Microbes reduce susceptibility of *Aedes aegypti* to Zika virus by enhancing blood digestion and limiting midgut cell infection

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Short Title: Microbes mediate Zika virus susceptibility in mosquitoes

Abstract

The worldwide expansion of mosquito-borne pathogens necessitates improved control measures, including approaches to restrict infection and 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 species and lower abundance of microbes. 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 reduce 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 single bacterial species in gnotobiotic mosquitoes is responsible for reducing ZIKV vector competence. *Ae. aegypti* with depleted microbes 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 microbe depletion via antibiotic treatment increases Zika virus infection and midgut permeability and impairs bloodmeal digestion. These data show that microbial exposure throughout the life of a mosquito restricts Zika virus infection by facilitating blood digestion and restricting midgut cell infection. These findings advance understanding of microbiota-mosquito-virus interactions by defining how microbes reduce susceptibility of *Aedes aegypti* to Zika virus.

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, especially in tropical regions. Absent licensed vaccines or specific treatments for these pathogens, 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 which species 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.*

79 mosquitoes primarily derive from the environment (6–8), reside dominantly in the midgut (8,9),
80 and can influence physiology and life history traits through nutritional supplementation
81 (6,7,10,11) and immune stimulation (12–14). Prior studies from our group and others show that
82 microbiota influence ZIKV competence for *Ae. aegypti* (15,16), and *Ae. albopictus* (17). We
83 discovered that when *Ae. aegypti* larvae reared in water with microbes sourced from cemeteries
84 were exposed orally to ZIKV as adults, they became infected, developed disseminated
85 infections, and transmitted virus less efficiently than cohorts where larvae were reared in
86 laboratory tap water with fewer numbers and species of microbes (15). We also determined that
87 the presence of microbes accelerates mosquito development. Another study demonstrated that
88 ablation of microbiota via oral treatment of adult *Ae. aegypti* with antibiotics prior to exposure to
89 ZIKV in an artificial bloodmeal did not affect vector competence (18). Together, these findings
90 suggest that the influence of microbiota on ZIKV vector competence may be specific to the
91 mosquito developmental stage (larval, pupal, adult) during which the microbes are present.
92 Beyond ZIKV, microbial effects on mosquito life-stage development and metabolism that
93 condition pathogen susceptibility may be generalizable to an array of mosquito-borne
94 pathogens. Depending on the bacterial species, larval gut microbiota can modify lipid
95 metabolism in *Ae. aegypti* which decreases DENV susceptibility, possibly via metabolism-driven
96 fitness changes (19). Bacterial infection in *Anopheles (An.) gambiae* larvae increases hemocyte
97 activity in adults, which correlates with elevated *Plasmodium falciparum* susceptibility in vectors
98 (20). To understand whether reduced ZIKV vector competence after larval development in
99 water containing microbes is conferred transstadially from larvae to pupae to adults, one goal of
100 this study was to determine whether exposure to microbes throughout life is required to reduce
101 ZIKV susceptibility in *Ae. aegypti*.
102
103 Microbes may modify vector competence of mosquitoes via multiple mechanisms, including to
104 alter immune system activation, by direct antiviral or antiparasitic activity, by modifying resource

105 competition, or by affecting physiological processes, including blood digestion. Many of these
106 processes occur in the mosquito midgut, which represents the first infection barrier for most
107 vector-borne pathogens. Microbes are likely also involved in maintaining physical barriers that
108 restrict access of an ingested virus or parasite to the mosquito midgut. The commensal
109 bacterium *Serratia marcescens* increases susceptibility of *Ae. aegypti* for CHIKV by secreting a
110 protein that digests membrane-bound mucins coating the gut epithelium, rendering the
111 epithelium more accessible to the virus (21). The bacterium *Bacillus thuringiensis* svar.
112 *israelensis* (Bti) expresses δ-endotoxins that create pores in midgut cells of larval mosquitoes,
113 which is lethal and has long been a mainstay of larvicidal vector control (22,23). Treatment of
114 the malaria vector *An. coluzzi* with antibiotics hinders the synthesis of the peritrophic matrix
115 (PM), a chitinous mesh that sequesters the bloodmeal and furnishes digestive enzymes used in
116 bloodmeal digestion (24). Blood digestion in the PM also coincides with immunological sensing
117 and sequestering of microbiota proliferating in it (25), similar to processes in the mucous layer in
118 a mammalian intestine (26). Disruption of the PM can also modulate vector competence.
119 Knockdown of heme peroxidase 1 (HPx1), which promotes PM assembly and increases
120 reactive oxygen species that restrict microbiota growth, reduces ZIKV or DENV infection of the
121 *Ae. aegypti* midgut (27). We hypothesized that an antibiotic-mediated reduction in microbes in
122 pupal and adult *Ae. aegypti* mosquitoes would decrease blood digestion, resulting in increased
123 ZIKV susceptibility by giving the virus more time to access the midgut since blood digestion is
124 thought to occur concomitantly with virus infection of the midgut epithelium (28). We reared *Ae.*
125 *aegypti* larvae in environmental water containing microbes. Half of the experimental cohorts
126 were administered antibiotics in water during the pupal stage to ablate microbes. Some cohorts
127 of adults were also treated with antibiotics administered in sugar water after eclosion and prior
128 to exposure to ZIKV-spiked bloodmeals. Since antibiotics create a dysbiotic environment and
129 may not eliminate resident antibiotic-resistant microbiota (29), we first verified that antibiotic
130 treatment of *Ae. aegypti* reared in laboratory water did not modify midgut integrity or ZIKV vector

131 competence. We also generated gnotobiotic mosquitoes where larvae and pupae were reared
132 in water with an *Elizabethkingia spp.* strain isolated from colonized mosquitoes to determine that
133 this single bacterial species is not sufficient to modulate vector competence, which suggests
134 that many species, similar to the composition we detected in environmental water sources(15),
135 are required. Next, we measured physiological differences in the midgut in blood engorged
136 mosquitoes across cohorts treated with or without antibiotics at different life stages. Microscopy
137 was used to evaluate midgut anatomy and integrity and to quantify ZIKV-infected midgut cells.
138 The magnitude and rate of bloodmeal digestion was measured by quantifying hemoglobin levels
139 over time. RNA sequencing (RNA-seq) was used to identify differential gene expression in
140 cohorts of *Ae. aegypti* treated with or without antibiotics at different life stages and in response
141 to blood with or without ZIKV. We observed that *Ae. aegypti* treated with antibiotics as pupae
142 and adults are more susceptible to ZIKV infection compared to mosquitoes that were not
143 antibiotic-treated. Microbe reduction by antibiotic treatment of pupae and adults also increased
144 midgut permeability, the number of ZIKV-infected midgut cells, and impaired bloodmeal
145 digestion. Antibiotic treatment itself did not reduce ZIKV vector competence or affect midgut
146 permeability, ruling out toxic or physiologic effects due to the antibiotics. Gene ontology
147 analyses of ZIKV-exposed mosquitoes revealed enrichment of PM-associated genes, with more
148 genes related to blood protein metabolism upregulated in mosquitoes harboring microbiota.
149 Together our results show that exposure to a microbes throughout the life of *Ae. aegypti*
150 facilitates blood digestion and reduces ZIKV midgut cell infection.
151

152 **Materials and Methods**

153

154 **Biosafety**

155 All ZIKV infection experiments were conducted in a biosafety level 3 (BSL3) laboratory and were
156 approved by the University of California, Davis Institutional Biosafety Committee under
157 Biological Use Authorization #R1863.
158
159 **Mosquito sources**
160 *Ae. aegypti* mosquitoes colonized from field collections from 2 locations in California were used
161 in this study. Mosquitoes were field-collected in 2016 as larvae (Los Angeles) and eggs (Clovis).
162 Mosquitoes from both locations were maintained for generations numbers noted in Figures
163 representing F_{20}, $F_{22,}$ or F_{26}, at 26°C, 80% humidity, and a 12 h:12 h light : dark (L : D) cycle with
164 10% sucrose provided to adults and periodic presentation of sheep blood to stimulate egg
165 laying, as described in our previous study (15).
166
167 **Mosquito generation and antibiotic treatments**
168 *Microbe source and preparation*. Microbes from water collected outdoors in cemeteries were
169 added to water in which *Ae. aegypti* larvae were reared, using an approach detailed in our
170 previous study (15). Briefly, 1 liter (L) of collected water was filtered through a 1 mm mesh to
171 remove large debris, centrifuged at 3000 g to pellet microbes, washed 3 times with sterile 1X
172 phosphate buffered saline (PBS, Thermo Fisher Scientific, Emeryville, CA), and frozen in
173 glycerol at -80°C. Microbe aliquots were thawed at room temperature and placed in laboratory
174 tap water prior to addition of mosquito eggs.
175 *Larval rearing.* Mosquito eggs were hatched in 1 L deionized (diH_2O) water spiked with 1 mL of
176 microbes from a glycerol stock aliquot. Approximately 1500 larvae were reared in three pans at
177 a density of ~500 larvae/pan. Each pan contained an agarose plug that was prepared by mixing
178 1% agarose (Sigma-Aldrich, St. Louis, MO) with pulverized fish food (final concentration of 100
179 g/L, or 10% [Tetra, Melle, Germany]) and rodent chow (final concentration of 80 g/L, or 8%
180 [Teklad Global 18% Protein Rodent Diet, Envigo, Indianapolis, IN]), which was autoclave-

181 sterilized before casting into 12-well plates. One to 2 plugs were added to larval pans every
182 other day until day 15 post-hatch. Larval trays were kept in environmental growth chambers
183 (Binder, Bohemia, NY) at 26°C, 80% humidity, and a 12 h:12 h L:D cycle.
184 *Antibiotic treatments to pupae and adults*. Pupae or adults were divided into three treatments: 1)
185 mosquitoes exposed to antibiotics (Abx) both as **p**upae and **a**dults (AbxPA), 2) mosquitoes
186 exposed to antibiotics as **a**dults (AbxA) only, and 3) mosquitoes not exposed to antibiotics (No
187 Abx), where the latter represents the control group. First, pupae were picked and randomly
188 divided into 2 batches each day. The AbxPA batch of pupae was washed in diH_2O containing an
189 antibiotic cocktail consisting of 50 U/mL each of penicillin, streptomycin, and kanamycin (Sigma-
190 Aldrich, St. Louis, MO) for 10 minutes before transfer to another water vessel containing fresh
191 antibiotic cocktail. This concentration is in the middle of the range of doses used previously in
192 similar studies with *Ae. aegypti* (21) and *An. stephensi* (30). The other batch of pupae, from
193 which the AbxA and No Abx groups were derived, was transferred to a separate cage without
194 washing or antibiotics. Adults in the AbxPA group were maintained on the antibiotic cocktail
195 dissolved in 10% sucrose (Thermo Fisher Scientific, Emeryville, CA) *ad libitum*. Adults in the
196 other batch were initially maintained on filter-sterilized 10% sucrose without antibiotics *ad libitum*
197 until eclosion was complete. Once adults reached a density of ~500, females were aspirated out
198 and transferred in cohorts of 80-120 mosquitoes to 32-ounce plastic containers (Amazon,
199 Seattle, WA) with mesh lids. At this point, the second non-antibiotic treated batch was further
200 divided into two treatments: AbxA was provided 10% sucrose *ad libitum* with the antibiotic
201 cocktail, and No Abx was fed 10% sucrose *ad libitum* without the antibiotic cocktail. As a control
202 for toxicity or physiological effects stemming from antibiotic treatment, we also reared
203 mosquitoes in laboratory tap water (conventional) with or without antibiotics, which were
204 provided as described above, to pupal and adult mosquitoes (conventional + Abx). Adult
205 mosquitoes were maintained at 26°C, 80% humidity, and 12 hour : 12 hour L : D cycle
206 throughout the experiments. All mosquitoes were housed in the same incubator. Antibiotic

207 treatment experiments with conventional mosquitoes were performed once and experiments
208 with mosquitoes provided environmental microbes were repeated twice and are represented as
209 the high and low ZIKV dose.
210
211 **Gnotobiotic mosquito generation**
212 An *Elizabethkingia spp.* strain isolated from colonized *Ae. aegypti* from Los Angeles was
213 cultured in LB broth at 37°C for 24 hours prior to inoculation into larval flasks. *Ae. aegypti* eggs
214 attached to their egg papers cut into strips were surface sterilized by submersion in 1% bleach
215 for 10 minutes. After 10 minutes, bleach was removed by vacuum filtration of eggs through 1000
216 mL of diH_2O and a 0.22 μm filter (Genesee Scientific, San Diego, CA). Eggs were then washed
217 twice in 70% ethanol and allowed to dry. To prepare larval flasks, 50 mL of diH_2O and one larval
218 nutrient plug were added to four T75 tissue culture flasks. Paper strips containing ~200 eggs
219 were placed into each tissue culture flask and spiked with 1 mL of 1.4 x 10^8 CFU/mL
220 *Elizabethkingia* culture before sealing the lid and incubating at 28°C with 80% humidity and 12
221 h:12 h L:D cycle for 5 days. When pupae developed, lids of the tissue culture flasks were
222 removed, and a sterilized fabric sleeve feeding into a makeshift plastic bucket with a mesh lid
223 was taped around the opening of the flask to allow adults to emerge into the new holding
224 container. Individual mosquitoes of each life stage were sampled, homogenized, and cultured
225 on LB plates to confirm *Elizabethkingia* colonization and the absence of other contaminating
226 bacteria, which was verified by colony morphology.
227
228 **Bacterial quantification by 16S qPCR**
229 We measured the efficacy of antibiotic treatment for removing bacteria from AbxA and AbxAP
230 groups by performing 16S qPCR on larvae, pupae, and adults 3-5 days post-eclosion (dpe) from
231 all groups, as well as in L4 larvae and pupae before antibiotic treatment. DNA from 5 L4 larvae,

232 5 pre-treatment pupae, 5 No Abx, 10 AbxA, and 10 AbxAP individuals was extracted with the
233 Quick-DNA Tissue/Insect Microprep Kit (Zymo Research, Irvine, CA, USA), according to the
234 manufacturer instructions and eluted in 30 µL elution buffer. Extracted DNA was PCR-amplified
235 in the V3-V4 (31) hypervariable region of the 16S rRNA gene, as previously described (15,31).
236 Quantification of bacteria was performed by SYBR Green Real-Time PCR (Thermo Fisher
237 Scientific, Emeryville, CA) to amplify the 16S rRNA gene in whole mosquitoes. Each sample
238 was tested in technical duplicates. 16S quantities were normalized to an *Ae. aegypti* reference
239 ribosomal protein S17 gene (RPS17) and reported as a 16S:RPS17 ratio (32).
240

241 **Hemolytic activity**

242 Hematophagous bacteria can directly lyse blood cells and confound hemoglobin digestion
243 measurements. Bacterial isolates from adult female *Ae. aegypti* were screened for hemolytic
244 activity via culturing on agar plates. Individual mosquitoes were homogenized (TissueLyser,
245 Retsch, Haan, Germany) in 500 µL PBS and 40 µL of supernatant was plated on either R2A
246 agar (Sigma-Aldrich, St. Louis, MO) or sheep blood agar plates (Biological Media Services,
247 University of California, Davis School of Veterinary Medicine) for 3 days at 37°C. Up to 10
248 colonies from each mosquito were streaked on media plates 2-3 additional times to generate
249 axenic cultures. Resulting colonies were streaked on sheep blood agar at both 30°C and 37°C
250 and screened for clearing zones around colonies over the course of 3 days. The viability of this
251 assay was tested in-house with hemolytic *Streptococcus spp.* isolates from the UC Davis
252 School of Veterinary Medicine.
253

254 **Virus source and titrations**

255 We used ZIKV strain BR15 (Brazil 2015, SPH2015, GenBank Accession # KU321639), the
256 same strain we used previously for mosquito infection experiments (15). This virus strain was
257 originally isolated from human serum from a patient in Brazil in 2015 and passaged 3 times in

