 hemoglobin concentration, like the kit standard (**Figure S3A**) and that absorbance decreases
649 reproducibly in serially diluted mosquitoes at both 560 and 580 nm wavelengths. The assay was
650 unaffected by the 10-minute 60°C heat treatment of homogenized fed mosquitoes used to
651 inactivate ZIKV infectivity in the samples (**Figure S3B**). Blood-engorged females were
652 homogenized and assayed for hemoglobin immediately after bloodfeeding at 0 dpf to assess
653 whether different groups ingested the same bloodmeal volume and to measure kinetics of
654 bloodmeal digestion 1, 2, and 3 dpf (**Figure 4A**). Although hemoglobin levels that approximate
655 bloodmeal size varied in individual mosquitoes, there was no significant difference in mean
656 ingested hemoglobin levels on 0 dpf across antibiotic-treated groups regardless of whether the
657 bloodmeal contained ZIKV, which shows that antibiotic treatment did not influence bloodmeal
658 size (KW test with multiple comparisons, P = 0.522) (**Figure 4B, C**). After ingestion of blood
659 without ZIKV, mean hemoglobin levels in the AbxPA group were significantly higher than in the
660 No Abx group 2 dpf (KW test, P = 0.0063). By 3 dpf, mean hemoglobin levels in the AbxPA
661 group were significantly higher than both AbxA and No Abx groups (KW test with multiple
662 comparisons, adjusted $P_{AbxPA\text{-}No\ Abx}$ = 0.0347, adjusted $P_{AbxPA\text{-}AbxA}$ = 0.051) (**Figure 4B**). The
663 same pattern was observed after ingestion of blood with ZIKV: AbxPA showed a significantly
664 reduced rate of blood digestion compared to No Abx 1 dpf (KW test with multiple comparisons,
665 adjusted $P_{AbxPA\text{-}No\ Abx}$ = 0.0368, adjusted $P_{AbxPA\text{-}AbxA}$ = 0.0035) and both No Abx and AbxA 2 dpf
666 (KW test with multiple comparisons, adjusted $P_{AbxPA\text{-}No\ Abx}$ = 0.0037, adjusted $P_{AbxPA\text{-}AbxA}$ =
667 0.0027) (**Figure 4C**). Hemoglobin levels at 3 dpf for individuals that ingested blood with ZIKV
668 was not assayed since uneven bloodfeeding rates across cohorts and the desire to maintain
669 statistically robust group sizes led us to prioritize use these mosquitoes for infection and
670 dissemination assessments. We also compared mean hemoglobin levels across treatments and
671 days post-feed for mosquitoes that ingested blood with or without ZIKV. Although levels did not

672 differ significantly after bloodmeal ingestion on 0 dpf, mean hemoglobin digestion was
673 significantly faster in No Abx and AbxA on 1 dpf (KW test, $P_{No\ Abx} = 0.0163$, $P_{AbxA} = 0.0009$) but
674 not AbxPA (KW test, P = 0.25) in ZIKV-exposed mosquitoes compared to mosquitoes that
675 ingested blood without ZIKV (**Figure 4D**). To evaluate whether these observations extend to *Ae.*
676 *aegypti* from a different genetic background than LA, CA, we performed the similar analyses
677 with *Ae. aegypti* from Clovis, CA. Genome sequencing shows that *Ae. aegypti* from LA and
678 Clovis are genetically distinct (49), and were probably derived from separate independent
679 introductions into CA since 2013 when the species became established in the state. Using a
680 similar design (**Figure S4A**), as for mosquitoes from LA, AbxA and AbxPA from Clovis showed
681 significantly lower blood digestion relative to No Abx 2 dpf (KW test with multiple comparisons,
682 adjusted $P_{AbxPA\text{-}No\ Abx} = 0.0013$, adjusted $P_{AbxPA\text{-}AbxA} = 0.027$) (**Figure S4B**). Clovis AbxPA
683 exhibited a marginally but not statistically significantly higher infection rate (34%) compared to
684 AbxA (26%) and No Abx (15%) groups (Chi-square [$\chi^2$] test, P = 0.106). The dissemination
685 rates and mean RNA levels in ZIKV-positive Clovis mosquitoes were not different for any
686 treatment (**Figure S4C**). These data show that disruption of larval and pupal microbes reduces
687 bloodmeal digestion in two genetic lineages of *Ae. aegypti*, and that blood with ZIKV is digested
688 more quickly than blood lacking virus.
689
690 **Figure 4. Bloodmeal digestion kinetics in antibiotic-treated microbe-disrupted *Ae. aegypti*. A)**
691 **Experimental design** for detection of hemoglobin in homogenized antibiotic treated mosquitoes that
692 ingested blood with or without ZIKV**.** *Aedes aegypti* from LA, CA were reared as in Figure 1A to
693 generate three groups of mosquitoes: no antibiotics (No Abx), antibiotics as adults (AbxA), and
694 antibiotics as larvae, pupae, and adults (AbxPA). Adult mosquitoes aged 3-5 days were presented
695 with a bloodmeal with or without 2.1 x $10^6$ PFU/mL ZIKV. On day 0 (within 1 hour of feeding) and at
696 1, 2 and 3 days dpf a subset of 10-15 mosquitoes were homogenized and heat treated to destroy
697 ZIKV infectivity for bloodmeal volume detection using a hemoglobin assay. Infection and


A
Los Angeles, CA
Ae. aegypti
No Abx
AbxA
AbxPA
sheep blood
sheep blood + ZIKV
0 dpf N = 10-12
1 dpf N = 10-12
2 dpf N = 10-12
3 dpf N = 10-12
0 dpf N = 12-15
1 dpf N = 12-15
2 dpf N = 12-15
homogenize + heat inactivate
hemoglobin assay
Generate groups
Blood feed + incubate
Whole body collection
Virus inactivation
Digestion assay
Adults 3-5 dpe
Adults 0-3 dpf
B
HEMOGLOBIN OVER TIME: BLOOD ONLY
Hemoglobin (µg/body)
0 dpf
1 dpf
2 dpf
3 dpf
C
HEMOGLOBIN OVER TIME: BLOOD + ZIKV
D
HEMOGLOBIN CHANGE: ZIKV VS. BLOOD ONLY
Percent hemoglobin relative to 0 dpf
ZIKV:
E
INFECTION AND DISSEMINATION AT 3 DPF
BODIES
LEGS/WINGS
ZIKV RNA levels (copies/mosquito)
LOD
17% (3/18)
50% (6/12)
33% (5/15)
66% (2/3)
33% (2/6)
100% (5/5)

698 dissemination were assessed 3 dpf. **Hemoglobin levels in *Ae. aegypti* as a proxy for bloodmeal**
699 **digestion in groups that were (B) bloodfed or (C) bloodfed with ZIKV.** The number of *Ae.*
700 *aegypti* that ingested blood that were assayed per day is: No Abx = 12, AbxA = 10, AbxPA 15. The
701 number of *Ae. aegypti* that ingested blood spiked with ZIKV that were assayed per day is: No Abx =
702 18, AbxA = 12, AbxPA = 15. **D) Hemoglobin levels in bloodfed versus ZIKV exposed *Ae.***
703 ***aegypti*.** Mosquitoes that ingested ZIKV that were not treated with antibiotics or treated only as
704 adults show increased blood digestion compared to mosquitoes treated with antibiotics as pupae
705 and adults. The bar graphs show the percent reduction in hemoglobin at 1 and 2 dpf compared to
706 the 0 dpf, calculated for all Abx groups and between ZIKV exposed and bloodfed only groups. **E)**
707 **Microbial disruption of pupae and adults does not impact ZIKV dissemination rates 3 dpf.**
708 Legs and wings of individual bloodfed females from the same cohort were assayed by ZIKV RT-
709 qPCR at three dpf. Each symbol in B, C and E shows the mean measurement for 1 mosquito. Bars
710 show mean and error bars indicate the standard deviation. * Denotes $P < 0.05$; ** denotes $P < 0.01$;
711 **** denotes $P<0.0001$, ns is not significantly different.
712
713 **Reduced bloodmeal digestion does not influence ZIKV dissemination from the *Ae.***
714 ***aegypti* midgut**
715 We next hypothesized that, in addition to disrupting midgut cell integrity, antibiotic mediated
716 reduction of microbes in mosquitoes and resulting reduced bloodmeal digestion provides the
717 virus more time to bind and enter midgut epithelial cells, manifest as increased ZIKV infection
718 and dissemination very early (3 dpf) after bloodfeeding. However, the rates and magnitude of
719 ZIKV RNA in bodies and legs/wings in antibiotic-treated groups was not different from the
720 mosquitoes that were not antibiotic treated that ingested ZIKV-spiked blood 3 days earlier
721 (Fisher's exact test, $P > 0.05$) (**Figure 4E**). Together with the midgut cell infectivity data, these
722 results suggest that increased ZIKV midgut cell infection observed in antibiotic treated pupae