258 Vero cells (CCL-81, ATCC, Manassas, VA) before freezing in stocks. Stocks were titrated on
259 Vero cells prior to bloodmeal presentation to approximate stock titers, and ZIKV-spiked sheep
260 blood was also titrated after bloodfeeding to confirm the dose presented in each bloodmeal. For
261 titrations, serial 10-fold dilutions of cell culture supernatant containing virus stock or ZIKV-spiked
262 blood were inoculated into multi-well plates containing confluent Vero cells at one dilution per
263 well and incubated for 1 hour at 37°C in 5% CO_2 with rocking every 10 minutes. After
264 incubation, each well was filled with an agar overlay (0.5% agarose [Thermo Fisher Scientific,
265 Emeryville, CA] in Dulbecco's modified Eagle's medium [DMEM, Thermo Fisher Scientific,
266 Emeryville, CA], + 2% fetal bovine serum [FBS, Genesee Scientific, San Diego, CA] +
267 penicillin/streptomycin [Thermo Fisher Scientific, Emeryville, CA]). The cells were incubated for
268 1 week at 37°C in 5% CO_2, then fixed with 4% formalin (Thermo Fisher Scientific, Emeryville,
269 CA). Fixed cells were stained with 0.025% crystal violet (Thermo Fisher Scientific, Emeryville,
270 CA) in 20% ethanol and visualized on a light table to count plaques. ZIKV bloodmeal titers are
271 represented as plaque-forming units (PFU) per mL of blood.
272
273 **ZIKV vector competence experiments**
274 Stock ZIKV in DMEM, or DMEM with no virus as a control, were diluted in 10-fold increments
275 with fresh heparinized sheep blood (HemoStat Laboratories, Dixon, CA) to achieve ZIKV titers
276 of 10^4 and 10^6 PFU/mL. Bloodmeals were presented to female *Ae. aegypti* at 3-5 days post-
277 eclosion in cohorts of 80-120 per container, at 2-3 containers per treatment. Cohort sizes were
278 very large to maximize the numbers of bloodfed mosquitoes in the case of low feeding rates.
279 Mosquitoes were sucrose-starved 18-24 hours before the presentation of bloodmeals.
280 Mosquitoes were offered bloodmeals for 60 minutes through a collagen membrane rubbed with
281 an artificial human scent (BG-Sweetscent mosquito attractant, Biogents USA) and heated to
282 37°C in a membrane feeder (Hemotek Limited, Blackburn, United Kingdom). Bloodmeals were

283 presented to mosquitoes inside a plexiglass glove box containing an open bottle of boiling water
284 to maximize humidity. After feeding, mosquitoes were immobilized with CO_2 for 5 seconds and
285 then immobilized on a chill table (Bioquip (R.I.P.), Compton, CA, USA) to assess their
286 bloodfeeding status using a microscope at 10X magnification. Fully engorged females (18-45
287 per treatment for each experiment) were sorted into fresh plastic containers at a density of 10-
288 20 mosquitoes per container and held at 28°C with 80% humidity and 12 h:12 h L:D cycle for 14
289 days. No Abx cohorts were provided 10% sucrose *ad libitum,* while AbxPA, AbxA, and
290 conventional + Abx cohorts were maintained on 10% sucrose with the antibiotic cocktail *ad*
291 *libitum*.
292
293 At 14 days post-bloodfeed (dpf), surviving mosquitoes were CO_2-anesthetized and immobilized
294 on a chill table. Bodies, legs/wings, and saliva were collected to assess infection, dissemination,
295 and transmission rates, respectively. Legs/wings were removed before collection of expectorate
296 into capillary tubes containing PBS by forced salivation, using an established approach (33). To
297 minimize cross-contamination, surgical tools were washed once in CaviCide® (Metrex, Orange,
298 CA) and twice in 70% ethanol between dissections. Each capillary tube was placed in a 1.5-mL
299 tube containing 250 µL PBS and pulse-centrifuged at 8,000 g to recover saliva. Bodies and
300 legs/wings were placed into 2-mL tubes containing 500 µL PBS and a 5-mm glass bead
301 (Thermo Fisher Scientific, Emeryville, CA), then homogenized at 30 hertz for 5 minutes in a
302 TissueLyser (Retsch, Haan, Germany). Viral RNA was extracted using a MagMax Viral RNA
303 Extraction Kit (Thermo Fisher Scientific, Emeryville, CA), into 60 µL elution buffer following the
304 manufacturer's recommendations.
305
306 Detection and quantification of viral RNA in mosquito tissues and saliva were performed by
307 quantitative reverse transcription polymerase chain reaction (RT-qPCR) using a TaqMan Fast
308 Virus 1-Step Master Mix and a ZIKV-specific primer set (ZIKV 1086F/1162c, probe: ZIKV 1107-

309 FAM) using established methodologies (33,34). Cycle threshold (Ct) values from the RT-qPCR
310 were converted to RNA genome copies using standard curves established with ZIKV RNA of
311 known concentration. Each sample was assayed in technical duplicates and converted to copies
312 per mosquito before calculating the average value. The limit of detection (LOD) was calculated
313 from the standard curve linear regression line where the Ct value = 40. Samples that did not
314 yield a detectable Ct of less than 40 were reported at the LOD. Infection rates are reported as
315 the number of ZIKV-positive individuals divided by the number that ingested ZIKV-spiked blood.
316 Dissemination rates are reported as the number of ZIKV-positive legs and wings divided by the
317 number of ZIKV-infected individuals. Transmission rates are reported as the number of ZIKV-
318 positive saliva samples divided by the number individuals with disseminated ZIKV in legs and
319 wings.
320
321 **Midgut permeability assessments**
322 A low molecular weight dextran-FITC (MW 4000, Sigma-Aldrich, St. Louis, MO) was co-spiked
323 into ZIKV-bloodmeals and used to measure midgut permeability in No Abx, AbxA, AbxPA,
324 conventional, conventional + Abx, and gnotobiotic groups that were evaluated within 1 hour
325 after bloodfeeding. Permeability was assessed by measuring the fluorescent intensity of
326 dextran-FITC inside versus outside at three locations in the midgut for each mosquito for a total
327 of 5 mosquitoes per treatment. A stock solution of 20 mg/mL dextran-FITC dissolved in PBS
328 was spiked into sheep blood at a 1:20 dilution to achieve 1 mg/mL, the concentration used in a
329 previous study (35). Dextran-spiked blood mixed with 10^5 PFU/ml ZIKV and presented to
330 mosquitoes as described above for vector competence assessments. Blood-engorged
331 mosquitoes were visualized by fluorescence microscopy to detect FITC in tissues. Samples
332 were covered in foil for all post-dissection steps to minimize photobleaching. Quantification of
333 FITC fluorescence was then performed on Fiji (ImageJ) (36) by measuring intensity relative to
334 background by subtracting fluorescence of an equal-sized region outside the sample area.

335 Fluorescence intensity ratios between the gut lumen and hemocoel were calculated at three
336 randomly drawn areas of equal size around the gut perimeter, then averaged together. Each
337 average was also conducted across three slices per sample to minimize focal plane variation in
338 fluorescence. At least 4 mosquitoes per group were included in each permeability assessment.
339

340 **Fluorescence imaging of mosquito midguts**

341 Mosquitoes in No Abx, AbxA, and AbxPA groups that ingested ZIKV-spiked bloodmeals were
342 imaged using immunofluorescence microscopy to examine midgut structure and to quantify the
343 number of ZIKV-infected midgut epithelial cells. Legless and wingless mosquito bodies or
344 dissected midguts from mosquitoes at 2 or 3 dpf were placed in 10% formalin in PBS for 24
345 hours, then prepared for various image-based assessments as described below. Mosquitoes
346 were imaged at the University of California, Davis Advanced Imaging Facility using a Leica TCS
347 SP8 STED 3X inverted microscope.

348 *Midgut structure*: Mosquito bodies were washed once in PBS, submerged in PBS with 30%
349 sucrose overnight, and then molded and frozen at -80°C in Tissue Plus OCT medium (Thermo
350 Fisher Scientific, Emeryville, CA). Tissue molds were cut into 10 μm slices at the median sagittal
351 plane (at a depth where the blood bolus reaches maximum diameter, determined with test
352 samples) using a Leica CM1860 Cryostat Microtome (Leica Biosystems, Deer Park, IL, USA)
353 and embedded onto charged microscope slides. A single drop of ProLong Gold Antifade
354 Mountant with DAPI (Thermo Fisher Scientific, Emeryville, CA) was used to mount each slide.

355 *ZIKV infection of midguts:* Dissected midguts in 10% formalin were washed twice in PBS.
356 Tissues were submerged in a blocking solution consisting of 1X PBS, 0.2% Triton X-100
357 (PBS+T) (Bio-Rad Laboratories, Hercules, CA), 1% bovine serum albumin (BSA, Sigma-Aldrich,
358 St. Louis, MO), and 10% normal goat serum (Lampire Biological Laboratories, Pipersville, PA)
359 for 10 minutes before staining with a primary monoclonal antibody that reacts with multiple
360 flaviviruses (Flavivirus treatment antigen antibody D1-4G2-4-15, Novus Biologicals, Littleton,

361 CO) at a 1:1000 dilution in blocking solution at 4°C overnight. Midguts were washed five times
362 via direct tube transfer in PBS+T before staining with the secondary antibody (Goat anti-mouse
363 Alexa Fluor 594, Abcam, Cambridge, United Kingdom) at a 1:1000 dilution in blocking solution
364 for 1 hour at room temperature. Midguts were washed 5X in PBS+T before mounting onto
365 microscope slides with ProLong Gold Antifade Mountant with DAPI. ZIKV detection and
366 quantification were done by manually counting distinct red patches, using blue DAPI as a
367 reference for individual cells across three slices of a single midgut, and averaging the values.
368 FITC, DAPI, and Alexa Fluor 594 were each visualized (and FITC was quantified) in their
369 respective channels before merging.
370
371 **Histologic analyses**
372 Histological images at 20X and 40X magnification were used to visualize mosquito anatomy and
373 to validate that sample preparation for immunofluorescence microscopy did not mechanically
374 disrupt the midgut, which could affect interpretations of midgut integrity. Blood-engorged
375 mosquitoes with their legs and wings removed were placed in 10% formalin in PBS for 24 h,
376 then encased within tissue cassettes (Thermo Fisher Scientific, Emeryville, CA) submerged in
377 70% ethanol. Samples were submitted to the University of California, Davis School of Veterinary
378 Medicine Anatomic Pathology Service, where whole mosquitoes were paraffin-embedded, cut
379 into 5 μm slices, and stained with hematoxylin and eosin (H&E). Slides were visualized at 20X
380 and 40X magnification under a Zeiss Axio Vert A1 inverted light microscope connected to an
381 Axiocam 208 color camera (Carl Zeiss, Jena, Germany). Although we evaluated sections from 5
382 individual mosquitoes each for No Abx, AbxA, and AbxPA, groups, we used histology readouts
383 as a qualitative approach to evaluate gross midgut anatomy. Anatomical structures were
384 identified by an expert in mosquito anatomy. Representative images for each of the three
385 groups are shown.
386

387 **Blood digestion analyses**

388 Quantification of hemoglobin in bloodfed mosquitoes using a hemoglobin colorimetric detection
389 kit (Thermo Fisher Scientific, Emeryville, CA) was used to measure blood digestion. At 0, 1, 2,
390 or 3 dpf, 10-12 individual mosquitoes each in No Abx, AbxA, and AbxPA, groups exposed to
391 blood only or ZIKV-spiked blood with their legs/wings removed were homogenized in 300 µL
392 hemoglobin diluent, 100 µL of which was used for the assay, per manufacturer's instructions.
393 Absorbance at 570 nm was measured from each sample once in 96-well plates using a
394 SpectraMax iD5 Multimode Microplate Reader (Molecular Devices, San Jose, CA). Absorbance
395 values were converted to hemoglobin concentrations via standard curves generated from
396 hemoglobin standards and serial dilutions of fresh sheep blood used for feeding. Output
397 absorbance values were normalized to homogenized, non-bloodfed mosquito negative controls,
398 and units were converted to µg per mosquito body. Before conducting the assay, we validated
399 and optimized this kit for bloodfed mosquitoes, including ensuring that heat incubation of
400 mosquitoes at 60°C for 10 minutes to destroy ZIKV infectivity did not affect hemoglobin
401 quantification (**Figure S1**).

402

403

404 **RNA sequencing and bioinformatics**

405 Mosquito messenger RNA was sequenced to determine differences in gene expression
406 associated with antibiotic treatments after ZIKV exposure. Total RNA was extracted from adult
407 female mosquitoes in triplicate pools of 5 individuals each for No Abx, AbxA, and AbxPA groups
408 using the Quick-RNA Tissue/Insect Microprep kit (Zymo Research, Irvine, CA, USA). RNA
409 extracts were eluted into 20 µL elution buffer and quantified using a Qubit fluorometer RNA
410 high-sensitivity (HS) assay (Thermo Fisher Scientific, Emeryville, CA). Approximately 1 µg of
411 total RNA per sample was submitted to Genewiz Inc. from Azenta Life Sciences (Chelmsford,
412 MA, USA), where poly-A selection, library preparation, and paired-end Illumina HiSeq 2x150 bp

413 were performed. Raw FASTQ data files were received and treated by filtering and trimming
414 reads with *cutadapt v3.2* (37) using default parameters (Quality cutoff >= 30, minimum length of
415 100 bp). Next, *FastQC* (38) was used to visualize quality scores. Processed reads were then
416 aligned to version AaegL5.2 of an *Ae. aegypti* transcriptome obtained from VectorBase (39)
417 using *bowtie2 v2.4.3* (40). Aligned reads were imported into R and analyzed with the package
418 Differential Expression Analysis using *DESeq2 v1.28.1* (41). Default parameters were used to
419 determine the differential abundance of each gene using an input experimental design of
420 [antibiotic treatment + bloodmeal status + interaction]. Genes with a significance (adjusted P
421 value) below 0.05 are reported. Significant genes were mapped to biological processes using
422 the Gene Ontology program *topGO* (42).
423
424 **Statistical analyses**
425 The 16S:RPS17 ratios to verify antibiotic efficacy from qPCR were compared using a Kruskal-
426 Wallis test with multiple comparisons for adult mosquito treatments and across life stages.
427 Differences in ZIKV infection, dissemination, and transmission rates between groups were
428 determined using Fisher's exact tests. Differences in ZIKV RNA levels in bodies, legs/wings,
429 and saliva across groups were assessed by Kolmogorov-Smirnov tests. Differences in
430 hemoglobin levels across groups were evaluated using Kruskal-Wallis tests with repeated
431 measures. Relative fluorescence values and numbers of nuclei were averaged in triplicate and
432 tested for significance by one-way ANOVA tests. Differences in numbers of ZIKV-infected
433 midgut epithelial cells were assessed using Fisher's exact tests. For RNA-Seq, statistical tests
434 built into the *DESeq2* package were used. In short, each sample was normalized by library size,
435 and the geometric mean for each gene was calculated. Negative binomial generalized linear
436 models (glm) were used for each gene to determine differential expression across antibiotic-
437 treated groups. The Wald test was used for statistical significance, with correction for the false
438 discovery rate (FDR) set to 0.05. For principal components analyses (PCA), variance stabilized

transformation was implemented before plotting. All statistical analyses, except RNA-Seq, were performed using GraphPad PRISM 9.0.2 (GraphPad Software, San Diego, CA). RNA-Seq analyses were conducted in R *v3.6* (43). P-values of less than 0.05 were considered statistically significant. For gene ontology (GO), Fisher's exact tests were used on GO terms and corrected for the false discovery rate.

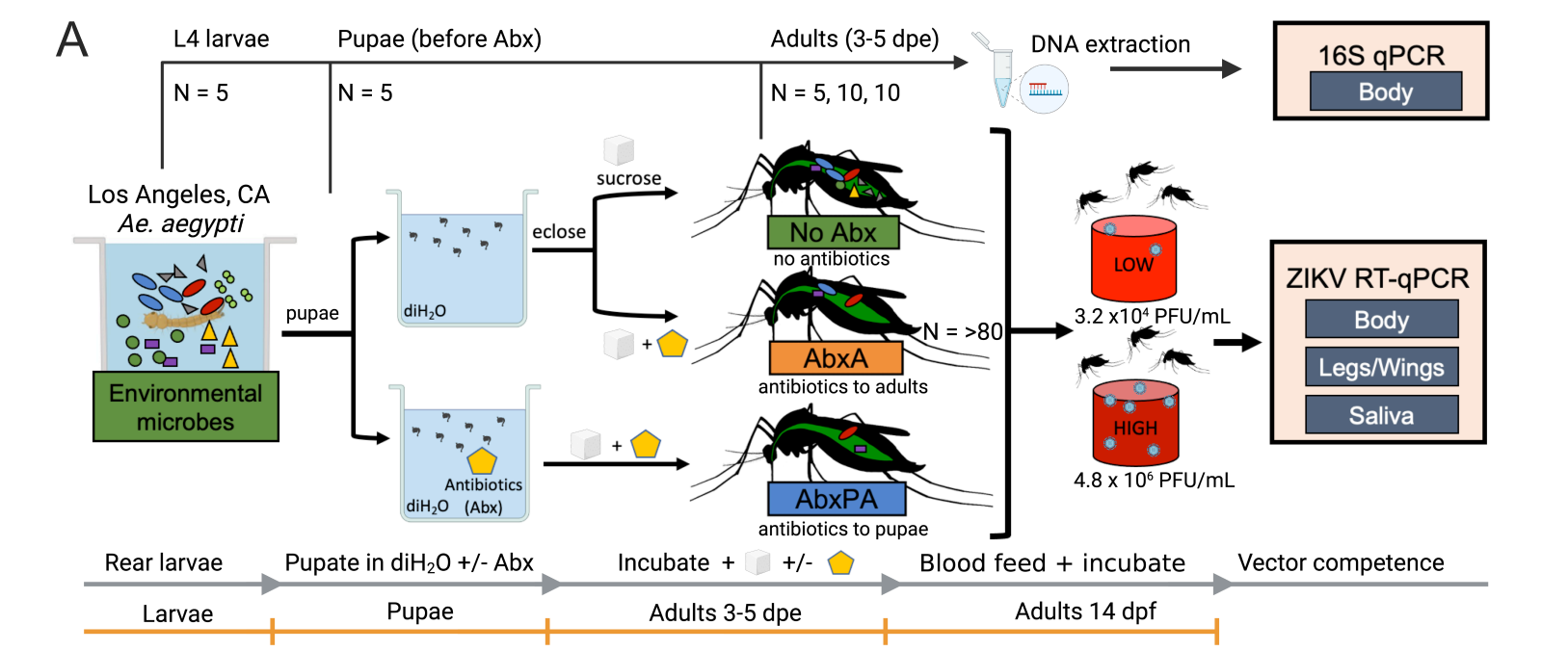
Data availability. Raw sequencing data are available from the NCBI Sequence Read Archive under BioProject entry PRJNA818687. Scripts for the RNA-Seq analysis are available on Github (DOI: 10.5281/zenodo.7259822).

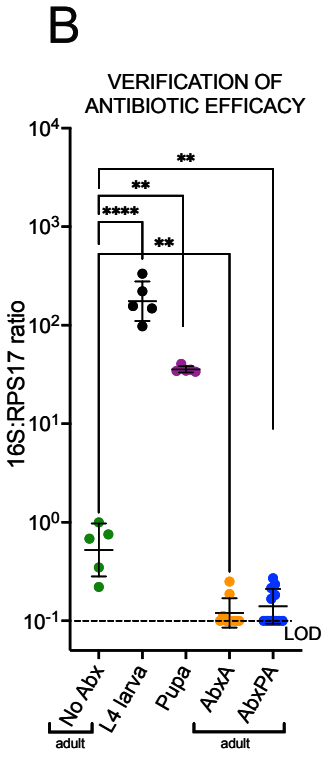
Results

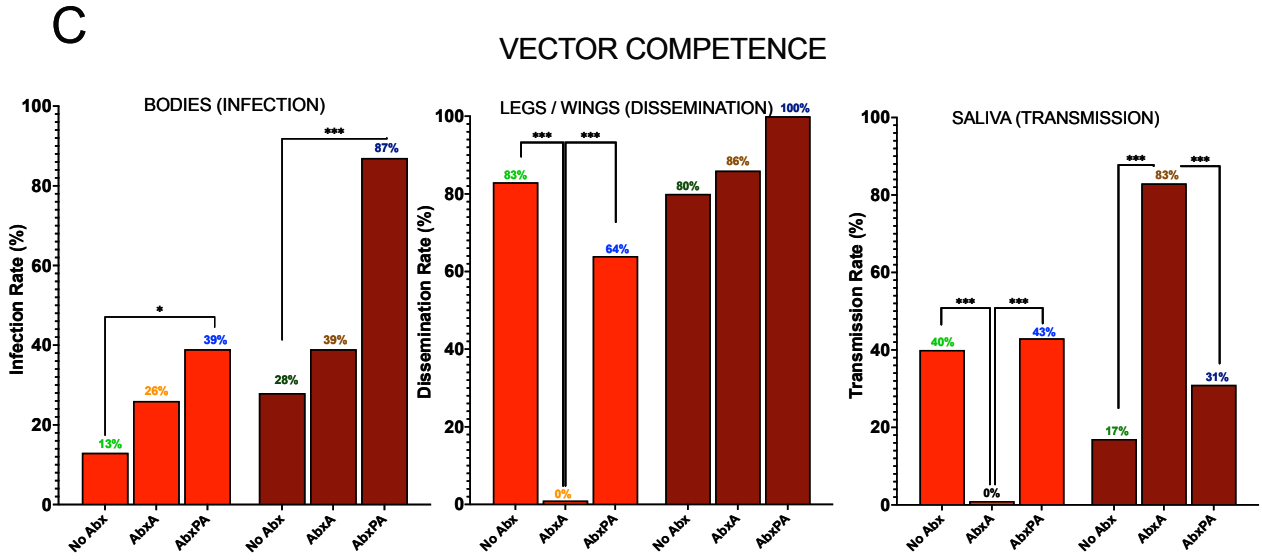
Reduced *Ae. aegypti* susceptibility to ZIKV conferred by microbial exposure is life stage-dependent

To understand whether reduced ZIKV vector competence after larval development in water with microbes that we observed previously (15) is conferred transstadially from larvae to pupae to adults, we first determined whether exposure to microbes throughout life (i.e. at larval, pupal, and adult stages) is required to reduce susceptibility of *Ae. aegypti* to ZIKV. *Ae. aegypti* larvae were reared in laboratory water spiked with microbes that were collected from water in cemeteries that we characterized in a previous study (15) which contained over 1000 bacterial species from the phyla *Bacteroides*, *Firmicutes*, and *Proteobacteria*. Control mosquitoes were not provided antibiotics (No Abx) at any life stage. To assess the effects of microbe depletion at pupal and adult life stages, cohorts of **p**upae and the **a**dults they developed into were administered an antibiotic (Abx) cocktail (AbxPA) that was provided to pupae in water and orally in sucrose to adults from eclosion to 3 days of age (**Figure 1A**). To study the effects of antibiotic treatment on adult mosquitoes that were not antibiotic treated at earlier life stages, we also

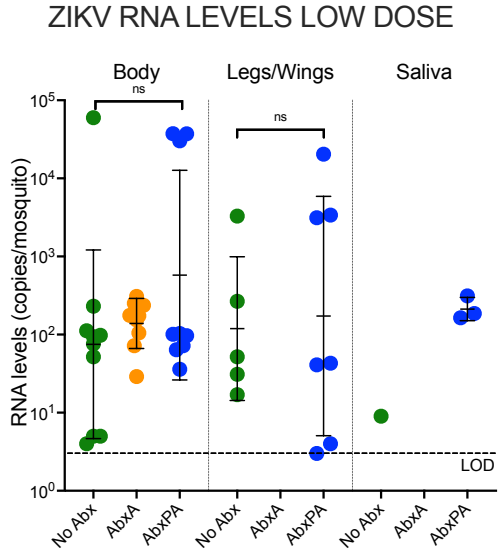
465 included cohorts where **a**dults were provided antibiotics (AbxA) orally for 3-5 days post-eclosion.
466 After each treatment, mosquitoes were orally presented once at 3-5 days post-eclosion with low
467 or high-dose ZIKV-spiked bloodmeals for vector competence assessments. To maximize
468 bloodfeeding success, sugar was withdrawn from mosquitoes 24 hours prior to bloodmeal
469 presentation. We first assessed the efficacy of antibiotic treatment via qPCR quantification of
470 16S DNA. Antibiotics significantly reduced the number of prokaryotes in all treated groups.
471 (Kruskal-Wallis [K-W] with multiple comparisons, P = 0.002 between No Abx and AbxA [adj. P =
472 0.002] and No Abx and AbxPA [adj. P = 0.006]) (**Figure 1B**). Significantly higher 16S:RPS17
473 ratios, our proxy for prokaryote levels, were observed in No Abx L4 larvae and pupae relative to
474 adult mosquitoes (K-W multiple comparisons, P = <0.0001 between No Abx and larva [adj. P =
475 0.0012] and No Abx and pupa [adj. P = 0.0079]). This is consistent with ours (15) and others'
476 (7,13) previous observations that microbial abundance decreases during mosquito
477 development. Antibiotic treatment of pupae and adults significantly increased ZIKV infection
478 rates at both low (3.2×10^4) and high (4.8×10^6) PFU/ml ZIKV bloodmeal doses compared to
479 no antibiotic treatment (Fisher's exact test, low dose: P 0.0398, high dose: P < 0.0001).
480 Antibiotic treatment of adults marginally but not significantly increased infection rates compared
481 to no antibiotic treatment (Fisher's exact test: P = >0.05) At the low ZIKV dose, antibiotic
482 treatment of adults resulted in no disseminated infections in mosquitoes whose bodies were
483 infected, which is significantly lower than 83% dissemination in mosquitoes that were not
484 antibiotic treated or that were antibiotic treated as pupae and adults (Fisher's exact test,
485 P<0.001). At the high ZIKV dose, dissemination rates were not significantly different across
486 antibiotic treatments (Fisher's exact test: P = >0.05). Significantly more mosquitoes treated with
487 antibiotics as adults that had disseminated infections transmitted ZIKV compared to mosquitoes
488 that were not antibiotic treated or that were antibiotic treated as both pupae and adults (Fisher's
489 exact test: P = 0.002) (**Figure 1C**). In the high ZIKV dose groups, levels of ZIKV RNA in saliva
490 were higher in AbxPA compared to AbxA (Kolmogorov-Smirnov test, P = 0.009), but not higher

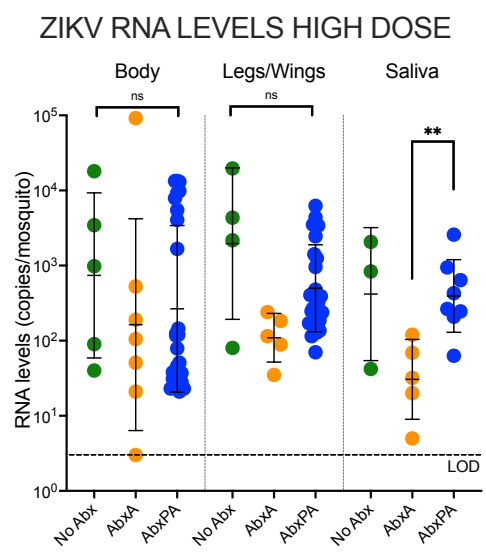






Generation	Group	ZIKV dose (PFU/mL)	Infection		Dissemination		Transmission	
			Rate	P value	Rate	P value	Rate	P value
F_{20}	No Abx	3.2×10^4	13% (6/45)	0.0398	83% (5/6)	<0.001	40% (2/5)	<0.001
	AbxA		26% (9/34)		0% (0/9)		NA	
	AbxPA		39% (11/28)		64% (7/11)		43% (3/7)	
F_{22}	No Abx	4.8×10^6	28% (5/18)	<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)	



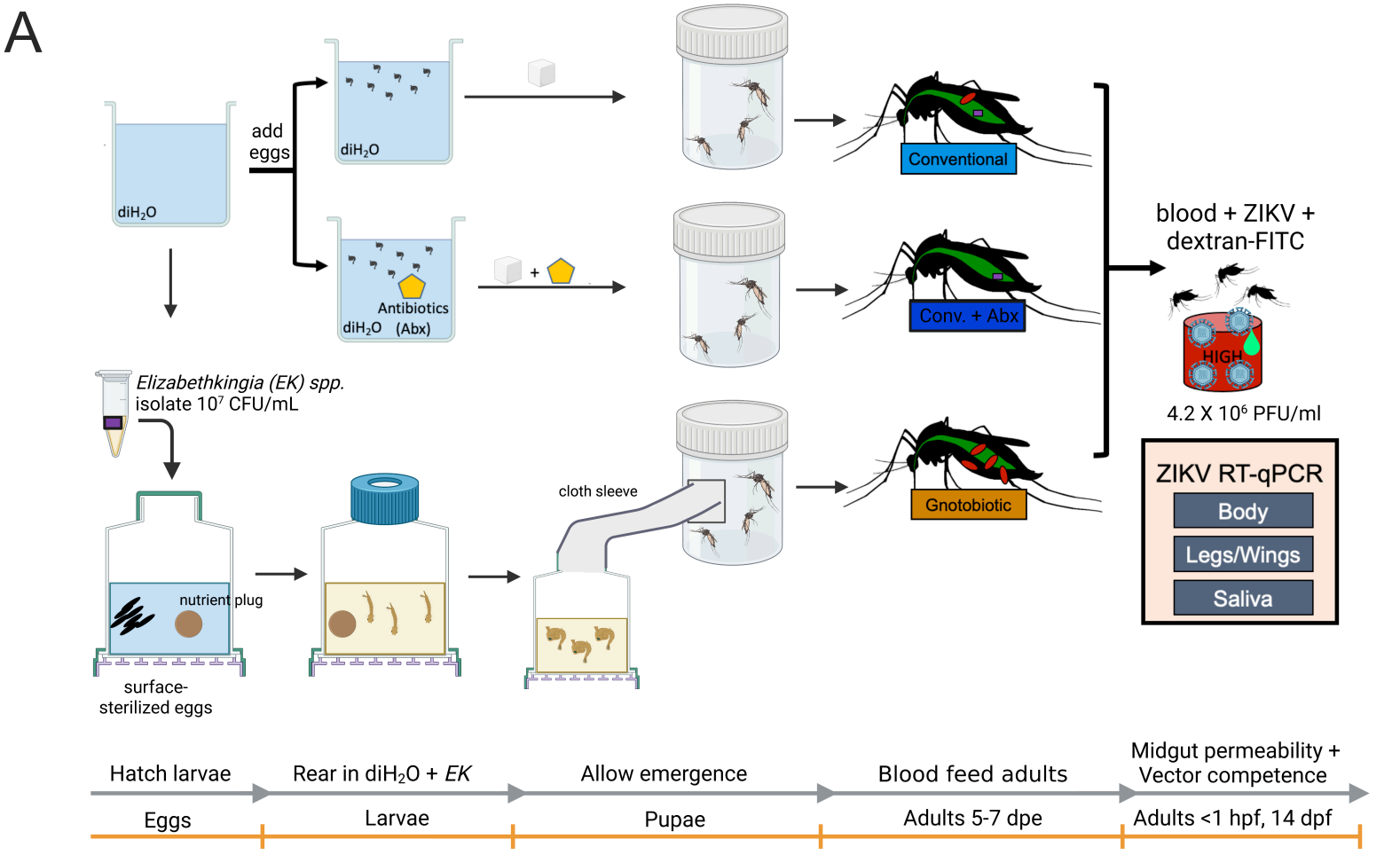


491 than No Abx (Kolmogorov-Smirnov test, P = 0.71) (**Figure 1E;** transmission in the low dose
492 [**Figure 1D**] was not evaluated statistically due to low numbers). Collectively, these results
493 show that antibiotic treatment during both pupal and adult *Ae. aegypti* life stages increases rates
494 of ZIKV infection after oral exposure to two ZIKV doses and, at the higher dose, the magnitude
495 of ZIKV transmitted in saliva. The larger effect of AbxPA compared to AbxA treatment on ZIKV
496 vector competence shows that microbes acquired by larvae must be maintained as adults to
497 reduce ZIKV susceptibility and suggest that microbe-mosquito interactions that influence ZIKV
498 susceptibility need to occur in the aqueous phase of mosquito life.
499
500 **Figure 1. Microbe depletion using antibiotic treatment of *Ae. aegypti* pupae and adults from**
501 **Los Angeles, California, increases ZIKV susceptibility. A) Experimental design**. Microbes from
502 cemetery headstones were added to tap water during rearing of *Ae. aegypti* larvae from Los
503 Angeles, CA. Pupae in the antibiotic (Abx) groups were treated by adding an antibiotic cocktail to
504 pupal water and orally in sugar to adults (AbxPA). Antibiotics were delivered orally in sugar to adult
505 Abx (AbxA) groups. No antibiotics (No Abx) groups received no antibiotics at any life stage.
506 Mosquitoes from each group and life stage were sequenced to verify the efficacy of antibiotic
507 treatment. Adult mosquitoes 3-5 days of age were presented with either of 2 doses of ZIKV spiked
508 into bloodmeals, and ZIKV vector competence was assessed by measuring ZIKV RNA levels in
509 bodies to determine infection, legs/wings of individuals with infected bodies to assess dissemination,
510 and in saliva of individuals with infected legs/wing to measure transmission, which are presented as
511 rates in cohorts and mean levels from individuals. **B) Antibiotic treatment reduces bacterial DNA**
512 **in mosquitoes.** Bacteria was quantified in mosquitoes by 16S qPCR. Each point represents a single
513 mosquito (N larvae, pupae, No Abx = 5; N AbxA, AbxPA = 10). 16S copies were normalized to the
514 *Ae. aegypti* RPS17 gene. The limit of detection (LOD) was a 0.1 16S:RPS17 ratio; below this value,
515 16S was not detected. Statistical significance across all groups was determined by a Kruskal-Wallis
516 test with multiple comparisons. **C) ZIKV vector competence of microbe-depleted antibiotic**