723 and adults establishes after 3 dpf and that augmented rates of infection in microbe-disrupted
724 mosquitoes do not augment early dissemination.
725
726 **Hemolytic bacteria do not augment bloodmeal digestion in *Ae. aegypti* that were not**
727 **microbe modulated via antibiotic treatment**
728 To determine whether bacteria with hemolytic activity contribute to enhanced bloodmeal
729 digestion in the No Abx groups, bacteria from 5 No Abx treated adult mosquitoes were cultured
730 on LB and R2A agar and then Sanger sequenced. Although different species of bacteria were
731 detected, none showed hemolytic activity (**Table S1**), suggesting that bloodmeal digestion is not
732 directly mediated by bacteria in the mosquito midgut in the *Ae. aegypti* studied with the assays
733 used here. Together, these data show that microbes enhance the ability of *Ae. aegypti* to digest
734 blood, likely via a mechanism not tied directly to bacteria mediated hemolysis since the
735 culturable bacteria recovered from mosquitoes that were not antibiotic treated were not
736 hemolytic.
737
738 **Blood ingestion and ZIKV exposure influence the *Ae. aegypti* transcriptome**
739 Absent increased dissemination from the midgut, to identify gene expression differences
740 associated with increased ZIKV infection in AbxPA, we employed RNA-Seq analyses on pooled
741 adult female *Ae. aegypti* from LA before bloodfeeding and at 2 dpf (**Figure 5A**). We also
742 analyzed females that ingested blood with our without ZIKV. On average, 94.5% (min: 92.7,
743 max: 95.7%) of reads from all samples aligned at least once to the Liverpool *Ae. aegypti*
744 transcriptome. Mosquitoes that had not bloodfed clustered separately from those that ingested
745 blood with or without ZIKV (**Figure 5B**, **Figure S5A**). Bloodmeal status correlated more strongly
746 with variance (accounting for 84%, $P<0.001$) in the overall transcriptomic profile compared to
747 antibiotic treatment (<5%, $P = 0.83$). ZIKV in the bloodmeal also yielded differentially expressed
748 genes (DEGs) for each antibiotic treated group. Prior to blood with or without ZIKV, 20

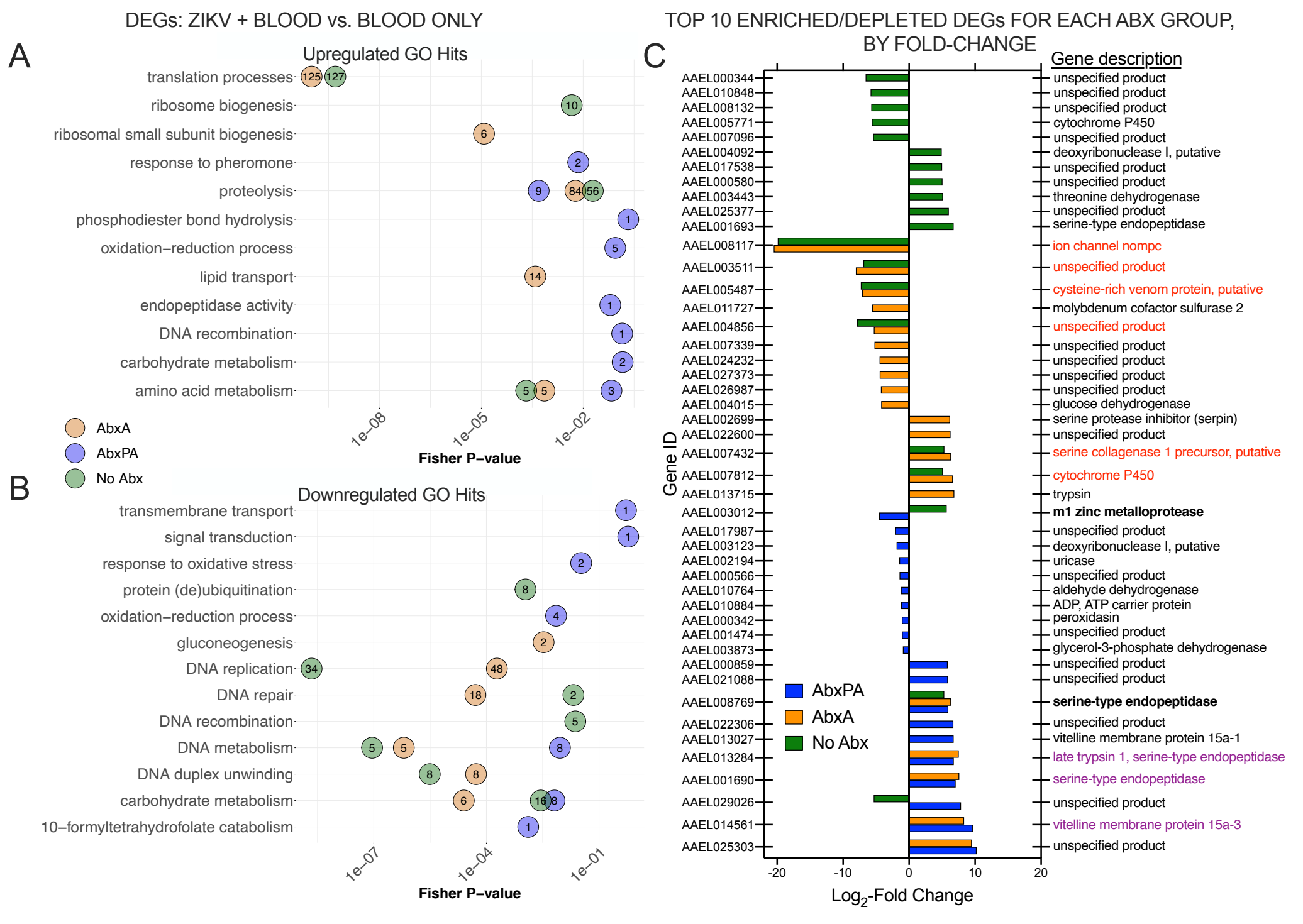

A
RNA-SEQ WORKFLOW
Los Angeles, CA
Ae. aegypti
No Abx
AbxA
AbxPA
only
blood only
sheep blood + ZIKV
pool of 5
x3 per treatment
Total RNA
Library prep + Sequence
Poly-A selection
Illumina HiSeq
Map reads
Generate groups
Blood feed/ Sugar
Whole body collection
RNA extraction
RNA-Seq
Align to transcriptome
Adults 3-5 dpe
Before blood-feed or 2 dpf
B
PCA: BLOOD MEAL STATUS & ABX TREATMENT
PC2: 11%
PC1: 73%
Bloodmeal content
before bloodfeed
blood only
blood + ZIKV
Antibiotic treatment
No Abx
AbxA
AbxPA
C
DEGs: ZIKV vs. BLOOD ONLY
AbxA
AbxPA
No Abx
953
6
49
5
1010
2
241
D
KEGG ASSIGNMENT OF DEGs
AbxPA
AbxA
No Abx
Number of DEGs
Thermogenesis
Metabolic processes
Glycan degradation
unknown
E
LACK OF IMMUNE GENES AMONG DEGs (AbxA vs. AbxPA)
AMPs
apoptosis
DENV H.F.
Imd
JAK–STAT
other
RNAi
ROS
Toll
Log2–Fold Change
padj
NonSig
Sig
cathepsin 1
defA
defC
defD
gam1
lysC
lysc11
ppo8
casps18
casps7
dronc
iap2
ddx
HMGB
n–gluc
tep22
vATPase–g
IKK2
rel2
sarm1
Dome
DVRF1
GPRMGL5
tbx6
Ukn566
Ukn7703
unspecified
CTLMA14
CTP4H32
HPx1
k–b ras
mmp
mosGCTL–1
par3
SVP
Tep3
unspecified
ago2
dcr1
dcr2
loqs2
piwi4
piwi5
piwi6
r2d2
cat1a
pxt
cact
CLIPB13A
CLIPB21
GNBPA2
GNBPA4
GNBPA6
PGRP
rel1A
Spatzle1A
Toll10
Toll11
Toll6
Toll9A
Gene name

749 significant DEGs were identified between AbxPA and No Abx (**Table S2**): the most
750 downregulated in AbxPA relative to No Abx was uncharacterized and other downregulated
751 genes in AbxPA included membrane components and ATP binding proteins. After bloodfeeding,
752 over a third of genes in the *Ae. aegypti* genome were differentially expressed, consistent with
753 other RNA-Seq studies (50). At 2 dpf, 7846 genes in No Abx, 9356 genes in AbxA, and 7846
754 genes in AbxPA were differentially expressed compared to sugar-fed only mosquitoes. Among
755 them, 6676 genes were shared among No Abx, AbxA, and AbxPA, representing an
756 overwhelming majority of the total DEGs (**Figure S5B**). Between bloodfed without and with
757 ZIKV, DEG differences were more apparent: AbxA had 1974 DEG, and No Abx had 1258, and
758 AbxPA had 62 (**Figure 5C**, only DEGs that differed significantly across antibiotic treatments are
759 shown [adjusted $P < 0.05$ by Wald test]). Most shared DEGs were between AbxA and No Abx
760 groups; AbxPA expressed a unique set of DEGs. Assignment of Kyoto Encyclopedia of Genes
761 and Genomes (KEGG) pathway identity (IDs) to DEGs identified many unknown genes.
762 Hypothetical protein products with no homology to genes with functional annotations were
763 common (No Abx: 526, AbxA: 792, AbxPA: 29), as were genes involved in metabolic processes
764 (No Abx: 371, AbxA: 599, AbxPA: 25) and thermogenesis (No Abx: 277, AbxA: 452, AbxPA: 9)
765 (**Figure 5D**). In ZIKV bloodfed mosquitoes, AbxPA reduced differential gene expression more
766 than AbxA or NoAbx. Taken together, these data suggest that bloodfeeding has a larger effect
767 than microbial exposure on *Ae. aegypti* gene expression and implicate microbe-mediated
768 effects on factors other than changes in transcription as modifiers of ZIKV vector competence.
769