517 **treated mosquitoes.** Bar graphs show ZIKV infection, dissemination, and transmission rates from
518 cohorts as determined by ZIKV RNA RT-qPCR. The summary table shows the same data in the bar
519 graphs. Rates are calculated as the number of bodies, legs/wings, or expectorates that yielded at
520 detectable ZIKV RNA, divided by the total number of individuals that ingested blood within a cohort,
521 were infected, or developed disseminated infections, respectively. Statistical significance across all
522 treatments was determined by Fisher's exact tests. Vector competence experiments at each dose
523 were conducted once where mosquitoes in different cohorts were presented the same bloodmeal.
524 **ZIKV RNA levels in mosquito tissues after ingestion of low (D) or high (E) dose ZIKV-spiked**
525 **bloodmeals**. Each dot shows the mean value for an individual mosquito based on triplicate
526 measurements. The average LOD across all experiments was 3 copies per mosquito and is
527 represented as the dotted line. Mosquitoes without detectable ZIKV RNA are not shown.
528 Kolmogorov-Smirnov tests were used to compare mean RNA levels. For B, D, and E, geometric
529 means are shown, and error bars show standard deviations. *Denotes $P < 0.05$ **denotes $P < 0.01$,
530 *** denotes $P < 0.001$, ns is not significant at $P<0.05$.
531
532 **Antibiotic treatment does not impact midgut permeability or ZIKV vector competence**
533 Next, to understand whether increased ZIKV vector competence in antibiotic treated compared
534 to untreated *Ae. aegypti* is due to antibiotic treatment that could impact mosquito physiology and
535 influence ZIKV susceptibility, or the dysbiotic environment created by antibiotics that results
536 from ablation of most microbes, we compared ZIKV vector competence and midgut integrity in
537 mosquitoes reared in water without environmental microbes that was either treated with
538 antibiotics (Conventional + Abx) or not antibiotic treated (Conventional), as well as in gnotobiotic
539 mosquitoes that were not antibiotic treated (Gnotobiotic) (**Figure 2A**). For the antibiotic treated
540 group, antibiotics were administered to both larval water and adults in sugar water using the
541 same design as for the AbxPA cohorts. Gnotobiotic mosquitoes have a limited microbiome
542 compared to mosquitoes reared in environmental water, and, unlike axenic mosquitoes that do

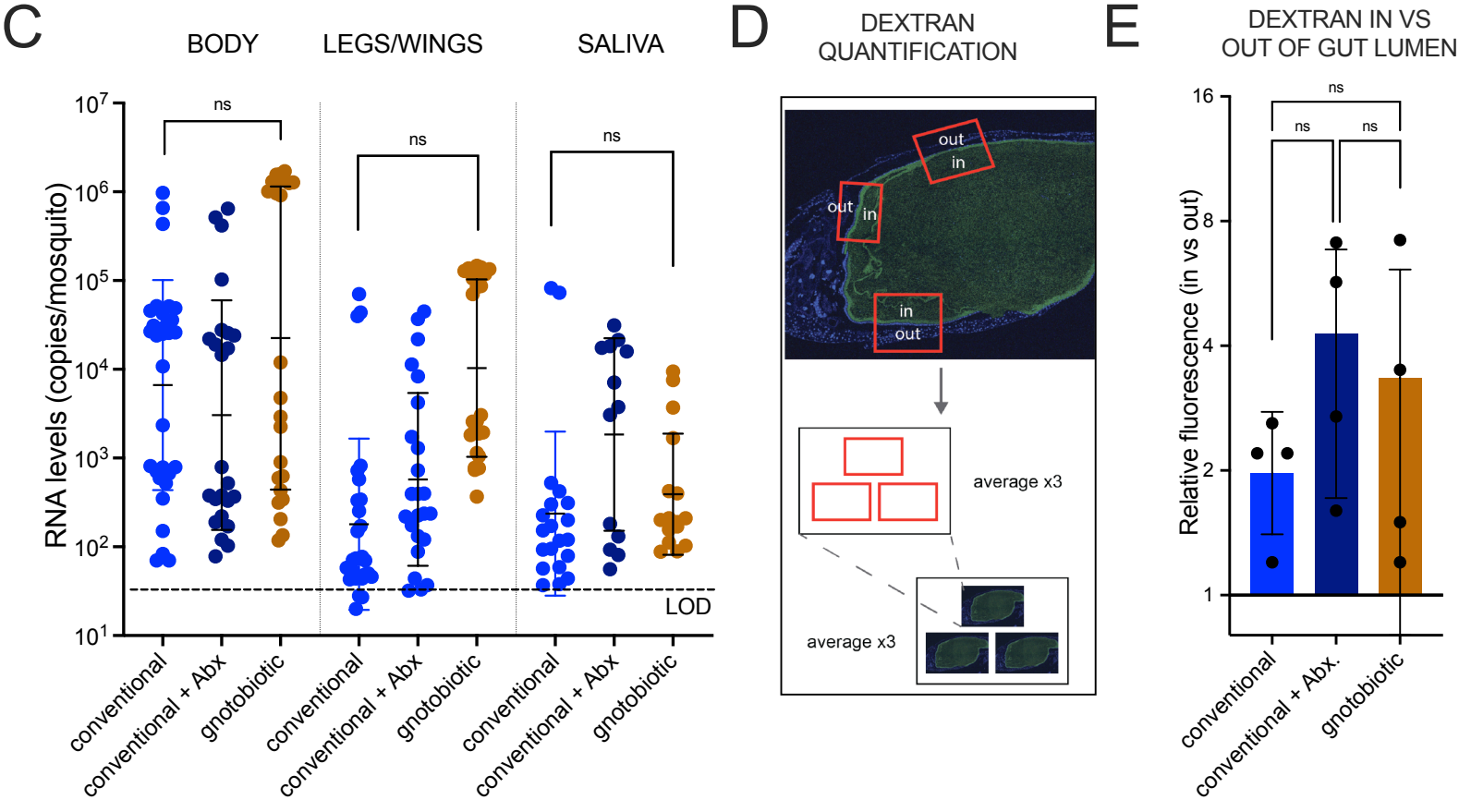
543 not possess any microbes, do not suffer from the lack of microbiome-derived essential nutrients
544 that can impact fitness (44). Gnotobiotic *Ae. aegypti* were generated by inoculating larval water
545 with an *Elizabethkingia spp.* culture isolated from our colonized *Ae. aegypti* from Los Angeles.
546 *Elizabethkingia spp*. was selected since it is ubiquitous in the environment (44) and represents a
547 dominant bacterial sequence in larval and adult *Ae. aegypti* (15). The three groups were
548 presented with high dose ZIKV-spiked blood with and without fluorescent dextran (FITC-
549 dextran). A subset of mosquitoes were evaluated within 1 hour of bloodfeeding for midgut
550 permeability assays and the remaining bloodfed individuals were assessed for vector
551 competence at 14 days. The purpose of adding FITC-dextran, which is a small molecule that
552 can traverse cell membranes, was to evaluate whether antibiotics or a gnotobiotic environment
553 influence midgut permeability, which is a proxy for midgut integrity. Infection, dissemination, and
554 transmission rates were not statistically different across any group (Fisher's exact test: P >0.09)
555 (**Figure 2B**). Mean ZIKV RNA levels in ZIKV-positive bodies, legs/wings, and saliva were also
556 not statistically different across groups (Kolmogorov-Smirnov test, P >0.05) (**Figure 2C**). We
557 visualized and quantified the relative fluorescent intensity of FITC-dextran inside versus outside
558 the midgut (**Figure 2D**), where higher fluorescence inside versus outside is indicative of greater
559 midgut integrity. For this assessment, four to five mosquitoes were examined per group, and
560 three separate sections for each mosquito were randomly selected, quantified, and averaged in
561 mosquitoes immediately after ingestion of a bloodmeal containing ZIKV. Dextran permeability
562 measurements showed no significant difference across groups in mean fluorescence inside
563 versus outside of the midgut (Kolmogorov-Smirnov test, P >0.05) (**Figure 2E**). These data
564 indicate that antibiotic treatment itself does not perturb midgut permeability or affect ZIKV vector
565 competence. The 100% infection and dissemination rates and high transmissibility (67%) of
566 ZIKV by gnotobiotic mosquitoes parallels our data from antibiotic-treated cohorts with reduced
567 microbial diversity and show that a spectrum of bacterial species not present in the gnotobiotic
568 mosquitoes is required to reduce vector competence. Together these data further implicate

569 mosquito microbes in modulating ZIKV susceptibility and justify use of antibiotics to reduce the
570 mosquito microbiome, which is the study design we employed for the experiments that follow to
571 further understand how microbial exposure in mosquito development impacts ZIKV vector
572 competence.
573
574 **Figure 2: A) Experimental design.** *Aedes aegypti* from Los Angeles, CA, were reared in laboratory
575 tap water without any antibiotics (Conventional, 'Conv.'), with antibiotics (Conv. + Abx), where
576 antibiotics were also provided orally to adults in sugar, or with *Elizabethkingia* (EK) *spp*.
577 (Gnotobiotic) bacteria isolated from our *Ae. aegypti* colony. Mosquito eggs in the Gnotobiotic group
578 were surface sterilized before hatching and reared in sterile flasks. Adult mosquitoes 3-5 days of age
579 were presented with high dose ZIKV spiked into bloodmeals, and ZIKV vector competence was
580 assessed by measuring ZIKV RNA levels in bodies to determine infection, legs/wings of individuals
581 with infected bodies to assess dissemination, and in saliva of individuals with infected legs/wing to
582 measure transmission, which are presented as rates in cohorts and mean levels from individuals.
583 The bloodmeals also contained 1 µg dextran-FITC (green drop) for midgut permeability
584 assessments. **B) ZIKV vector competence** data comparing infection, dissemination, or
585 transmission rates in Conventional, Conventional + Abx, and Gnotobiotic *Ae. aegypti* groups**.** Vector
586 competence experiments were conducted once where mosquitoes in different cohorts ingested the
587 same bloodmeal. Statistical significance across all treatments was determined by Fisher's exact
588 tests. **C) ZIKV RNA levels in mosquito tissues after ingestion of high dose ZIKV-spiked**
589 **bloodmeals**. Each dot shows the mean value for an individual mosquito based on triplicate
590 measurements. The average LOD across all experiments was 62 copies per mosquito and is
591 represented as the dotted line. Mosquitoes without detectable ZIKV RNA are not shown. **D) Dextran**
592 **fluorescence quantification approach** that was used to generate data in panel E. Fluorescence
593 ratios were calculated in triplicate from randomly selected areas in a single midgut slice. Dextran
594 permeation was calculated as the ratio of fluorescence intensity inside the gut lumen to fluorescence
595 intensity outside the gut lumen for randomly selected regions of the midgut (red rectangles). **E)**



B

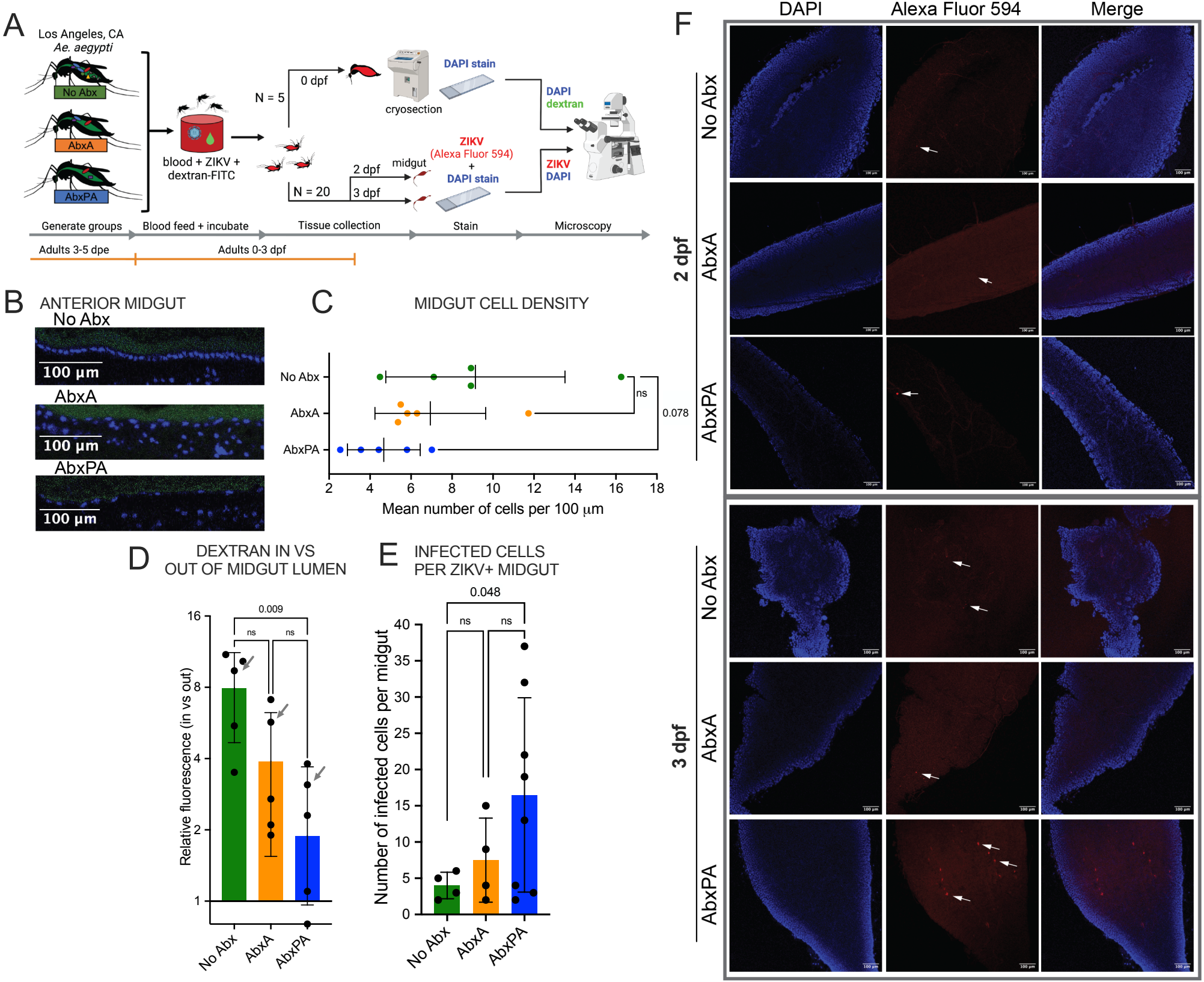
Generation	Group	ZIKV dose (PFU/mL)	Infection		Dissemination		Transmission	
			Rate	P value	Rate	P value	Rate	P value
F_{26}	conv.	4.2×10^6	94% (31/33)	0.87	90% (28/31)	0.09	71% (20/28)	0.41
	conv. + Abx		92% (24/26)		100% (24/24)		54% (13/24)	
	gnotobiotic		100% (24/24)		100% (24/24)		67% (16/24)	



596 **Mosquito midgut permeability assessed by relative dextran inside versus outside the midgut**
597 **lumen**. Each symbol represents 9 measurements from one mosquito, N=4 mosquitoes per
598 treatment. For C and E geometric means are shown, and error bars show standard deviations.
599 Kolmogorov-Smirnov tests were used to compare mean RNA or fluorescence levels. ns is not
600 significant at P<0.05.

601 **Microbial depletion via antibiotic treatment increases *Aedes aegypti* midgut permeability**

602 To understand how microbial exposure of larvae, pupae, and adults reduce susceptibility of *Ae.*
603 *aegypti* to ZIKV, we assessed permeability by directly visualizing and enumerating midgut
604 epithelial cell density in DAPI-stained midgut sections. We also applied the midgut integrity
605 assays (**Figure 2D**) used to evaluate ZIKV-exposed mosquitoes that were microbe-exposed or
606 microbe-reduced via antibiotic treatment. Our rationale for focusing on the midgut is that it
607 represents the first barrier to infection of *Ae. aegypti* by ZIKV after oral exposure. We asked
608 whether higher infection rates in AbxPA and AbxA groups compared to the No Abx group are
609 explained by modified epithelial structure or reduced midgut integrity resulting from microbe
610 depletion. We compared fluorescent intensity across antibiotic-treated groups immediately after
611 ingestion of a bloodmeal with ZIKV (**Figure 3A**). All mosquitoes had only bloodfed once,
612 eliminating potential confounding effects of repeated bloodfeeding on midgut integrity (45) and
613 were fully engorged; hemoglobin measurements (see below) also revealed no statistically
614 significant differences in mean ingested bloodmeal volumes across groups. The midgut
615 epithelium was less organized, as evidenced by reduced contiguity in DAPI-stained midgut
616 epithelial cell nuclei in both AbxA and AbxPA compared to No Abx groups (**Figure 3B**). These
617 qualitative observations of structural disorganization of the midgut were also supported
618 quantitatively, where marginally fewer cells in a 100 μm length of midgut epithelium were
619 detected in AbxPA compared to No Abx groups (**Figure 3C**, P = 0.078). The relative dextran-
620 FITC fluorescence inside versus outside of the midgut lumen for the AbxPA group was
621 significantly lower than the No Abx group (P = 0.009), and the AbxA group trended lower


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
ANTERIOR MIDGUT
No Abx
AbxA
AbxPA
100 µm
C
MIDGUT CELL DENSITY
No Abx
AbxA
AbxPA
ns
0.078
Mean number of cells per 100 µm
D
DEXTRAN IN VS OUT OF MIDGUT LUMEN
0.009
ns
Relative fluorescence (in vs out)
E
INFECTED CELLS PER ZIKV+ MIDGUT
0.048
Number of infected cells per midgut
F
DAPI
Alexa Fluor 594
Merge
2 dpf
3 dpf

622 (although not significantly) than the No Abx group (**Figure 3D**). To rule out physical rupture or
623 other disruptions to the midgut during sample preparation that may have reduced structural
624 integrity, we treated and stained midgut sections from a separate cohort of similarly treated
625 mosquitoes with DAPI or H&E. At 10X, 20X, and 40X magnification, midgut epithelia across
626 treatments did not appear structurally different (**Figures 3F**, **S1**). Together these data support a
627 process where antibiotic treatment to ablate microbes in pupal and adult *Ae. aegypti* life stages
628 disrupts midgut cell architecture and increases midgut permeability in adult mosquitoes after
629 ingestion of ZIKV-spiked bloodmeals.
630
631 **Figure 3. Antibiotic-mediated microbe depletion increases *Aedes aegypti* midgut**
632 **permeability. A) Schematic of bloodfeeding experiment and sample preparation for**
633 **immunofluorescence microscopy.** *Aedes aegypti* from Los Angeles, CA were reared as in Figure
634 1A to generate three groups of mosquitoes: no antibiotics (No Abx), antibiotics as adults (AbxA), and
635 antibiotics as larvae, pupae, and adults (AbxPA). Adult mosquitoes aged 3-5 days were presented
636 with a bloodmeal containing 1 mg/mL dextran-FITC (green) and 3.3 x 10^6 PFU/mL ZIKV. Whole
637 mosquitoes were harvested within 1 hour of feeding at 0 days post-feed (dpf), cryosectioned, DAPI
638 stained, and examined using fluorescence microscopy. Midguts from the same cohorts were
639 harvested at 2 and 3 dpf, and ZIKV antigen and DAPI were stained before fluorescence microscopy.
640 **B) Microbe depletion qualitatively disrupts regularity of DAPI staining in midgut epithelium of**
641 **AbxA and AbxPA compared to No Abx mosquitoes.** Anterior-zoomed cross sections of whole,
642 *Ae. aegypti* at 0 dpf 30 to 60 minutes post-engorgement visualized at 10X magnification. **C)**
643 **Antibiotic treatment of pupae and adults marginally reduces midgut cell density after AbxPA**
644 **compared to no Abx treatments.** Cell density was calculated as the number of cell nuclei (blue)
645 per 100 μm length of gut. Each symbol represents one mosquito. **D) Microbe depletion of pupae**
646 **and adults increases midgut permeability, evidenced by reduced relative dextran inside**
647 **versus outside the gut lumen in AbxPA compared to No Abx mosquitoes**. Dextran permeation

648 was calculated as the ratio of fluorescence intensity inside the gut lumen to fluorescence intensity
649 outside the gut lumen using the same approach as in Figure 2D. Each symbol represents 9
650 measurements from one mosquito. Grey arrows indicate the same mosquito represented in panel B.
651 **E) Antibiotic treated pupae and adults have more ZIKV-infected midgut epithelial cells**. The
652 number of ZIKV-infected cells, as determined by ZIKV antigen staining, were counted for each ZIKV-
653 positive midgut on 2 and 3 dpf. Data from both days is shown. Each symbol represents one
654 mosquito. **F) Antibiotic treated ZIKV-positive midguts at 2 and 3 dpf.** Midguts examined under
655 immunofluorescence at 10X magnification, at 2 and 3 dpf. Cell nuclei (blue) and ZIKV antigen (red)
656 are shown with the combined (merged) channels. Images reflect representative observations for
657 each antibiotic treatment. Arrows point to ZIKV antigen positive cells. For C lines show means and
658 for C, D and E error bars show standard deviations. ns is not significantly different at $p<0.05$.
659

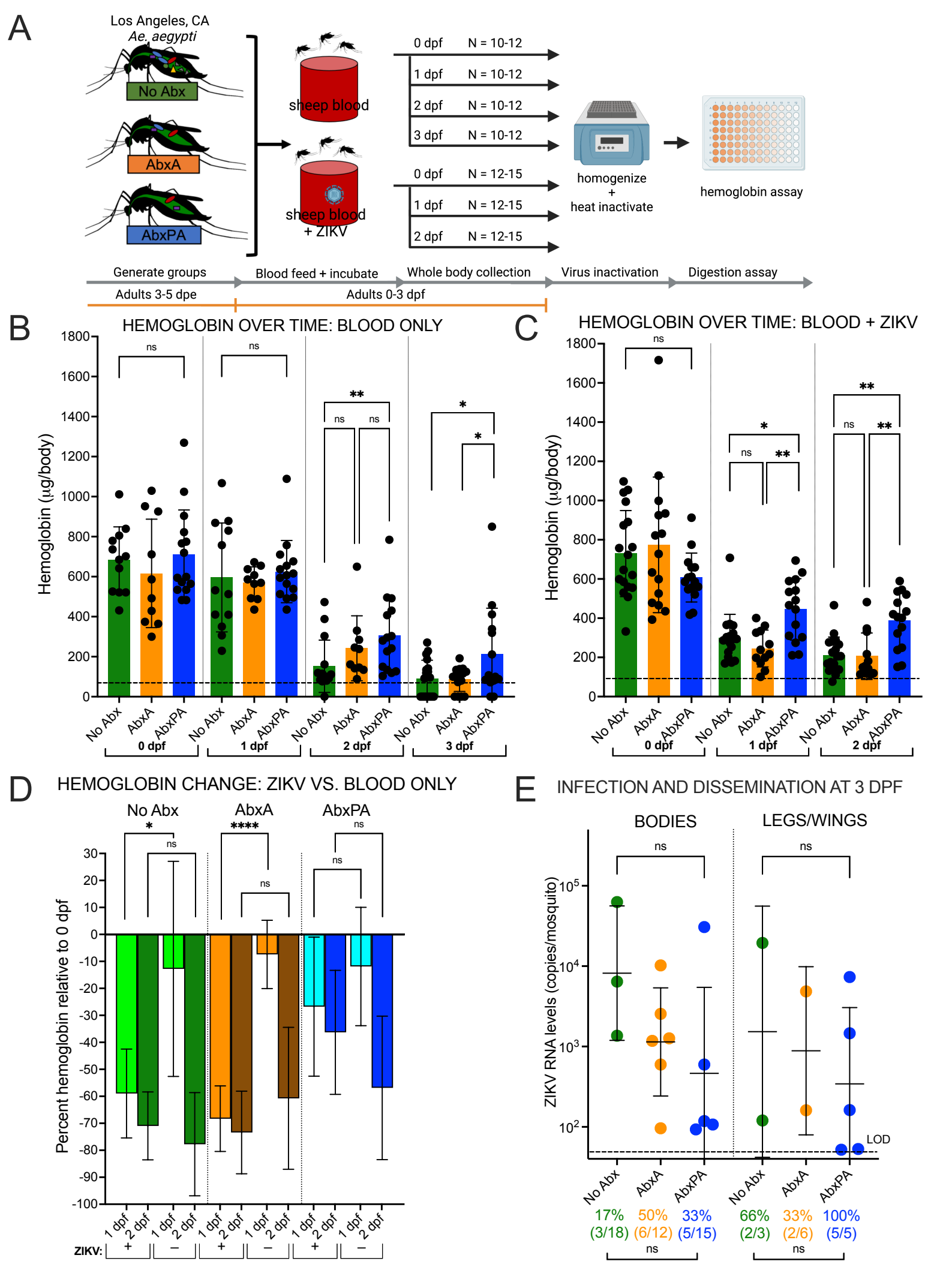
660 **Microbe depletion via antibiotic treatment increases ZIKV infection of the midgut**
661 **epithelium**

662 Next, we asked whether disruptions to midgut integrity with antibiotic treatment translate to
663 greater infection success, measured as higher numbers of ZIKV-infected midgut epithelial cells.
664 We detected ZIKV in midguts by staining sections with a flavivirus binding antibody 2 or 3 dpf
665 and counting the total number of cells in each midgut that were stained with fluorescent anti-
666 ZIKV antibody (**Figure 3A**). The number of ZIKV-positive cells in midguts was significantly
667 higher in the AbxPA group compared to the No Abx group (Brown-Forsythe ANOVA test with
668 Dunnett's multiple comparisons test, $P = 0.048$) (**Figure 3E**). These data support the role of
669 microbes in larvae and pupae in maintaining the integrity of the mosquito midgut which
670 associates with reduced midgut epithelium infection by ZIKV.
671

672 **Microbial depletion via antibiotic treatment impairs blood digestion**

673 Since midgut integrity can affect physiology of blood digestion in addition to ZIKV susceptibility,
674 we next assessed whether elevated midgut permeability stemming from microbe reduction by
675 antibiotic treatment affects blood digestion. We employed a commercially available hemoglobin
676 colorimetric assay as a proxy for blood protein digestion by quantifying the reduction of
677 hemoglobin levels in blood-engorged mosquitoes on 1 and 2 dpf compared to immediately after
678 feeding on 0 dpf. Before performing the assay, which is typically used for human blood, we first
679 validated and optimized the approach for use with homogenized mosquitoes that ingested
680 sheep bloodmeals. We observed that sheep blood absorbance varies linearly as a function of
681 hemoglobin concentration, like the kit standard (**Figure S2A**) and that absorbance decreases
682 reproducibly in serially diluted mosquitoes at both 560 and 580 nm wavelengths. The assay was
683 unaffected by the 10-minute 60°C heat treatment of homogenized fed mosquitoes used to
684 inactivate ZIKV infectivity in the samples (**Figure S2B**). Blood-engorged females were
685 homogenized and assayed for hemoglobin immediately after bloodfeeding at 0 dpf to assess
686 whether different groups ingested the same bloodmeal volume, as well to measure the kinetics
687 of bloodmeal digestion 1, 2, and 3 dpf (**Figure 4A**). Although hemoglobin levels that
688 approximate bloodmeal size varied in individual mosquitoes, there was no significant difference
689 in mean ingested hemoglobin levels on 0 dpf across antibiotic-treated groups regardless of
690 whether the bloodmeal contained ZIKV, which shows that antibiotic treatment did not influence
691 bloodmeal size (K-W test with multiple comparisons, P = 0.522) (**Figure 4B, C**). After ingestion
692 of blood without ZIKV, mean hemoglobin levels in the AbxPA group were significantly higher
693 than in the No Abx group 2 dpf (K-W test, P = 0.0063). By 3 dpf, mean hemoglobin levels in the
694 AbxPA group were significantly higher than both AbxA and No Abx groups (K-W test with
695 multiple comparisons, adjusted $P_{AbxPA\text{-}No\ Abx}$ = 0.0347, adjusted $P_{AbxPA\text{-}AbxA}$ = 0.051) (**Figure 4B**).
696 The same pattern was observed after ingestion of blood with ZIKV: AbxPA showed a
697 significantly reduced rate of blood digestion compared to No Abx 1 dpf (K-W test with multiple
698 comparisons, adjusted $P_{AbxPA\text{-}No\ Abx}$ = 0.0368, adjusted $P_{AbxPA\text{-}AbxA}$ = 0.0035) and both No Abx

699 and AbxA 2 dpf (K-W test with multiple comparisons, adjusted $P_{AbxPA\text{-}No\ Abx}$ = 0.0037, adjusted
700 $P_{AbxPA\text{-}AbxA}$ = 0.0027) (**Figure 4C**). Hemoglobin levels at 3 dpf for individuals that ingested blood
701 with ZIKV was not assayed since uneven bloodfeeding rates across cohorts and the desire to
702 maintain statistically robust group sizes led us to prioritize use these mosquitoes for infection
703 and dissemination assessments. We also compared mean hemoglobin levels across treatments
704 and days post-feed for mosquitoes that ingested blood with or without ZIKV. Although levels did
705 not differ significantly immediately after bloodmeal ingestion on 0 dpf, mean hemoglobin
706 digestion was significantly faster in No Abx and AbxA on 1 dpf (K-W test, $P_{No\ Abx}$ = 0.0163, P_{AbxA}
707 = 0.0009) but not AbxPA (K-W test, P = 0.25) in ZIKV-exposed mosquitoes compared to
708 mosquitoes that ingested blood without ZIKV (**Figure 4D**). These data show that ablation of
709 larval and pupal microbes reduce bloodmeal digestion, and that blood with ZIKV is digested
710 more quickly than blood lacking virus.
711
712
713 **Figure 4. Bloodmeal digestion kinetics in antibiotic-treated microbe-depleted *Ae. aegypti*. A)**
714 **Experimental design** for detection of hemoglobin in homogenized antibiotic treated mosquitoes that
715 ingested blood with or without ZIKV**.** *Aedes aegypti* from Los Angeles, CA were reared as in Figure
716 1A to generate three groups of mosquitoes: no antibiotics (No Abx), antibiotics as adults (AbxA), and
717 antibiotics as larvae, pupae, and adults (AbxPA). Adult mosquitoes aged 3-5 days were presented
718 with a bloodmeal with or without high dose (2.1 x 10^6 PFU/mL) ZIKV. On day 0 within 1 hour of
719 feeding and at 1, 2 and 3 days post feeding (dpf) a subset of 10-15 mosquitoes were homogenized
720 and heat treated to destroy ZIKV infectivity for bloodmeal volume detection using a hemoglobin
721 assay. Infection and dissemination were assessed 3 dpf. **Hemoglobin levels in *Ae. aegypti* as a**
722 **proxy for bloodmeal digestion in groups that were (B) bloodfed or (c) bloodfed with ZIKV.**
723 Despite ingesting equivalent bloodmeal sizes, microbe-reduction via antibiotic treatment reduced
724 blood digestion in the absence of ZIKV at 2 and 3 dpf. The number of *Ae. aegypti* that ingested


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)

725 blood that were assayed per day is: No Abx = 12, AbxA = 10, AbxPA 15. In mosquitoes that also
726 ingested ZIKV, despite ingesting equivalent bloodmeal sizes, antibiotic treatment reduces blood
727 digestion at 1 and 2 dpf. The number of *Ae. aegypti* that ingested blood spiked with ZIKV that were
728 assayed per day is: No Abx = 18, AbxA = 12, AbxPA = 15. **D) Hemoglobin levels in bloodfed**
729 **versus ZIKV exposed *Ae. aegypti*.** Mosquitoes that ingested ZIKV that were not treated with
730 antibiotics or treated only as adults show increased blood digestion compared to mosquitoes treated
731 with antibiotics as pupae and adults. The bar graphs show the percent reduction in hemoglobin at 1
732 and 2 dpf compared to the 0 dpf, calculated for all Abx groups and between ZIKV exposed and
733 bloodfed only groups. **E) Microbe depletion of pupae and adults does not impact ZIKV**
734 **dissemination rates 3 dpf.** Legs and wings of individual bloodfed females from the same cohort
735 were assayed by ZIKV RT-qPCR at three dpf. Each symbol in B, C and E shows the mean
736 measurement for 1 mosquito. Bars show mean and error bars indicate the standard deviation. *
737 Denotes $P < 0.05$; ** denotes $P < 0.01$; **** denotes $P<0.0001$, ns is not significantly different.
738

739 **Reduced bloodmeal digestion does not influence ZIKV dissemination from the *Ae.***
740 ***aegypti* midgut**

741 We next hypothesized that, in addition to disrupting midgut cell integrity, antibiotic mediated
742 reduction of microbes in mosquitoes and resulting reduced bloodmeal digestion provides the
743 virus more time to bind and enter midgut epithelial cells, manifest as increased ZIKV infection
744 and dissemination very early (3 dpf) after bloodfeeding. However, the rates and magnitude of
745 ZIKV RNA in bodies and legs/wings in antibiotic-treated groups was not different from the
746 mosquitoes that were not antibiotic treated that ingested ZIKV-spiked blood 3 days earlier
747 (Fisher's exact test, $P > 0.05$) (**Figure 4E**). Together with the midgut cell infectivity data, these
748 results suggest that increased ZIKV midgut cell infection observed in antibiotic treated pupae
749 and adults establishes after 3 dpf and that augmented rates of infection in microbe-depleted
750 mosquitoes do not augment early dissemination.