770 **Figure 5. Differentially expressed genes between antibiotic-treated adult *Ae. aegypti* females**
771 **that had or had not taken a bloodmeal. A) Experimental design.** For each antibiotic treatment
772 and bloodfeeding state, triplicate pools of 5 whole, legless mosquito bodies were RNA-extracted,
773 pre-processed, and sequenced. **B) Transcriptome profile differs more by bloodmeal status than**
774 **by antibiotic treatment.** PCA plot after variance stabilized transformation. Shapes indicate the

775 bloodmeal status and colors represent antibiotic treatments. Separate PCAs for individual variables,
776 are shown in **Figure S5**. **C) ZIKV-responsive DEGs are shared between *Ae. aegypti* that were**
777 **not antibiotic-treated or antibiotic-treated as adults.** Venn diagram of DEGs in non-bloodfed and
778 bloodfed *Ae. aegypti* for each treatment. **D) DEGs correspond to metabolic processes,**
779 **thermogenesis, and unknown genes.** KEGG Pathway ID assignment of DEGs in panel C. **E)**
780 **DEGs between *Ae. aegypti* mosquitoes treated with antibiotics as pupae and adults**
781 **compared to adults only and between mosquitoes that ingested ZIKV in blood or blood only**
782 **do not correspond to known immune response genes.** Relative expression of select immune-
783 related genes from literature (51–66). Expression is higher in AbxA if the $\log_2$-fold change is positive,
784 while expression is higher in AbxPA if $\log_2$-fold change is negative. Points are considered significant
785 (red) if the adjusted P value is < 0.01. Comparisons of the same genes between AbxA or AbxPA and
786 the control treatment, No Abx, are shown in **Figure S6**.
787
788 Provided that microbes mediate immunomodulatory effects in mosquitoes (13,67,68), we
789 theorized their presence could influence ZIKV susceptibility by modifying the expression of
790 immune response pathways where some respond to both bacteria and viruses. We identified
791 immunity-related DEGs across antibiotic-treated mosquitoes 2 dpf in the RNA-Seq data.
792 Transcripts representing components of various immune pathways were accessioned from
793 existing literature and used to filter the dataset before analyses of DEG profiles. Representative
794 genes examined include regulators from Toll (51–53), RNAi (54–57), JAK-STAT (52,58,59), and
795 Imd pathways (60,69), as well as general immune and immune-adjacent genes such as
796 hormones (61–63,70), antimicrobial peptides (AMPs) (59,64), reactive oxygen species (ROS)
797 (65,66), and known DENV host factors (59,64). There were few DEGs demonstrating significant
798 changes in transcript abundance in immune pathways between AbxA and AbxPA, No Abx and
799 AbxA, or No Abx and AbxPA groups (**Figures 5E, S5**). Comparison of immune genes transcript
800 abundance differences between No Abx and AbxA and between No Abx and AbxPA revealed

801 several JAK-STAT pathway genes (**Figure S6**). These data suggest that gene expression
802 changes after microbe disruption via antibiotic treatment of *Ae. aegypti* that show different
803 susceptibility to ZIKV infection are not related to known immune response pathways.
804
805 **Gene ontology assignments reveal the role of microbiota in stimulating expression of**
806 **protein digestion-associated genes in *Ae. aegypti* orally exposed to ZIKV**
807 Given that RNA-Seq revealed no major differences in expression of immune response genes
808 after microbe disruption, we next performed Gene Ontology (GO) analyses to predict enriched
809 functions in non-immune associated DEGs. We identified DEGs displaying significant changes
810 in abundance between antibiotic treated mosquitoes that ingested ZIKV in blood or that
811 ingested blood only. The AbxA and No Abx groups that ingested ZIKV in blood showed
812 increased expression of genes enriched in general translation processes (AbxA: 125, adjusted
813 $P < 10^{-10}$; No Abx: 127, adjusted $P < 10^{-20}$) and proteolysis (AbxA: 84, adjusted P = 0.0006; No
814 Abx: 56, adjusted P = 0.015). The AbxPA group showed few upregulated genes resulting in a
815 lack of enriched functions (**Figure 6A**). Conversely, enrichments in genes downregulated in
816 mosquitoes that ingested ZIKV in blood versus mosquitoes that ingested blood showed reduced
817 transcript abundance for genes associated with DNA metabolism (8) and redox processes (4) in
818 the ZIKV exposed AbxPA mosquitoes. AbxA and No Abx were depleted in genes for DNA
819 replication (N=48 and 34, respectively) and DNA repair (N=18 and 2, respectively). All treatment
820 groups that ingested blood with ZIKV compared to blood only exhibited downregulation of
821 carbohydrate metabolism genes (AbxPA: N=8, adjusted P = 0.006; AbxA: N=6, adjusted P =
822 0.0002; No Abx: N=16, adjusted P = 0.017) (**Figure 6B**). These data suggest that microbiota
823 during early ZIKV infection promote expression of genes associated with proteolysis and
824 translation while downregulating genes relating to DNA synthesis. The magnitude of change in
825 DEGs in antibiotic treated mosquitoes that ingested ZIKV in blood or blood only were also
826 compared. Gene AAEL008117, the most depleted transcript in AbxA and No Abx groups (~20


DEGs: ZIKV + BLOOD vs. BLOOD ONLY
A
Upregulated GO Hits
translation processes
ribosome biogenesis
ribosomal small subunit biogenesis
response to pheromone
proteolysis
phosphodiester bond hydrolysis
oxidation−reduction process
lipid transport
endopeptidase activity
DNA recombination
carbohydrate metabolism
amino acid metabolism
1e−08
1e−05
1e−02
Fisher P−value
AbxA
AbxPA
No Abx
B
Downregulated GO Hits
transmembrane transport
signal transduction
response to oxidative stress
protein (de)ubiquitination
oxidation−reduction process
gluconeogenesis
DNA replication
DNA repair
DNA recombination
DNA metabolism
DNA duplex unwinding
carbohydrate metabolism
10−formyltetrahydrofolate catabolism
1e−07
1e−04
1e−01
Fisher P−value
TOP 10 ENRICHED/DEPLETED DEGs FOR EACH ABX GROUP, BY FOLD-CHANGE
C
Gene description
Gene ID
AAEL000344 unspecified product
AAEL010848 unspecified product
AAEL008132 unspecified product
AAEL005771 cytochrome P450
AAEL007096 unspecified product
AAEL004092 deoxyribonuclease I, putative
AAEL017538 unspecified product
AAEL000580 unspecified product
AAEL003443 threonine dehydrogenase
AAEL025377 unspecified product
AAEL001693 serine-type endopeptidase
AAEL008117 ion channel nompc
AAEL003511 unspecified product
AAEL005487 cysteine-rich venom protein, putative
AAEL011727 molybdenum cofactor sulfurase 2
AAEL004856 unspecified product
AAEL007339 unspecified product
AAEL024232 unspecified product
AAEL027373 unspecified product
AAEL026987 unspecified product
AAEL004015 glucose dehydrogenase
AAEL002699 serine protease inhibitor (serpin)
AAEL022600 unspecified product
AAEL007432 serine collagenase 1 precursor, putative
AAEL007812 cytochrome P450
AAEL013715 trypsin
AAEL003012 m1 zinc metalloprotease
AAEL017987 unspecified product
AAEL003123 deoxyribonuclease I, putative
AAEL002194 uricase
AAEL000566 unspecified product
AAEL010764 aldehyde dehydrogenase
AAEL010884 ADP, ATP carrier protein
AAEL000342 peroxidasin
AAEL001474 unspecified product
AAEL003873 glycerol-3-phosphate dehydrogenase
AAEL000859 unspecified product
AAEL021088 unspecified product
AAEL008769 serine-type endopeptidase
AAEL022306 unspecified product
AAEL013027 vitelline membrane protein 15a-1
AAEL013284 late trypsin 1, serine-type endopeptidase
AAEL001690 serine-type endopeptidase
AAEL029026 unspecified product
AAEL014561 vitelline membrane protein 15a-3
AAEL025303 unspecified product
AbxPA
AbxA
No Abx
-20
-10
0
10
20
Log2-Fold Change

827 $log_2$-fold reduced), corresponds to nompC, a mechanosensitive ion channel that senses balance
828 and touch in *Drosophila melanogaster* (71). The most common DEGs that changed in response
829 to ZIKV exposure matched to proteolytic and PM-associated enzymes, including
830 endopeptidases, collagenases, and dehydrogenases (72). Multiple serine-type endopeptidases
831 were differentially expressed depending on antibiotic treatment (**Figure 6C**). In addition, some
832 vitellogenesis associated proteins were more enriched in antibiotic treated mosquitoes
833 compared to the No Abx group. In AbxPA, the gene demonstrating the biggest reduction in
834 transcript abundance corresponds to a m1 zinc metalloprotease, showed increased expression
835 in the No Abx group. Overall, GO analyses revealed *Ae. aegypti* with disrupted microbiota are
836 deficient in genes that influence blood protein metabolism.
837
838 **Figure 6. Gene Ontology assignment of DEGs and identity of most enriched/depleted genes**
839 **in *Ae. aegypti* that were** antibiotic treated and that ingested ZIKV in bloodmeals or blood only. **A)**
840 **Upregulated GO hits for mosquitoes treated with antibiotics as adults or not antibiotic treated**
841 **map to general translational processes and proteolysis.** Mosquitoes treated with antibiotics as
842 pupae and adults demonstrate a reduced complement of proteolysis associated genes, and **B) GO**
843 **enrichments in genes showing reduced transcript abundance for all antibiotic treatments**
844 **primarily map to DNA synthesis and repair mechanisms.** GO terms for the DEGs (determined
845 from Figures 6C-D) and their descriptions on the y-axis, and statistical significance (P-values,
846 Fisher's exact test) of the GO term on the x-axis, with the exact number of DEGs within the colored
847 circles. DEGs farther to the left (lower P-values) are more significant. **C) ZIKV-responsive DEGs**
848 **demonstrating the highest magnitude fold change are distinct between AbxPA and No Abx**
849 **treatment groups.** The top 10 upregulated and top 10 downregulated DEGs, by magnitude $log_2$-fold
850 change for each antibiotic treatment. A positive $log_2$-fold change refers to more transcripts after a
851 ZIKV-spiked bloodmeal, while a negative value refers to fewer transcripts. The right y-axis is the
852 gene description from annotations retrieved from transcriptome metadata as well as VectorBase