751

752 **Hemolytic bacteria do not augment bloodmeal digestion *Ae. aegypti* that were not**
753 **microbe reduced via antibiotic treatment**

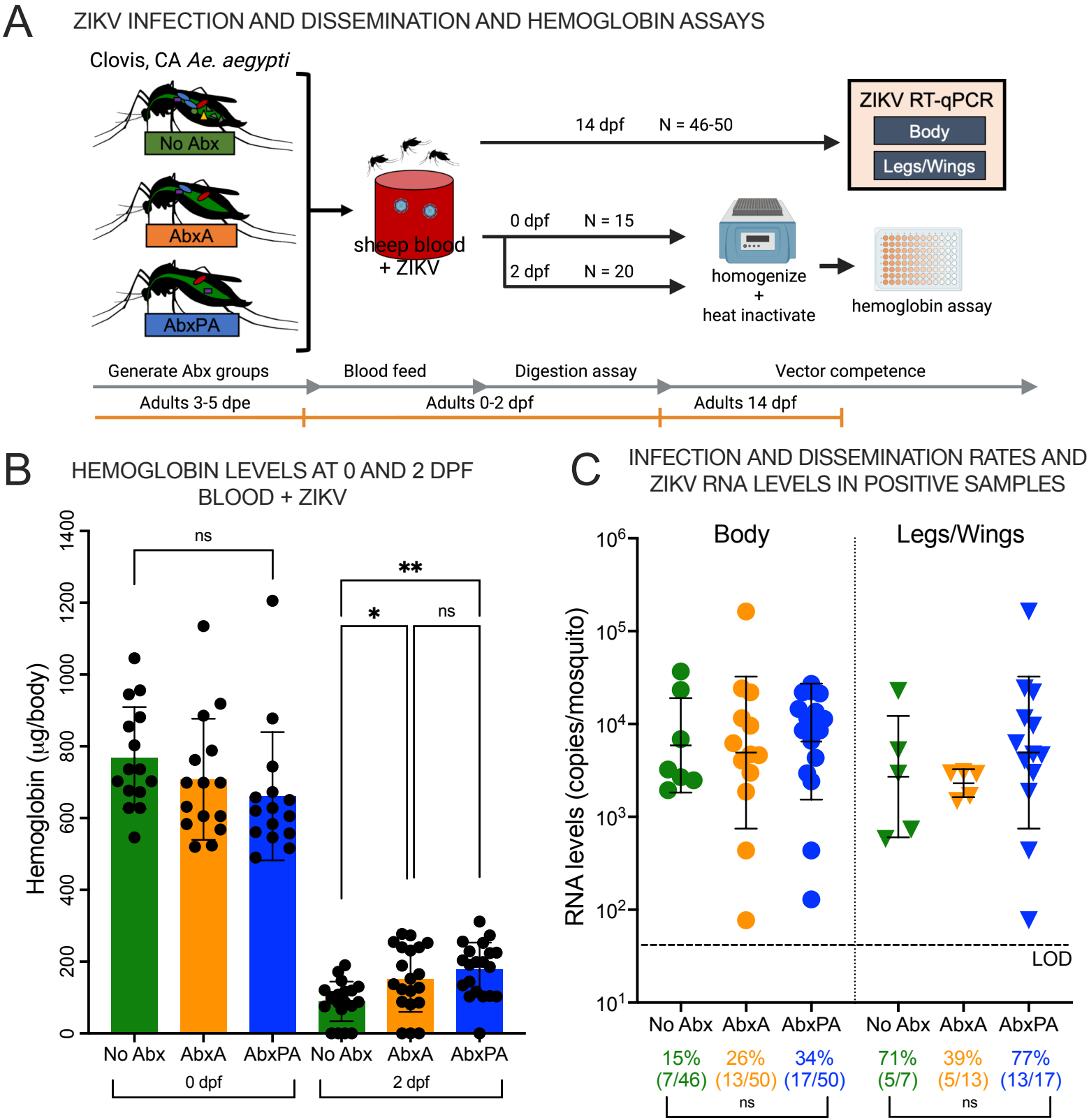
754 To determine whether bacteria with hemolytic activity contribute to enhanced bloodmeal
755 digestion in the No Abx groups, bacteria from 5 No Abx treated adult mosquitoes were cultured
756 on two types of agar and then Sanger sequenced. Although different species of bacteria were
757 detected, none showed hemolytic activity (**Table S1**), suggesting that bloodmeal digestion is not
758 directly mediated by bacteria in the mosquito midgut. Together, these data show that microbes
759 enhance the ability of *Ae. aegypti* to digest blood, likely via a mechanism not tied directly to
760 bacteria mediated hemolysis since the culturable bacteria recovered from mosquitoes that were
761 not antibiotic treated were not hemolytic.

762

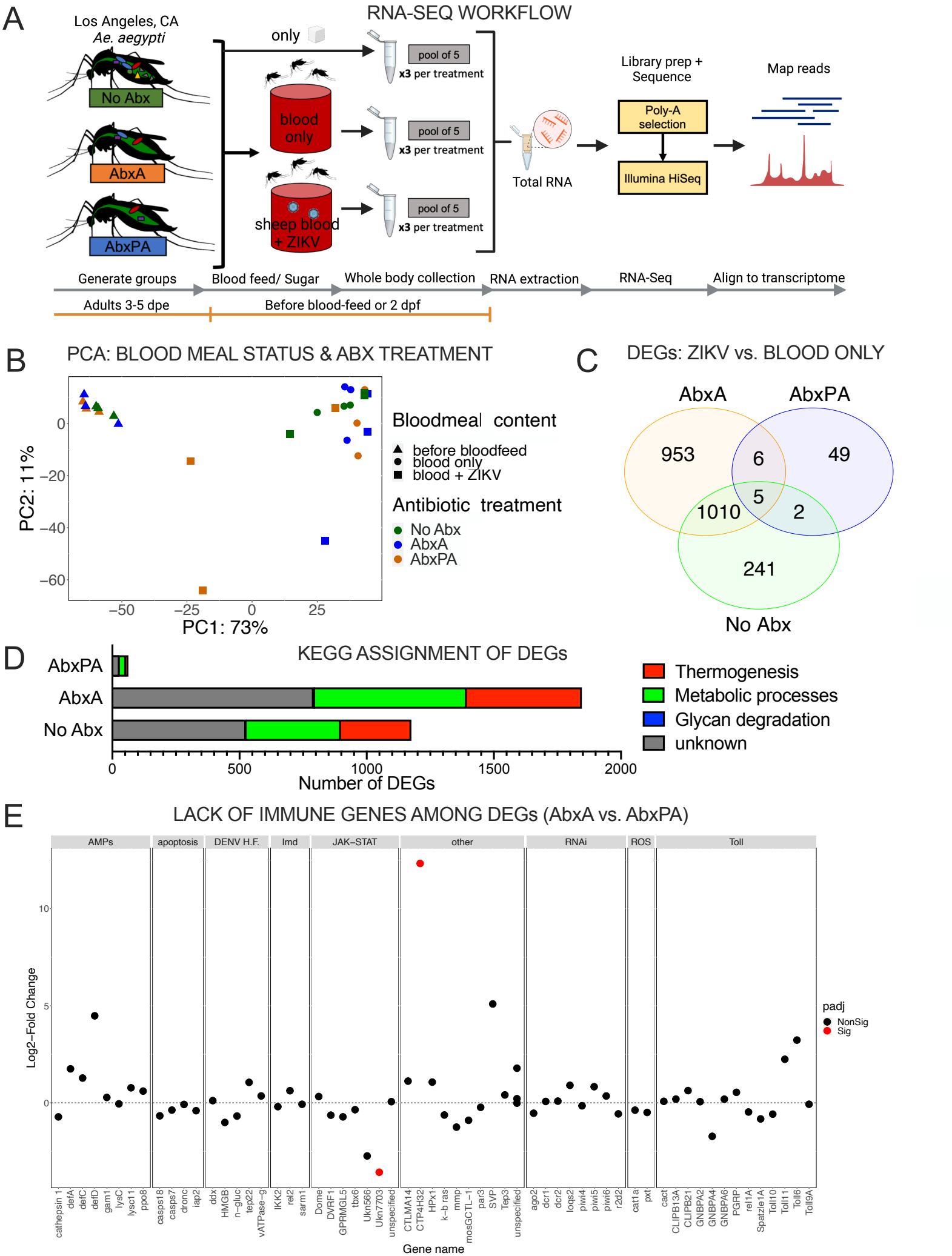
763 **Impaired blood digestion in the presence of microbes extends to genetically distinct *Ae.***
764 ***aegypti***

765 To evaluate whether our observations of impaired blood digestion with antibiotic treatment are
766 generalizable to *Ae. aegypti* from a different genetic background than Los Angeles, CA, we
767 performed the similar analyses with *Ae. aegypti* from Clovis, CA. Genome sequencing shows
768 that *Ae. aegypti* from Los Angeles and Clovis are genetically distinct (46), and were probably
769 derived from separate independent introductions into California since 2013 when the species
770 became established in the state. *Ae. aegypti* from Clovis were reared under the same antibiotic
771 treatments as for Los Angeles mosquitoes. Blood digestion kinetics and ZIKV vector
772 competence were quantified in Clovis mosquitoes (**Figure 5A**). As observed for mosquitoes
773 from Los Angeles, AbxA and AbxPA groups from Clovis demonstrated significantly lower blood
774 digestion relative to the No Abx group 2 dpf (K-W test with multiple comparisons, adjusted
775 $P_{AbxPA\text{-}No\ Abx} = 0.0013$, adjusted $P_{AbxPA\text{-}AbxA} = 0.027$) (**Figure 5B**). In parallel to Los Angeles
776 mosquitoes, the Clovis AbxPA group exhibited a marginally but not statistically significantly

777 higher infection rate (34%) compared to both the Clovis AbxA (26%) and No Abx (15%) groups
778 (Chi-square [χ^2] test, P = 0.106). The dissemination rates and mean RNA levels in ZIKV-positive
779 Clovis mosquitoes were not different for any treatment (**Figure 5C**). These results from
780 genetically distinct *Ae. aegypti* support the generalizability of our observations wherein microbes
781 augment blood digestion in *Ae. aegypti* from more than one genetic lineage.
782
783 **Figure 5. Bloodmeal digestion in antibiotic-mediated microbe depleted *Ae. aegypti* pupae**
784 **and adults from Clovis, California. A) Experimental design** using the same groups as in Figure 1
785 and hemoglobin assessments as in Figure 4 where an *Ae. aegypti* colony from Clovis, CA was used
786 instead of *Ae. aegypti* from Los Angeles, CA. Mosquitoes were harvested 14 dpf for vector
787 competence assessments. **B) Hemoglobin levels in *Ae. aegypti* as a proxy for bloodmeal**
788 **digestion in groups that were ZIKV exposed.** Despite ingesting equivalent bloodmeal sizes,
789 antibiotic treatment reduced blood digestion with ZIKV at 2 dpf in Clovis mosquitoes. Error bars
790 show the means and standard deviations. N = 15 for each treatment on 0 dpf, 20 per treatment on 2
791 dpf. **C) Microbe depletion of pupae and adults does not impact ZIKV infection or**
792 **dissemination in Clovis mosquitoes.** Rates of infection and mean ZIKV RNA levels from bloodfed
793 Clovis *Ae. aegypti* subjected to different antibiotic treatments were not significantly different at 14
794 dpf. Error bars denote the geometric means and standard deviations. The dotted line denotes the
795 average LOD which was 62 RNA copies/mosquito. ZIKV levels in bodies and legs/wings with
796 detectable ZIKV RNA are shown in panel C. (* is $P < 0.05$; ** is $P < 0.01$; ns is not significantly
797 different). Each symbol in B and C shows the mean value for 1 mosquito based on triplicate ZIKV
798 RT-qPCR measurements.
799
800 **Blood ingestion and ZIKV exposure influence the *Ae. aegypti* transcriptome**
801 Absent increased dissemination from the midgut, to identify gene expression differences
802 underlying increased susceptibility to ZIKV infection after antibiotic treatment of pupae and