853 (42). Red text indicates the top genes shared between No Abx and AbxA. Purple text indicates the
854 top genes shared between AbxPA and AbxA.
855
856 **Discussion**
857 This study shows that microbial exposure confers resistance to ZIKV infection of *Ae. aegypti*
858 and that microbes enhance bloodmeal digestion and midgut integrity and reduce midgut cell
859 infection. Reduced microbial colonization during both pupal and adult *Ae. aegypti* life stages
860 produced the largest increases in ZIKV susceptibility. These effects were not due to antibiotic
861 treatment itself and our studies with gnotobiotic mosquitoes show that exposure to more than
862 *Elizabethkingia spp.* is required to reduce ZIKV susceptibility. The lack of modified ZIKV
863 susceptibility in microbe-reduced Clovis *Ae. aegypti* contrasts with our observations from Los
864 Angeles mosquitoes and highlights the need for further work to understand whether microbes
865 have differential impacts on ZIKV competence in *Aedes* mosquitoes from different regions,
866 which is supported by a recent study showing mosquito genotype effects in Senegalese *Ae.*
867 *aegypti* (18).
868
869 Augmented rates of ZIKV infection but not dissemination in *Ae. aegypti* after microbe reduction
870 via antibiotic treatment suggest that microbes act at the midgut infection barrier. Augmented
871 transmission by AbxA but not AbxPA could be explained by a stochastic effect due to the
872 smaller group size of AbxA versus AbxPA or another mechanism we do not yet understand.
873 Hematophagous feeding introduces a unique problem in that engorgement immensely distends
874 the midgut. Midgut distention can result in breaches in the integrity of the epithelial barrier,
875 presenting an opportunity for a virus ingested with blood to bypass infection of the epithelium.
876 Prior studies detected bloodmeal-induced perforations in the midgut basal lamina, which do not
877 fully recover after digestion, supporting a process where successive bloodmeals increase rates
878 of ZIKV dissemination (48). Considering that we observed a reduced magnitude of and rate of

879 bloodmeal digestion in antibiotic-treated mosquitoes as pupae and adults, we envision a
880 process where microbes accelerate digestion, decreasing the window of time the virus has to
881 escape the bloodmeal and access the midgut epithelium. We did not observe gross structural
882 differences in the midgut epithelium with DAPI or H&E staining, so we theorize that microbes do
883 not impact midgut permeability by physically modifying the midgut epithelium, although electron
884 microscopy studies could yield more insight into structural changes. We acknowledge that
885 increased midgut permeability may also allow for extracellular ZIKV traversion of the midgut
886 epithelium and that this is a process we did not measure in these studies. Our 0 dpf midgut
887 permeability assays were performed within 1 hour of feeding, which could be too early to
888 observe structural differences in the midgut epithelium. Future studies focusing on midgut
889 integrity at higher temporal resolution are warranted.
890
891 Given that there are conserved elements of mosquito antiviral defenses and gut microbial
892 homeostasis, including immune signaling and off-target suppression by ROS and AMPs (12,67),
893 we were initially surprised that gene expression analyses did not detect differences in antiviral
894 immune responses in response to antibiotic treatment. Rather, our RNA-Seq analyses confirm
895 prior observations that show protein digestion and vitellogenesis dominate mosquito
896 physiological processes after bloodfeeding (50), in parallel with other work showing upregulation
897 of proteases and trypsins 2 days post-ZIKV exposure (73). Blood digestion and vitellogenesis
898 may be hampered without microbial symbionts. Midgut symbionts may also indirectly reduce
899 mosquito vector competence in multiple ways. These include supplementation of larval nutrition
900 and development to increase mosquito fitness (10,74–77), stimulation of bloodfeeding behavior
901 resulting in larger bloodmeals (although bloodmeal size differences were not observed in our
902 studies) and increased nutrition (78), and regulation of midgut epithelial cell proliferation, where
903 the physical microbe-midgut interface can block virus access to midgut infection (79) [although
904 this may be pathogen specific as increased midgut infection has also been observed (20,80)].

905

906 Both microbes and ZIKV in bloodmeals resulted in increased blood digestion relative to
907 microbe-reduced antibiotic treated mosquitoes that ingested blood without virus. It is unclear
908 why ZIKV enhances digestion; this may result from the virus modulating cell metabolism to
909 benefit viral replication, a pattern shown for many viruses (81). In human cells, infection with
910 DENV elevates glycolysis to meet cellular energy needs (82). ZIKV infection of mosquito midgut
911 cells may increase energy demand, which can be met by increasing blood metabolism. Absent
912 ZIKV in the bloodmeal, antibiotic-treated mosquitoes exhibited digestive deficiencies during late
913 digestion, defined here as periods beyond 2 dpf. Since blood ingestion is followed by rapid
914 proliferation of midgut microbes that results from a heme-mediated reduction in ROS (83), the
915 delayed digestion kinetics in antibiotic treated mosquitoes could be influenced by the disrupted
916 microbial environment that promotes digestion.

917

918 Determining how microbes modulate the susceptibility of mosquitoes to arboviruses can help
919 identify approaches to reduce transmission and decrease human disease. Microbial effects may
920 also explain variability in vector competence across laboratories and geographic origins where
921 each has different microbial communities (84), together with conventionally acknowledged
922 factors like inbreeding during colonization (85,86). Our previous work, together with a growing
923 body of mosquito microbiome studies, suggests that mosquito-microbe-virus interactions are
924 environmentally variable and warrant investigation in more field-relevant contexts (15,77,87,88).
925 Our study has several limitations. These include use of a single microbial community, ZIKV
926 strain and dose, where disparate microbial species, community structures, and other ZIKV
927 strains could impact susceptibility differently. Changes in microbiome composition due to
928 antibiotic modification may differentially impact microbes with more than one copy of 16S rRNA,
929 which could bias our evaluation of antibiotic efficacy. Unculturable bacterial species could
930 potentially play a role in mediating hemolytic activity that could have impacted blood digestion

931 assays. Our use of antibiotics, which incompletely eliminates microbiota, most closely
932 represents a dysbiotic phenotype. Additionally, our gnotobiotic studies were limited to use of a
933 single bacterial species. Newer tools including auxotrophic symbionts (89) and axenic
934 mosquitoes (10) can be used to further investigate the metabolic roles of microbes in influencing
935 arboviral infection of mosquitoes. The addition of ZIKV to comprise 5% of the bloodmeal volume
936 could have resulted in augmented bloodmeal digestion compared to blood without ZIKV;
937 mechanistic studies to determine how ZIKV impacts bloodmeal digestion are warranted. Future
938 studies of infection dynamics at the microbe-mosquito midgut interface could prove useful in
939 reconciling disparate vector competence outcomes and discovering novel vector control
940 approaches.
941
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954
955 **Author Contributions**
956 Conceptualization: WL, LLC

Data Curation: WL, LKM, ETK

Formal Analysis: WL, LKM, ETK

Funding Acquisition: WL, LLC

Investigation: WL, LC

Methodology: WL, ALR, RL, LLC, GMA

Project Administration: LLC

Resources: LLC

Supervision: LLC, GMA

Validation: WL

Visualization: WL, ALR, LLC

Writing-Original Draft: WL

Writing-Reviewing and Editing: WL, ALR, LLC, LKM, ETK, GMA

972 **Figure S1. Histology of bloodfed female midguts.** Cross sections of whole, bloodfed female
973 *Ae. aegypti* along the median sagittal plane. H&E staining and bright field microscopy with a
974 focus on the blood bolus perimeter was used to assess and identify potential structural artifacts
975 during sample preparation that could confound immunofluorescence observations. Both 20X
976 and 40X magnification of the same section are shown side-by-side. MG epithelium = midgut
977 epithelium. PM region = peritrophic matrix region (where the fully formed PM would be seen).
978 RBCs = red blood cells, from bloodmeal. BL side = basal laminal side.
979
980 **Figure S2. Bacterial relative abundance.** Only bacteria with assigned taxonomy are shown.
981 Each group shown represents all adult mosquitoes sequenced (N=10 Conventional, N=10
982 Conventional + Abx, N=8 Gnotobiotic). ASVs were agglomerated into their respective genera,
983 represented by the colored bars.
984
985 **Figure S3. Validation of hemoglobin colorimetric assay kit for bloodfed mosquitoes. A)**
986 Serial 1:2-fold dilutions of heparinized sheep blood were tested in parallel with the kit
987 hemoglobin standard at the absorbance extremes of 560nm and 580nm. Lines indicate
988 standard curves generated from linear regression that were used in the hemoglobin assays. **B)**
989 Absorbance values of sheep blood hemoglobin after heat-treating at 60°C for 10 minutes in
990 duplicates (rep = replicate).
991
992 **Figure S4. Bloodmeal digestion in microbe-disrupted *Ae. aegypti* as pupae and adults from**
993 **Clovis, California. A) Experimental design** using the same groups as in Figure 1 and hemoglobin
994 assessments as in Figure 4 where an *Ae. aegypti* colony from Clovis, CA was used instead of *Ae.*
995 *aegypti* from LA, CA. Mosquitoes were harvested 14 dpf for vector competence assessments. **B)**
996 **Hemoglobin levels in *Ae. aegypti* as a proxy for bloodmeal digestion in groups that were ZIKV**
997 **exposed.** Despite ingesting equivalent bloodmeal sizes, antibiotic treatment reduced blood digestion

998 with ZIKV at 2 dpf in Clovis mosquitoes. Error bars show the means and standard deviations. N = 15
999 for each treatment on 0 dpf, 20 per treatment on 2 dpf. **C) Microbial disruption of pupae and**
1000 **adults does not impact ZIKV infection or dissemination in Clovis mosquitoes.** Rates of
1001 infection and mean ZIKV RNA levels from bloodfed Clovis *Ae. aegypti* subjected to different
1002 antibiotic treatments were not significantly different at 14 dpf. Error bars denote the geometric means
1003 and standard deviations. The dotted line denotes the average LOD which was 62 RNA
1004 copies/mosquito. ZIKV levels in bodies and legs/wings with detectable ZIKV RNA are shown in panel
1005 C. (* is $P < 0.05$; ** is $P < 0.01$; ns is not significantly different). Each symbol in B and C shows the
1006 mean value for 1 mosquito based on triplicate ZIKV RT-qPCR measurements.
1007
1008 **Figure S5. DEGs from bloodfed antibiotic-treated mosquitoes that were not exposed to**
1009 **ZIKV. A) Individual PCA plots** by a single variable representing the same data in Figure 6B.
1010 Shapes indicate the bloodmeal status while colors represent the antibiotic treatments. B) **DEGs**
1011 **between blood- and sugar-fed *Ae. aegypti***.
1012
1013 **Figure S6. Change in immune response gene expression across antibiotic treated groups**
1014 **of *Ae. aegypti*.** A positive $\log_2$-fold change indicates enrichment in AbxA or AbxPA, while a
1015 negative $\log_2$-fold change indicates enrichment in No Abx.
1016
1017

1018

| Closest bacterial species to sequences from *Ae. aegypti* not treated with antibiotics | E-value | Percent identity | Accession Number | Culture medium | Hemolytic activity? |
|---|---|---|---|---|---|
| *Elizabethkingia ursingii* strain EM514-03 | 3.00E-124 | 99% | ON714894.1 | R2A agar, sheep blood agar | no |
| *Elizabethkingia bruuniana* strain KMUH55 | 3.00E-124 | 99% | ON714884.1 | R2A agar, sheep blood agar | no |
| *Elizabethkingia miricola* strain BM10 | 7.00E-56 | 85% | CP011059.1 | R2A agar, sheep blood agar | no |
| *Pseudomonas sp.* MF0 | 3.00E-125 | 99% | AY331340.1 | R2A agar | NA |
| *Pseudacidovorax intermedius* strain HMF4787 | 1.00E-124 | 99% | MN595029.1 | R2A agar | NA |
| *Salmonella* clone (uncultured) | 8.00E-126 | 99% | EF605247.1 | R2A agar, sheep blood agar | no |

1019

1020 **Table S1. Bacterial isolates from adult *Ae. aegypti* that were not treated with antibiotics**
1021 **(No Abx).** Cultured and isolated bacterial colonies from adult female *Ae. aegypti*, 3-5 dpe were
1022 Sanger sequenced at the V4 region of the 16S gene. Sequences were input into nucleotide
1023 BLAST, and the closest hits are reported. Hemolytic activity was tested by presence of ring of
1024 clearance around colonies (from lysed red blood cells) when cultured on sheep blood agar at
1025 37°C. NA indicates hemolytic activity was not evaluated.

1026

1027

| Gene | $Log_2$Fold Change | Adjusted P | GO accession | GO term | Gene description |
|---|---|---|---|---|---|
| AAEL021374 | -4.99 | 0.019 | NA | NA | NA |
| AAEL020586 | -2.88 | 0.000 | GO:0016020 | membrane | NA |
| AAEL003626 | -1.27 | 0.047 | GO:0016021 | integral component of membrane | sodium/chloride dependent amino acid transporter |
| AAEL019793 | -1.00 | 0.026 | GO:0005524 | ATP binding | NA |
| AAEL010711 | -1.00 | 0.005 | GO:0005524 | ATP binding | eph receptor tyrosine kinase |
| AAEL020123 | -0.95 | 0.040 | NA | NA | NA |
| AAEL019444 | -0.87 | 0.022 | GO:0003723 | RNA binding | NA |
| AAEL004715 | -0.86 | 0.041 | GO:0008285 | negative regulation of cell population proliferation | b-cell translocation protein |
| AAEL017280 | -0.85 | 0.040 | GO:0003677 | DNA binding | NA |
| AAEL007041 | -0.83 | 0.033 | GO:0005509 | calcium ion binding | low-density lipoprotein receptor (ldl) |
| AAEL027453 | 1.49 | 0.019 | GO:0004550 | nucleoside diphosphate kinase activity | NA |
| AAEL012832 | 1.88 | 0.026 | GO:0031902 | late endosome membrane | cytochrome B561 |
| AAEL000384 | 3.58 | 0.000 | GO:0055085 | transmembrane transport | vesicular acetylcholine transporter |
| AAEL012340 | 4.56 | 0.026 | GO:0016788 | hydrolase activity, acting on ester bonds | lipase 1 precursor |
| AAEL029026 | 8.16 | 0.026 | NA | NA | NA |
| AAEL014561 | 8.47 | 0.040 | GO:0005576 | extracellular region | NA |

1028

1029 **Table S2. DEGs between sugar-fed *Ae. aegypti* without antibiotics or antibiotic treated as**

1030 **pupae and adults.** A negative $Log_2$fold-change refers to downregulation in AbxPA vs No Abx,

1031 while a positive $Log_2$fold-change refers to upregulation in AbxPA vs No Abx.