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

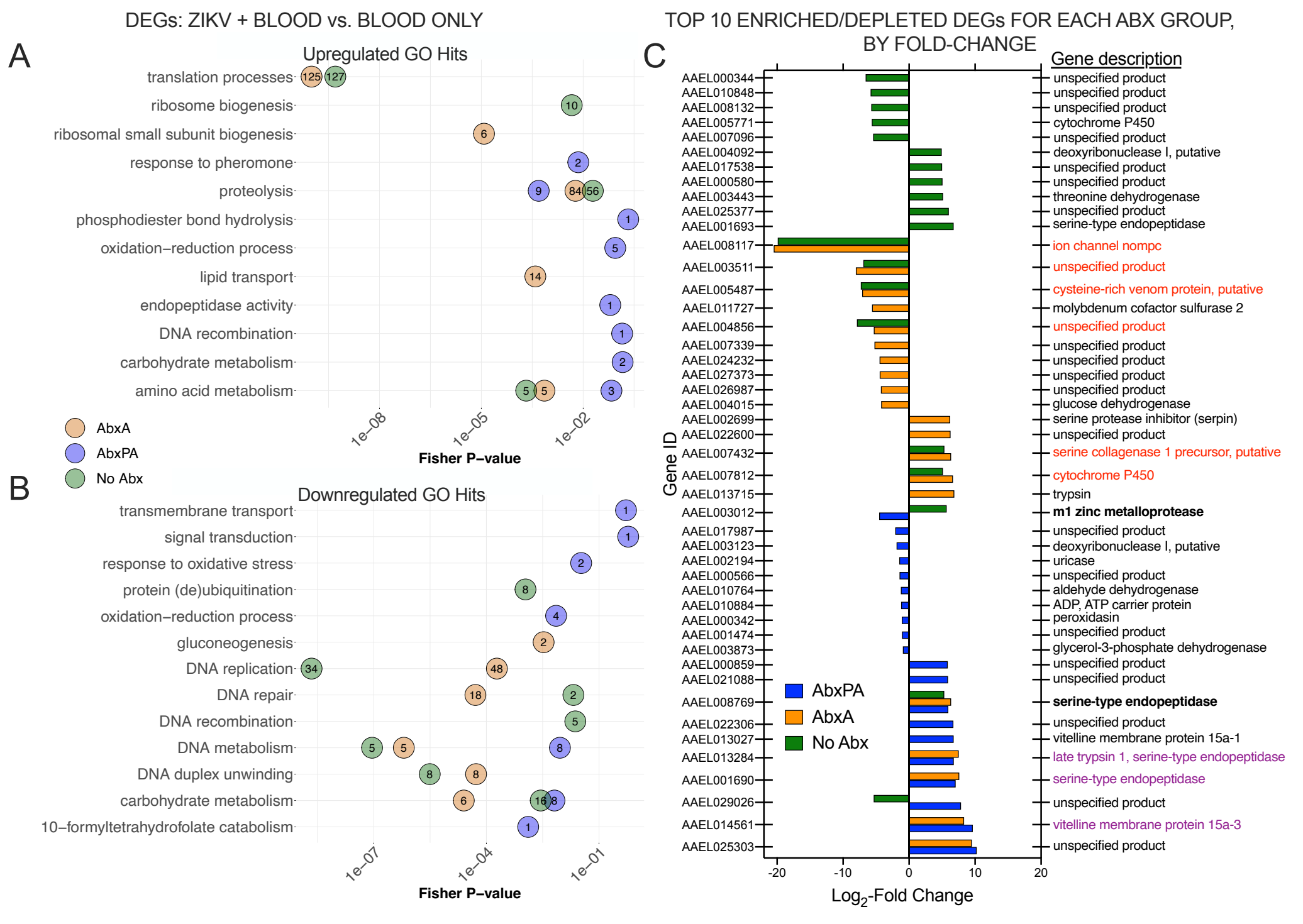
803 adults, we employed RNA-Seq analyses on pooled adult female *Ae. aegypti* from Los Angeles
804 before bloodfeeding and at 2 dpf. We also analyzed females that ingested blood only or ZIKV-
805 spiked blood (**Figure 6A**). On average, 94.5% (min: 92.7, max: 95.7%) of reads from all
806 samples aligned at least once to the Liverpool *Ae. aegypti* (LVP_WG) transcriptome. PCA
807 showed clustering of mosquitoes prior to bloodfeeding that was distinct from mosquitoes that
808 ingested blood with or without ZIKV (**Figure 6B**, **Figure S3A**). Using constrained analysis of
809 principal coordinates, we found that bloodmeal status correlated more strongly with variance in
810 the overall transcriptomic profile than antibiotic treatment, accounting for 84% of the variance (P
811 < 0.001) compared to <5% (P = 0.83), respectively. While most of the differences in gene
812 expression related to bloodfeeding status, the presence of ZIKV in the bloodmeal also yielded
813 differentially expressed genes (DEGs) for each antibiotic treatment. At baseline (prior to blood
814 or blood with ZIKV), 20 significant DEGs were found between AbxPA and No Abx (**Table S2**).
815 The most downregulated gene in AbxPA relative to No Abx was uncharacterized (AAEL021374,
816 adjusted P = 0.019). Other downregulated genes in AbxPA included membrane components
817 (AAEL020586, adjusted P = 0; AAEL003626, adjusted P = 0.047) and ATP binding proteins
818 (AAEL019793, adjusted P = 0.026; AAEL010711, adjusted P = 0.005). After bloodfeeding, over
819 a third of genes in the *Ae. aegypti* genome were differentially expressed, consistent with other
820 RNA-Seq studies (47). At 2 dpf, 7846 genes in No Abx, 9356 genes in AbxA, and 7846 genes in
821 AbxPA were differentially expressed compared to sugar-fed only mosquitoes. Among them,
822 6676 genes were shared among No Abx, AbxA, and AbxPA, representing an overwhelming
823 majority of the total DEGs (**Figure S3B**). Between bloodfed and bloodfed with ZIKV, differences
824 were more apparent. The AbxA and No Abx groups each had more DEGs between bloodfed
825 and bloodfed with ZIKV (No Abx: 1258 DEGs, AbxA: 1974) than AbxPA (62 DEGs) (**Figure 6C**,
826 only DEGs that differed significantly across antibiotic treatments are shown [adjusted P < 0.05
827 by Wald test]). Most shared DEGs were between the AbxA and No Abx groups; AbxPA
828 expressed a unique set of DEGs. Assignment of Kyoto Encyclopedia of Genes and Genomes


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

829 (KEGG) pathway identity (IDs) to DEGs identified many unknown genes. Hypothetical protein
830 products with no homology to genes with functional annotations were common (No Abx: 526,
831 AbxA: 792, AbxPA: 29), as were genes involved in metabolic processes (No Abx: 371, AbxA:
832 599, AbxPA: 25) and thermogenesis (No Abx: 277, AbxA: 452, AbxPA: 9) (**Figure 6D**). In ZIKV
833 bloodfed mosquitoes, antibiotic treatment of both pupal and adult stages reduces differential
834 gene expression more than treatment of adults only or no antibiotic treatment, indicating that
835 pupal-derived microbes have a greater effect on the gene expression profile than adult-derived
836 microbes. Taken together, these data suggest that bloodfeeding has a larger effect than
837 microbial exposure on *Ae. aegypti* gene expression and implicate microbe-mediated effects on
838 factors other than changes in transcription as modifiers of ZIKV vector competence.
839
840 **Figure 6. Differentially expressed genes between antibiotic-treated adult *Ae. aegypti* females**
841 **before and after a bloodmeal. A) Experimental design showing *Ae. aegypti* from Los Angeles,**
842 **CA, pooling and RNA sequencing approach.** For each antibiotic treatment and each bloodfeeding
843 state, triplicate pools of 5 whole, legless mosquito bodies were RNA-extracted, pre-processed, and
844 sequenced. **B) Transcriptome profile differs more by bloodmeal status than by antibiotic**
845 **treatment.** PCA plot after variance stabilized transformation. Shapes indicate the bloodmeal status
846 while colors represent the antibiotic treatments. Separate PCAs for individual variables, for ease of
847 visibility, are shown in Figure S3. **C) ZIKV-responsive DEGs are shared among between *Ae.***
848 ***aegypti* not antibiotic-treated and antibiotic-treated as adults.** Venn diagram of DEGs before
849 and after bloodfeeding by *Ae. aegypti* for each antibiotic treatment. **D) DEGs correspond to**
850 **metabolic processes, thermogenesis, and unknown genes for all antibiotic treatments.** KEGG
851 Pathway ID assignment of DEGs in panel C. **E) DEGs between mosquitoes treated with**
852 **antibiotics as pupae and adults compared to as adults only and ZIKV-bloodmeals or blood**
853 **only do not correspond to known immune response genes.** Comparison of immune response
854 gene expression between AbxA and AbxPA groups. Relative expression of select known and

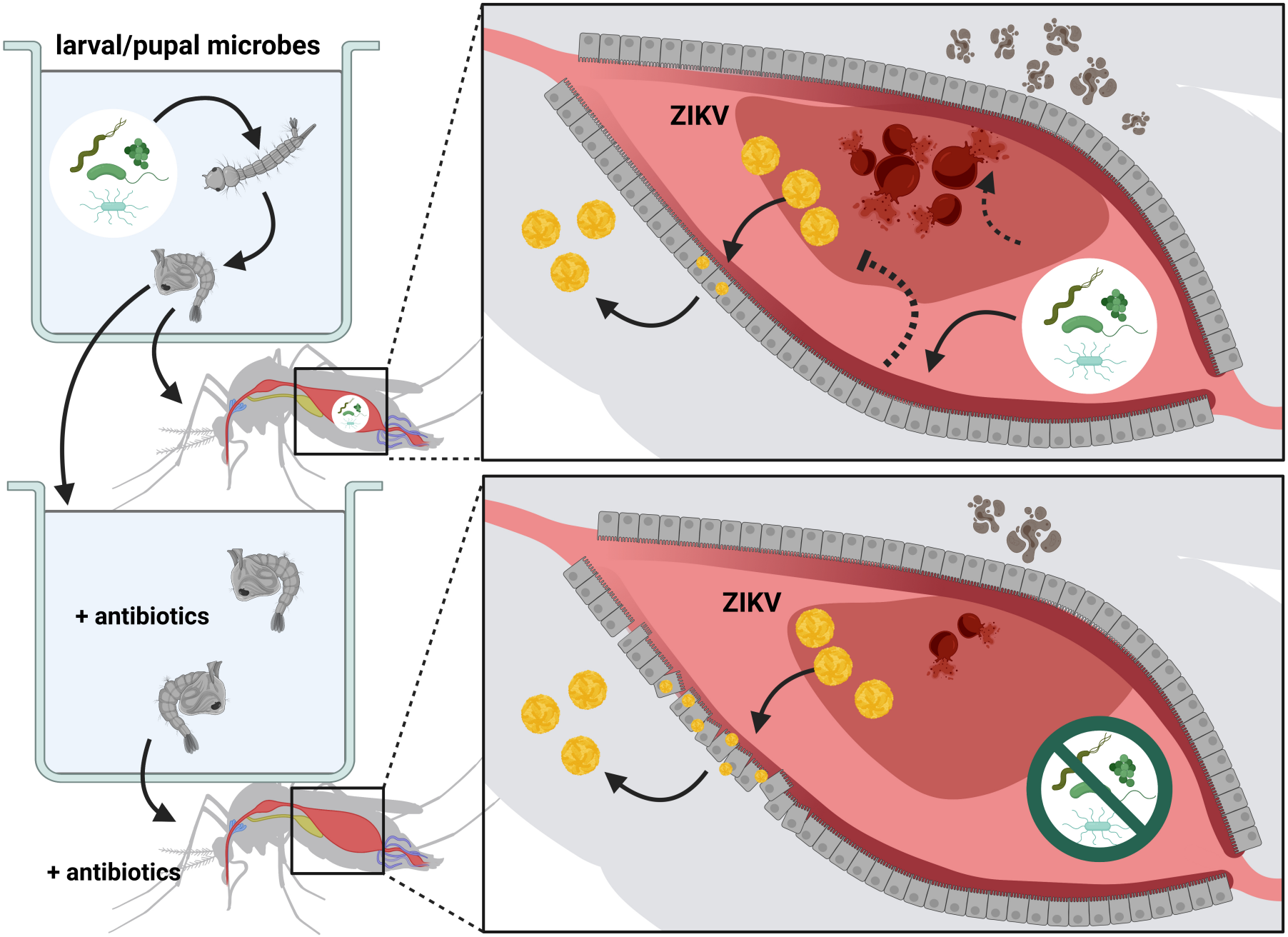
855 suspected immune-related genes from relevant literature (48–63). Expression is higher in AbxA if the
856 log_2-fold change is positive, while expression is higher in AbxPA if log_2-fold change is negative.
857 Points are considered significant (red) if the adjusted P value is < 0.01. Comparisons of the same
858 genes between AbxA or AbxPA and the control treatment, No Abx, are shown in Figure S4.
859
860 Provided that microbes mediate immunomodulatory effects in mosquitoes (13,64,65), we
861 theorized their presence could influence ZIKV susceptibility by modifying the expression of
862 immune response pathways where some respond to both bacteria and viruses. We identified
863 immunity-related DEGs across antibiotic-treated mosquitoes 2 dpf in the RNA-Seq data.
864 Transcripts representing components of various immune pathways were accessioned from
865 existing literature and used to filter the dataset before analyses of DEG profiles. Representative
866 genes examined include regulators from Toll (48–50), RNAi (51–54), JAK-STAT (49,55,56), and
867 Imd pathways (57,66), as well as general immune and immune-adjacent genes such as
868 hormones (58–60,67), antimicrobial peptides (AMPs) (56,61), reactive oxygen species (ROS)
869 (62,63), and known DENV host factors (56,61). There were few DEGs demonstrating significant
870 changes in transcript abundance in immune pathways between AbxA and AbxPA, No Abx and
871 AbxA, or No Abx and AbxPA groups (**Figures 6E, S4**). Exceptions are gene CTP4H32
872 (AAEL007812, cytochrome p450 (47)) which was elevated in AbxA, and Unkn566
873 (AAEL000566, transmembrane protein in the JAK-STAT pathway (56)) which was elevated in
874 AbxPA (**Figure 6E**). Comparison of immune genes between No Abx and AbxA and between No
875 Abx and AbxPA, revealed a handful of genes that passed the statistical threshold; these are in
876 the JAK-STAT pathway (Ukn566 between No Abx and AbxA; Ukn566 and Ukn7703 between No
877 Abx and AbxPA) (**Figure S4**). These data suggest that gene expression changes after microbe
878 depletion via antibiotic treatment *Ae. aegypti* that show different susceptibility to ZIKV infection
879 are not related to known immune response pathways.
880

881 **Gene ontology assignments reveal the role of microbiota in stimulating expression of**
882 **protein digestion-associated genes in *Ae. aegypti* orally exposed to ZIKV**
883 Given that RNA-Seq revealed no major differences in expression of immune response genes
884 with antibiotic treatment, we next performed Gene Ontology (GO) analyses to predict enriched
885 functions in non-immune associated DEGs. We identified DEGs displaying significant changes
886 in abundance between the antibiotic treatments in mosquitoes that ingested ZIKV in blood or
887 that ingested blood only. The AbxA and No Abx groups that ingested ZIKV in blood showed
888 increased expression of genes enriched in general translation processes (AbxA: 125, adjusted
889 $P < 10^{-10}$; No Abx: 127, adjusted $P < 10^{-20}$) and proteolysis (AbxA: 84, adjusted P = 0.0006; No
890 Abx: 56, adjusted P = 0.015). In contrast, the AbxPA group showed few upregulated genes
891 resulting in a lack of enriched functions (**Figure 7A**). Conversely, enrichments in genes
892 downregulated in mosquitoes that ingested ZIKV in blood versus mosquitoes that ingested
893 blood only revealed that the ZIKV the AbxPA treatment group showed reduced transcript
894 abundance for genes associated with DNA metabolism (8) and redox processes (4). AbxA and
895 No Abx were depleted in genes for DNA replication (N=48 and 34, respectively) and DNA repair
896 (N=18 and 2, respectively). All treatment groups that ingested blood with ZIKV compared to
897 blood only exhibited downregulation of carbohydrate metabolism genes (AbxPA: N=8, adjusted
898 P = 0.006; AbxA: N=6, adjusted P = 0.0002; No Abx: N=16, adjusted P = 0.017) (**Figure 7B**).
899 These data suggest that microbiota during early ZIKV infection promote expression of genes
900 associated with proteolysis and translation while downregulating genes relating to DNA
901 synthesis. The magnitude of change in DEGs in mosquitoes that ingested ZIKV in blood or
902 blood only for each antibiotic treatment were also compared. Gene AAEL008117, the most
903 depleted transcript in AbxA and No Abx groups (~20 $\log_2$-fold reduced), corresponds to nompC,
904 a mechanosensitive ion channel that senses balance and touch in *Drosophila melanogaster*
905 (68). The most common DEGs that changed in response to ZIKV exposure matched to
906 proteolytic and PM-associated enzymes, including endopeptidases, collagenases, and


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

907 dehydrogenases (69). Multiple serine-type endopeptidases were differentially expressed
908 depending on antibiotic treatment (No Abx: AAEL001693, all three: AAEL008769, AbxA, and
909 AbxPA: AAEL013284, AAEL001690) (**Figure 7C**). In addition, some vitellogenesis associated
910 proteins were more enriched in antibiotic treated mosquitoes compared to the No Abx group
911 (AbxA and AbxPA: AAEL014561 [vitelline membrane protein 15a-3], AbxPA: AAEL013027
912 [vitelline membrane protein 15a-1]). In AbxPA, the gene demonstrating the biggest reduction in
913 transcript abundance corresponds to a m1 zinc metalloprotease (AAEL003012), showed
914 increased expression in the No Abx group. Overall, GO analyses revealed *Ae. aegypti* with
915 disrupted microbiota are deficient in genes that influence blood protein metabolism.
916
917 **Figure 7. Gene Ontology assignment of DEGs and identity of most enriched/depleted genes.**
918 Gene Ontology (GO) analysis of significant DEGs comparing antibiotic treatment mosquitoes that
919 ingested ZIKV in bloodmeals versus blood only. **A) Upregulated GO hits for mosquitoes treated**
920 **with antibiotics as adults or untreated primarily map to general translational processes and**
921 **proteolysis.** Mosquitoes treated with antibiotics as pupae and adults demonstrate a reduced
922 complement of proteolysis associated genes, and **B) GO enrichments in genes showing reduced**
923 **transcript abundance for all antibiotic treatments primarily map to DNA synthesis and repair**
924 **mechanisms.** GO terms for the DEGs (determined from Figures 6C-D) and their descriptions on the
925 y-axis, and statistical significance (P-values, Fisher's exact test) of the GO term on the x-axis, with
926 the exact number of DEGs within the colored circles. DEGs farther to the left (lower P-values) are
927 more significant. **C) ZIKV-responsive DEGs demonstrating the highest magnitude fold change**
928 **are distinct between AbxPA and No Abx treatment groups.** The top 10 upregulated and top 10
929 downregulated DEGs, by magnitude $\log_2$-fold change for each antibiotic treatment. A positive $\log_2$-
930 fold change refers to more transcripts after a ZIKV-spiked bloodmeal, while a negative value refers
931 to fewer transcripts. The right y-axis is the gene description from annotations retrieved from