1032

1033

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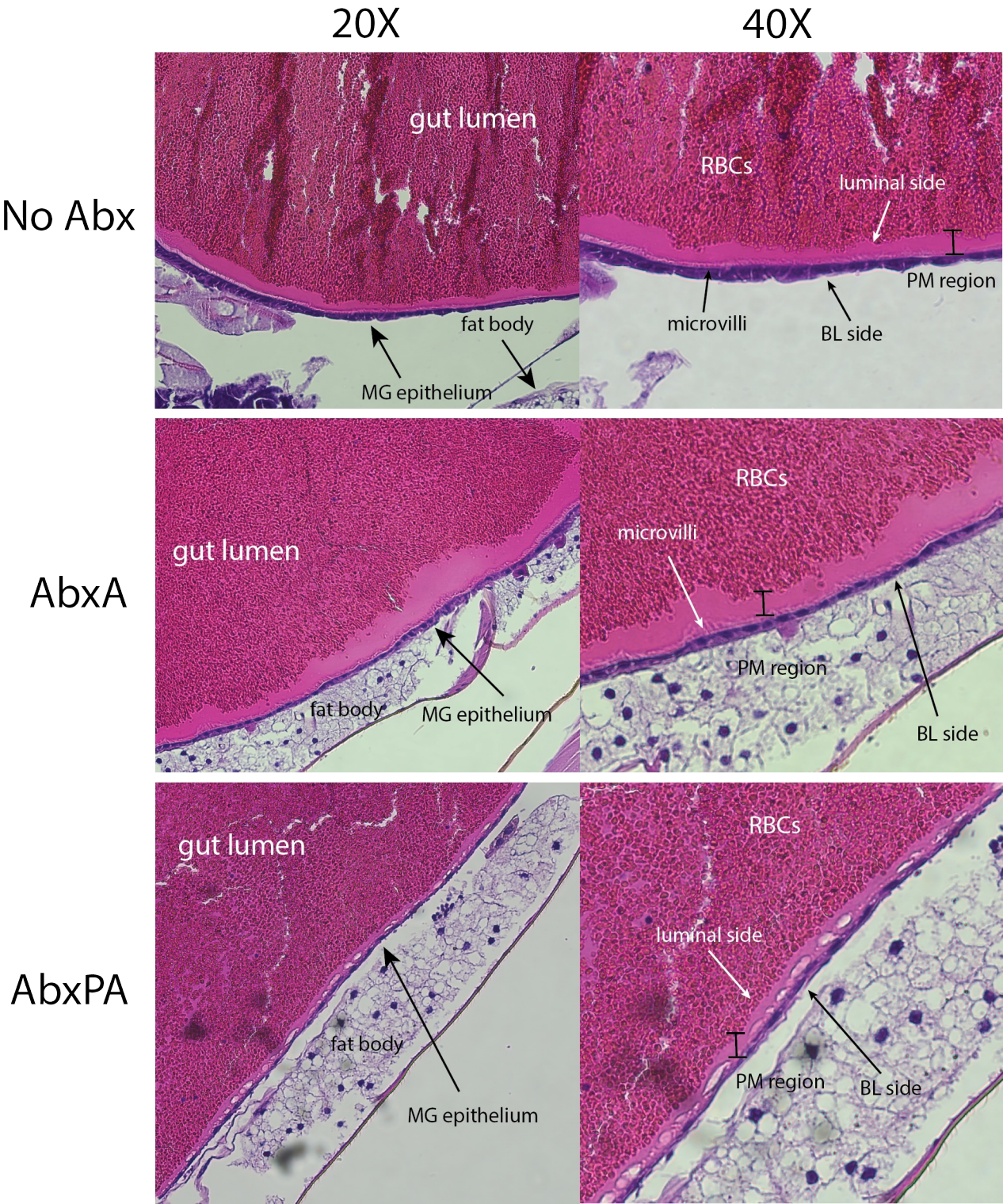
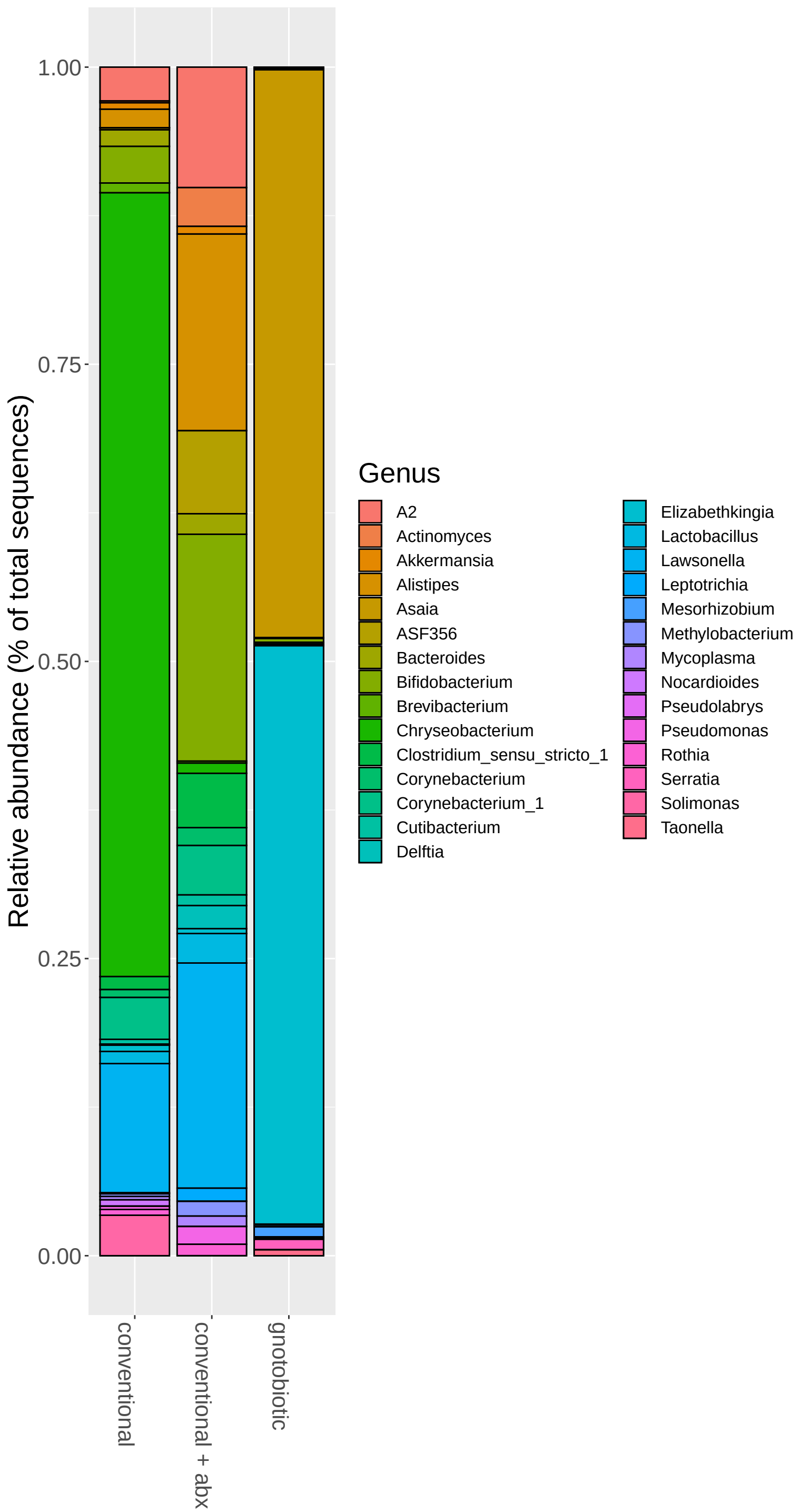
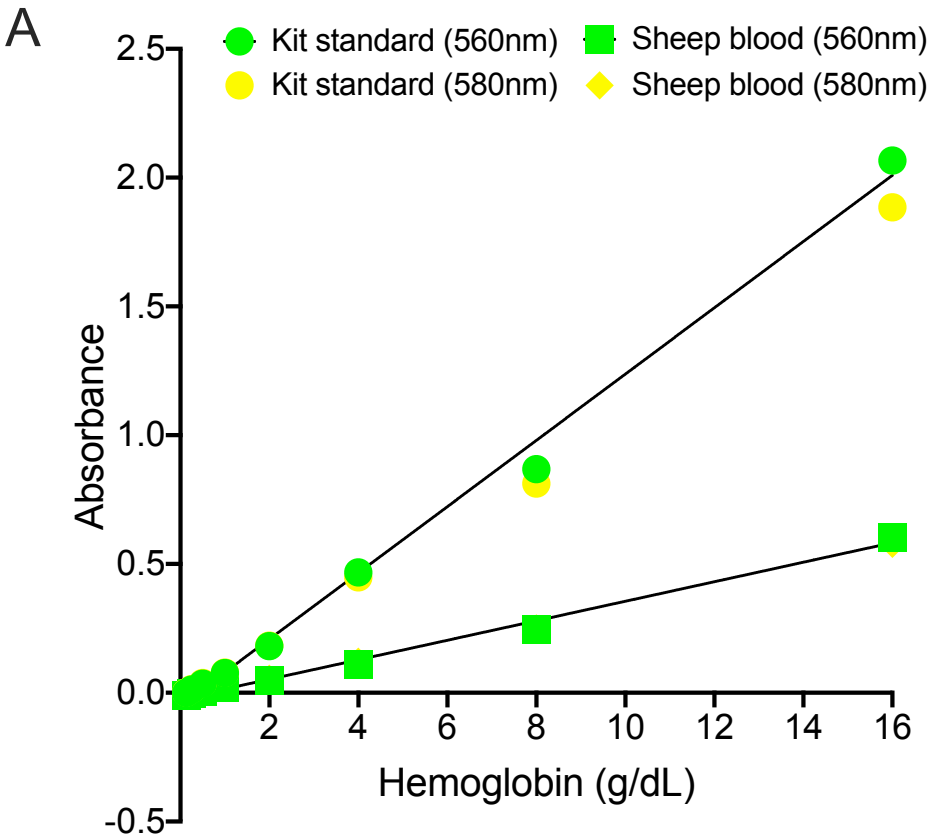
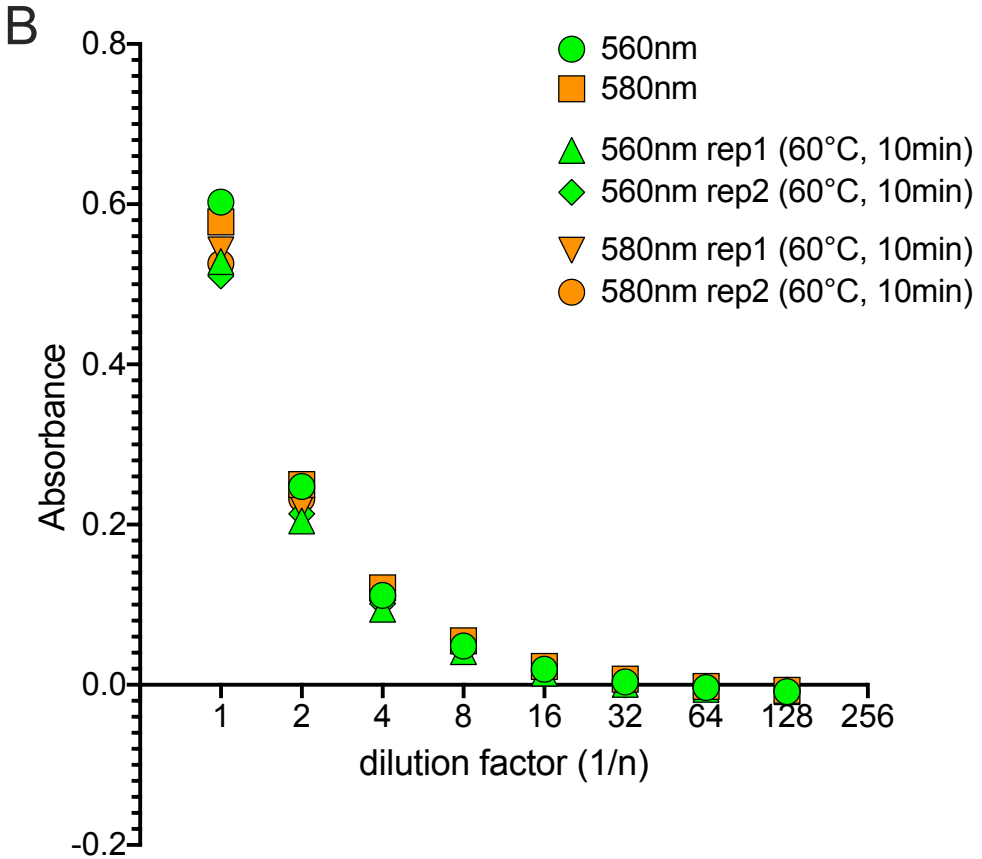
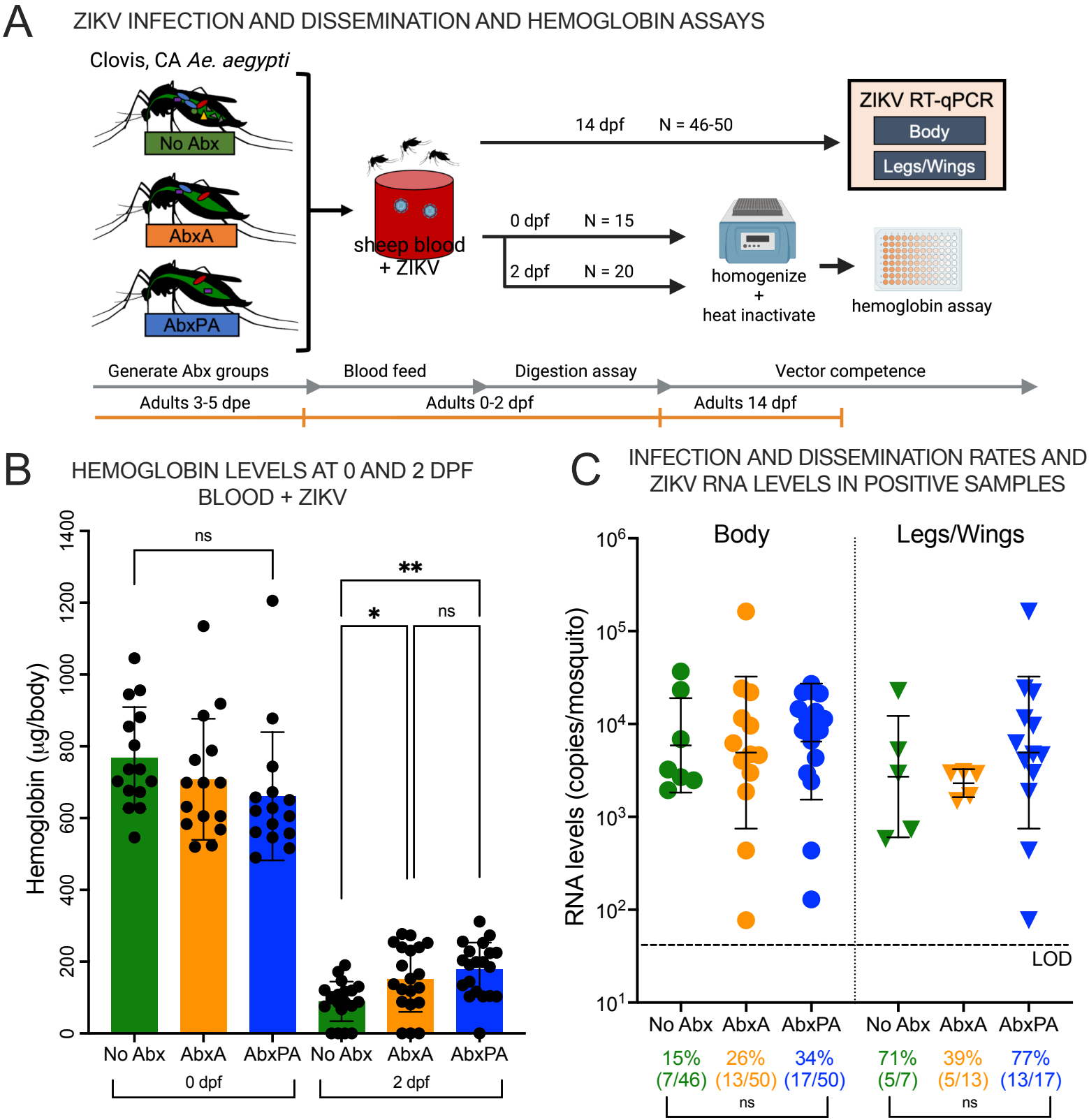

20X
40X
No Abx
gut lumen
fat body
MG epithelium
RBCs
luminal side
PM region
microvilli
BL side
AbxA
gut lumen
fat body
MG epithelium
RBCs
microvilli
PM region
BL side
AbxPA
gut lumen
fat body
MG epithelium
RBCs
luminal side
PM region
BL side

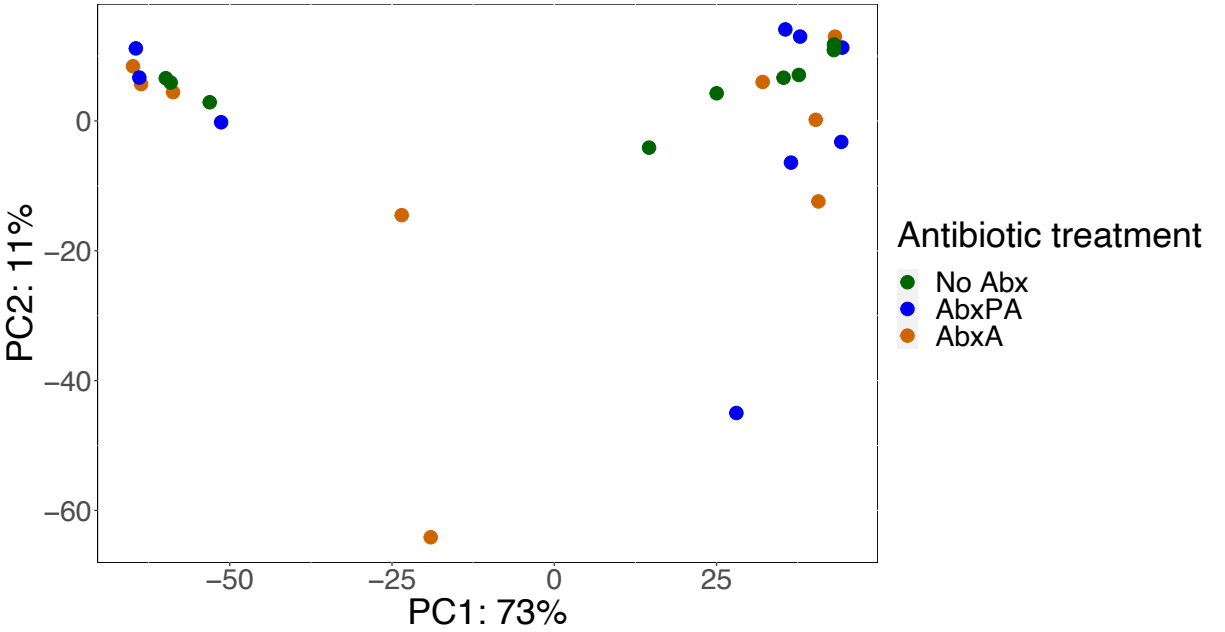

Relative abundance (% of total sequences)
1.00
0.75
0.50
0.25
0.00
conventional
conventional + abx
gnotobiotic
Genus
A2
Actinomyces
Akkermansia
Alistipes
Asaia
ASF356
Bacteroides
Bifidobacterium
Brevibacterium
Chryseobacterium
Clostridium_sensu_stricto_1
Corynebacterium
Corynebacterium_1
Cutibacterium
Delftia
Elizabethkingia
Lactobacillus
Lawsonella
Leptotrichia
Mesorhizobium
Methylobacterium
Mycoplasma
Nocardioides
Pseudolabrys
Pseudomonas
Rothia
Serratia
Solimonas
Taonella

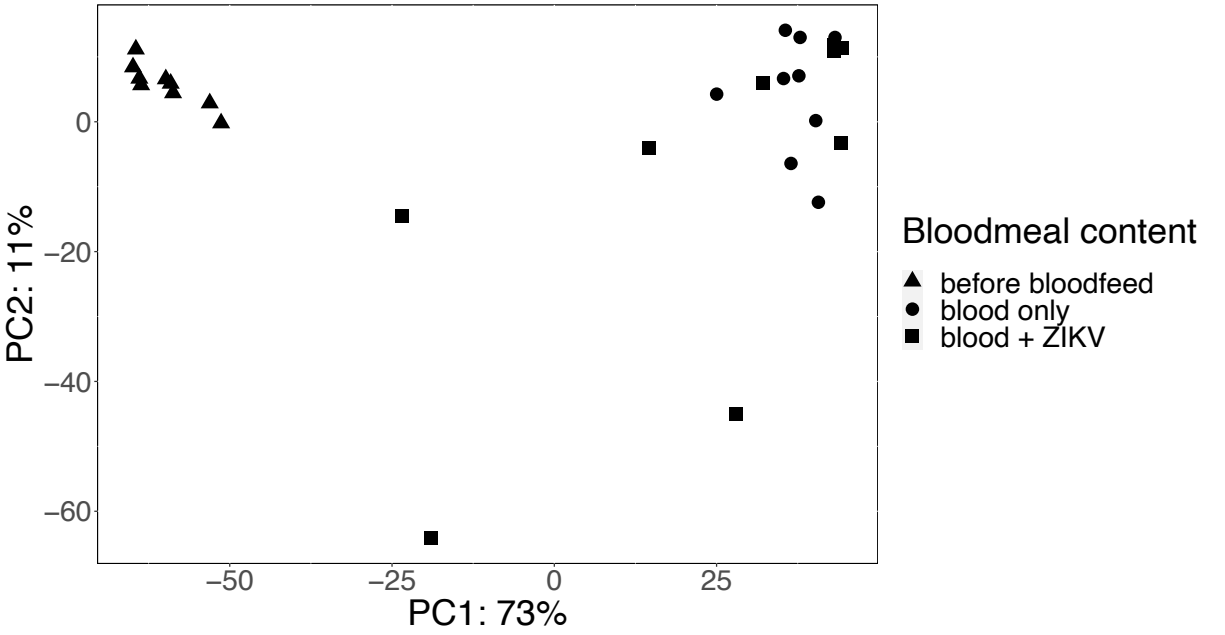

A
Kit standard (560nm)
Kit standard (580nm)
Sheep blood (560nm)
Sheep blood (580nm)
Absorbance
Hemoglobin (g/dL)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
2
4
6
8
10
12
14
16


B
560nm
580nm
560nm rep1 (60°C, 10min)
560nm rep2 (60°C, 10min)
580nm rep1 (60°C, 10min)
580nm rep2 (60°C, 10min)
Absorbance
dilution factor (1/n)
0.8
0.6
0.4
0.2
0.0
-0.2
1
2
4
8
16
32
64
128
256

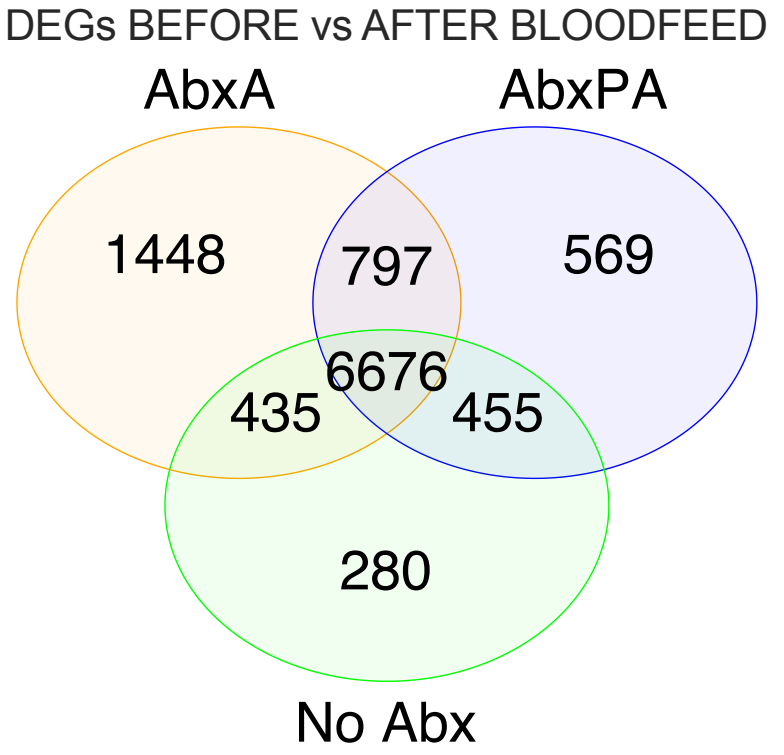

A
ZIKV INFECTION AND DISSEMINATION AND HEMOGLOBIN ASSAYS
Clovis, CA Ae. aegypti
No Abx
AbxA
AbxPA
sheep blood
+ ZIKV
14 dpf
N = 46-50
ZIKV RT-qPCR
Body
Legs/Wings
0 dpf
N = 15
2 dpf
N = 20
homogenize
+
heat inactivate
hemoglobin assay
Generate Abx groups
Blood feed
Digestion assay
Vector competence
Adults 3-5 dpe
Adults 0-2 dpf
Adults 14 dpf
B
HEMOGLOBIN LEVELS AT 0 AND 2 DPF
BLOOD + ZIKV
Hemoglobin (µg/body)
0
200
400
600
800
1000
1200
1400
ns
**
*
ns
No Abx
AbxA
AbxPA
No Abx
AbxA
AbxPA
0 dpf
2 dpf
C
INFECTION AND DISSEMINATION RATES AND
ZIKV RNA LEVELS IN POSITIVE SAMPLES
Body
Legs/Wings
RNA levels (copies/mosquito)
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
LOD
No Abx
AbxA
AbxPA
No Abx
AbxA
AbxPA
15%
(7/46)
26%
(13/50)
34%
(17/50)
71%
(5/7)
39%
(5/13)
77%
(13/17)
ns
ns

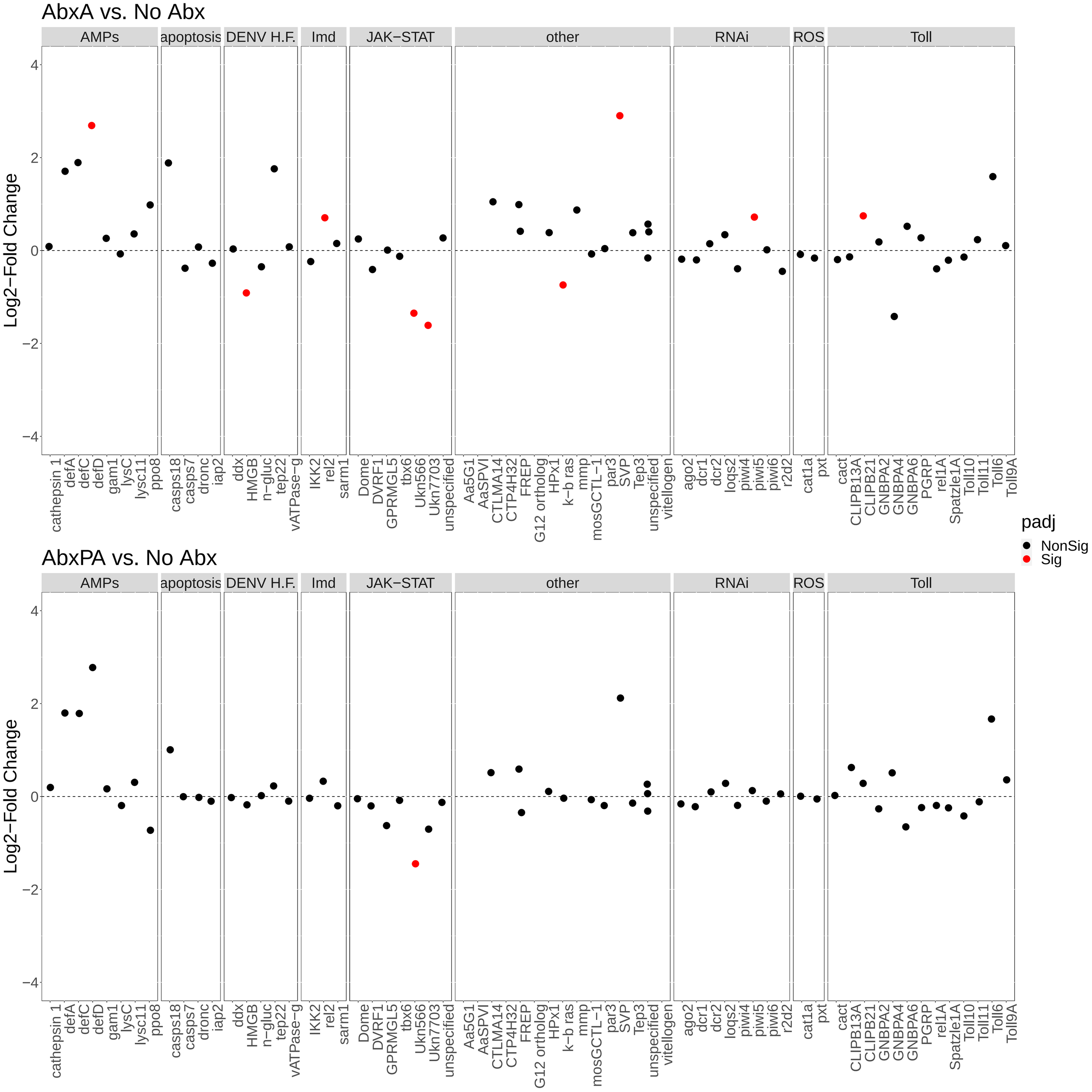
A


PC2: 11%
PC1: 73%
0
−20
−40
−60
−50
−25
0
25
Antibiotic treatment
No Abx
AbxPA
AbxA


PC2: 11%
PC1: 73%
0
−20
−40
−60
−50
−25
0
25
Bloodmeal content
before bloodfeed
blood only
blood + ZIKV

B


DEGs BEFORE vs AFTER BLOODFEED
AbxA
AbxPA
No Abx
1448
797
569
6676
435
455
280


AbxA vs. No Abx
AbxPA vs. No Abx
AMPs
apoptosis
DENV H.F.
Imd
JAK−STAT
other
RNAi
ROS
Toll
Log2−Fold Change
4
2
0
−2
−4
cathepsin 1
defA
defC
defD
gam1
lysC
lysc11
ppo8
casps18
casps7
dronc
iap2
ddx
HMGB
n−gluc
tep22
vATPase−g
IKK2
rel2
sarm1
Dome
DVRF1
GPRMGL5
tbx6
Ukn566
Ukn7703
unspecified
Aa5G1
AaSPVI
CTLMA14
CTP4H32
FREP
G12 ortholog
HPx1
k−b ras
mmp
mosGCTL−1
par3
SVP
Tep3
unspecified
vitellogen
ago2
dcr1
dcr2
loqs2
piwi4
piwi5
piwi6
r2d2
cat1a
pxt
cact
CLIPB13A
CLIPB21
GNBPA2
GNBPA4
GNBPA6
PGRP
rel1A
Spatzle1A
Toll10
Toll11
Toll6
Toll9A
padj
NonSig
Sig

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