932 transcriptome metadata as well as VectorBase (39). Red text indicates the top genes shared
933 between No Abx and AbxA. Purple text indicates the top genes shared between AbxPA and AbxA.
934
935 **Discussion**
936
937 Our data show that larval microbial exposure confers developmental resistance to ZIKV
938 infection of the mosquito midgut. We observed that microbial exposure in two genetically distinct
939 populations of *Ae. aegypti* enhances bloodmeal digestion via increased midgut integrity.
940 Together these effects reduce ZIKV infection of the midgut epithelium (**Figure 8**). Reduced
941 microbial colonization during both pupal and adult *Ae. aegypti* life stages, together with higher
942 bloodmeal titers, produced the largest increases in ZIKV susceptibility. These effects were not
943 due to antibiotic treatment itself and our studies with gnotobiotic mosquitoes show that exposure
944 to more than a single bacterial species (*Elizabethkingia spp.)* is required to reduce ZIKV
945 susceptibility. The lack of modified ZIKV susceptibility in microbe-reduced Clovis *Ae. aegypti*
946 contrasts with our observations from Los Angeles mosquitoes and highlights the need for further
947 work to understand whether microbes have differential impacts on *Aedes* mosquitoes from
948 different regions.
949
950 **Figure 8. Schematic representation showing how microbes across mosquito life stages**
951 **promote midgut permeability, increase bloodmeal digestion, and increase ZIKV infection in**
952 **the *Ae. aegypti* midgut.**
953
954 Augmented rates of ZIKV infection but not dissemination or transmission in *Ae. aegypti* after
955 microbe reduction via antibiotic treatment suggest that microbial effects act at the midgut
956 infection barrier but not at midgut escape or salivary gland infection or escape barriers.
957 Hematophagous feeding introduces a unique problem in that engorgement immensely distends


larval/pupal microbes
ZIKV
+ antibiotics
ZIKV
+ antibiotics

958 the midgut. Midgut distention can result in breaches in the integrity of the epithelial barrier,
959 presenting an opportunity for a virus ingested with blood to bypass infection of the epithelium.
960 Prior studies detected bloodmeal-induced perforations in the midgut basal lamina, which do not
961 fully recover after digestion, supporting a process where successive bloodmeals increase rates
962 of ZIKV dissemination (45). Considering that we observed an increased magnitude of and rate
963 of bloodmeal digestion in ZIKV-exposed mosquitoes without antibiotics compared to antibiotic-
964 treated pupae and adults, we envision a process where microbes accelerate digestion,
965 decreasing the window of time the virus has to escape the bloodmeal, traverse the PM, and
966 access the midgut epithelium. We did not observe gross structural differences in the midgut
967 epithelium with 10-40X DAPI and H&E staining, so we theorize that microbes do not impact
968 midgut permeability by physically modifying the midgut epithelium, although electron microscopy
969 studies could yield more insight into structural changes. Studies with the Plasmodium vector *An.*
970 *stephensi* show that antibiotic treatment disrupts the PM 2 dpf (70); our future work will assess
971 whether a similar phenomenon occurs in *Ae. aegypti*, as changes in the PM could also impact
972 ZIKV vector competence. We acknowledge that increased midgut permeability may also allow
973 for extracellular ZIKV traversion of the midgut epithelium and that this is a process we did not
974 measure in these studies.
975
976 Given that there are conserved elements of mosquito antiviral defenses and gut microbial
977 homeostasis, including immune signaling and off-target suppression by ROS and AMPs (12,64),
978 we were initially surprised that gene expression analyses did not detect differences in antiviral
979 immune responses in response to antibiotic treatment. Rather, our RNA-Seq analyses confirm
980 prior observations that show protein digestion and vitellogenesis dominate mosquito
981 physiological processes after bloodfeeding (47). Our results are also similar to another
982 transcriptomic study which found immense upregulation of proteases and trypsins 2 days post-
983 ZIKV exposure (71). It stands to reason that blood digestion and vitellogenesis are hampered

984 without the presence of microbial symbionts. Midgut symbionts may also indirectly reduce
985 mosquito vector competence in multiple ways. These include supplementation of larval nutrition
986 and development to increase mosquito fitness (10,72–75), stimulation of bloodfeeding behavior
987 resulting in larger bloodmeals (although bloodmeal size differences were not observed in our
988 studies) and increased nutrition (76), and regulation of midgut epithelial cell proliferation, where
989 the physical microbe-midgut interface can block virus access to midgut infection (77) [although
990 this may be pathogen specific as increased midgut infection has also been observed (21,78)].
991
992 Both microbes and ZIKV in bloodmeals resulted in increased blood digestion relative to
993 microbe-reduced antibiotic treated mosquitoes that ingested blood without virus. It is unclear
994 why ZIKV enhances digestion; this may result from the virus modulating cell metabolism to
995 benefit viral replication, a pattern shown for many viruses (79). In human cells, for example,
996 infection with DENV elevates glycolysis to meet cellular energy needs (80). Conceivably, a
997 similar trend is true for ZIKV-infected mosquito midgut cells, where increased energy demand
998 produced by viral infection is met by increasing blood metabolism. Absent ZIKV in the
999 bloodmeal, antibiotic-treated mosquitoes exhibited digestive deficiencies during late digestion,
1000 defined here as periods beyond 2 dpf. Since blood ingestion is followed by rapid proliferation of
1001 midgut microbes that results from a heme-mediated reduction in ROS (25), the delayed
1002 digestion kinetics in antibiotic treated mosquitoes could be influenced by the near absence of
1003 microbes to promote digestion. Since blood digestion occurs in 2 phases where disparate
1004 enzymes dominate each phase (81), it is also possible that the digestive enzymes that have
1005 different efficiencies were differentially expressed across antibiotic treatments. We observed
1006 late trypsins and serine-type endopeptidases (enzyme types commonly found in the latter phase
1007 of digestion) (81–84) in all bloodfed antibiotic groups at 2 dpf; greater resolution of how
1008 digestion enzymes change over time is warranted to test this hypothesis. While over 300 serine
1009 protease-like proteins have been described in *Aedes spp.* (85,86), very few have been studied

1010 experimentally, with the exception of HPx1 (25), which was not differentially regulated in
1011 mosquitoes in our study. One study implicated matrix metalloproteases and collagenase activity
1012 in degrading the basal lamina, which increased dissemination of CHIKV from the midgut (87).
1013 Our finding of a downregulated metalloprotease (AAEL003012) in mosquitoes with disrupted
1014 microbiota as pupae and adults, as well as an elevated serine collagenase precursor
1015 (AAEL007432) in mosquitoes that ingested ZIKV in blood supports a role of digestive enzymes
1016 in promoting arboviral dissemination. The potential interaction between blood protein
1017 metabolism and arboviral infection of mosquitoes warrants further study, including gene
1018 silencing or knockout studies to confirm functional roles of candidate genes that affect vector
1019 competence.
1020
1021 Whether blood digestion genes other than HPx1 (25), including those that are differentially
1022 upregulated in mosquitoes exposed to antibiotics, can also modulate mosquito immune
1023 responses is currently unclear. A link between metabolism and immunity is possible (88). Some
1024 proteases have immune functions (89,90), including cleavage of proenzymes that activate the
1025 prophenoloxidase pathway required for melanization (91). Metabolites influence and regulate
1026 immune pathways like Toll and Imd, and metabolic regulators balance microbial resistance and
1027 tolerance to gut symbionts in arthropods, including mosquitoes (71,88). In *Ae. aegypti*, blood-
1028 derived heme induces iron- and ROS-dependent signaling that also regulates immune genes,
1029 affecting DENV susceptibility (92). Metabolism of *Bacteroides* associates with elevated ZIKV
1030 susceptibility and conditions insecticide resistance (93), implicating a complex metabolic
1031 network that can also influence arbovirus susceptibility. Further investigation of the relationship
1032 between blood digestion and immunity could uncover novel determinants of mosquito vector
1033 competence.
1034

1035 Determining how microbes modulate the susceptibility of mosquitoes to arboviruses can help
1036 identify approaches to reduce transmission and decrease human disease. Microbial effects may
1037 also explain variability in vector competence across laboratories and geographic origins where
1038 each has different microbial communities (94), together with conventionally acknowledged
1039 factors like inbreeding during colonization (95,96). Our previous work, together with a growing
1040 body of mosquito microbiome studies, suggests that mosquito-microbe-virus interactions are
1041 environmentally variable and warrant investigation in more field-relevant contexts (15,19,75,97).
1042 A limitation of our studies is the use of a single microbial community and ZIKV strain. We
1043 acknowledge that disparate microbial species and community structures and other ZIKV strains
1044 could impact susceptibility differently. We also acknowledge that unculturable bacterial species
1045 could potentially play a role in mediating hemolytic activity that could have impacted blood
1046 digestion assays. Our use of antibiotics, which incompletely eliminates microbiota, most closely
1047 represents a dysbiotic phenotype. Additionally, our gnotobiotic studies were limited to use of a
1048 single bacterial species. Newer tools including auxotrophic symbionts (98) and axenic
1049 mosquitoes (10) can be used to further investigate the metabolic roles of microbes in influencing
1050 arboviral infection of mosquitoes. Future studies of infection dynamics at the microbe-mosquito
1051 midgut interface could prove useful in reconciling disparate vector competence outcomes and
1052 discovering novel vector control approaches.
1053
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Visualization: WL, ALR, LLC

Writing-Original Draft: WL

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

1083 **Figure S1. Histology of bloodfed female midguts.** Cross sections of whole, bloodfed female
1084 *Ae. aegypti* along the median sagittal plane. H&E staining and bright field microscopy with a
1085 focus on the blood bolus perimeter was used to assess and identify potential structural artifacts
1086 during sample preparation that could confound immunofluorescence observations. Both 20X
1087 and 40X magnification of the same section are shown side-by-side. MG epithelium = midgut
1088 epithelium. PM region = peritrophic matrix region (where the fully formed PM would be seen).
1089 RBCs = red blood cells, from bloodmeal. BL side = basal laminal side.
1090
1091 **Figure S2. Validation of hemoglobin colorimetric assay kit for bloodfed mosquitoes. A)**
1092 Serial 1:2-fold dilutions of heparinized sheep blood were tested in parallel with the kit
1093 hemoglobin standard at the absorbance extremes of 560nm and 580nm. Lines indicate
1094 standard curves generated from linear regression that were used in the hemoglobin assays. **B)**
1095 Absorbance values of sheep blood hemoglobin after heat-treating at 60°C for 10 minutes in
1096 duplicates (rep = replicate).
1097
1098 **Figure S3. DEGs from bloodfed antibiotic-treated mosquitoes that were not exposed to**
1099 **ZIKV. A) Individual PCA plots** by a single variable representing the same data in Figure 6B.
1100 Shapes indicate the bloodmeal status while colors represent the antibiotic treatments. B) **DEGs**
1101 **between blood- and sugar-fed *Ae. aegypti***.
1102
1103 **Figure S4. Change in immune response gene expression across antibiotic treated groups**
1104 **of *Ae. aegypti*.** A positive log_2-fold change indicates enrichment in AbxA or AbxPA, while a
1105 negative log_2-fold change indicates enrichment in No Abx.
1106

1107

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

1108

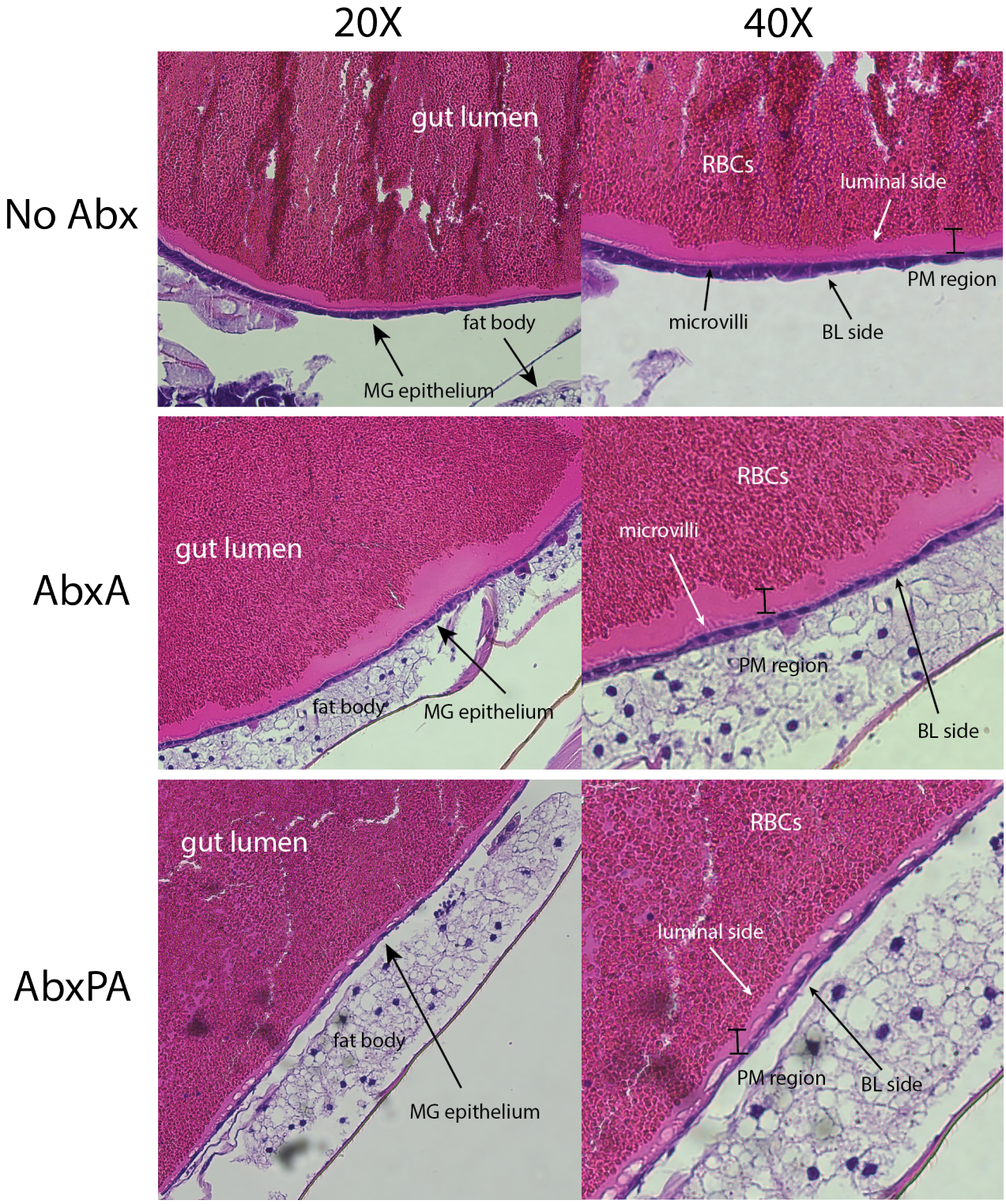
1109 **Table S1. Bacterial isolates from adult *Ae.* aegypti that were not treated with antibiotics**
1110 **(No Abx).** Cultured and isolated bacterial colonies from adult female *Ae. aegypti*, 3-5 dpe were
1111 Sanger sequenced at the V4 region of the 16S gene. Sequences were input into nucleotide
1112 BLAST, and the closest hits are reported. Hemolytic activity was tested by presence of ring of
1113 clearance around colonies (from lysed red blood cells) when cultured on sheep blood agar at
1114 37°C. NA indicates hemolytic activity was not evaluated.

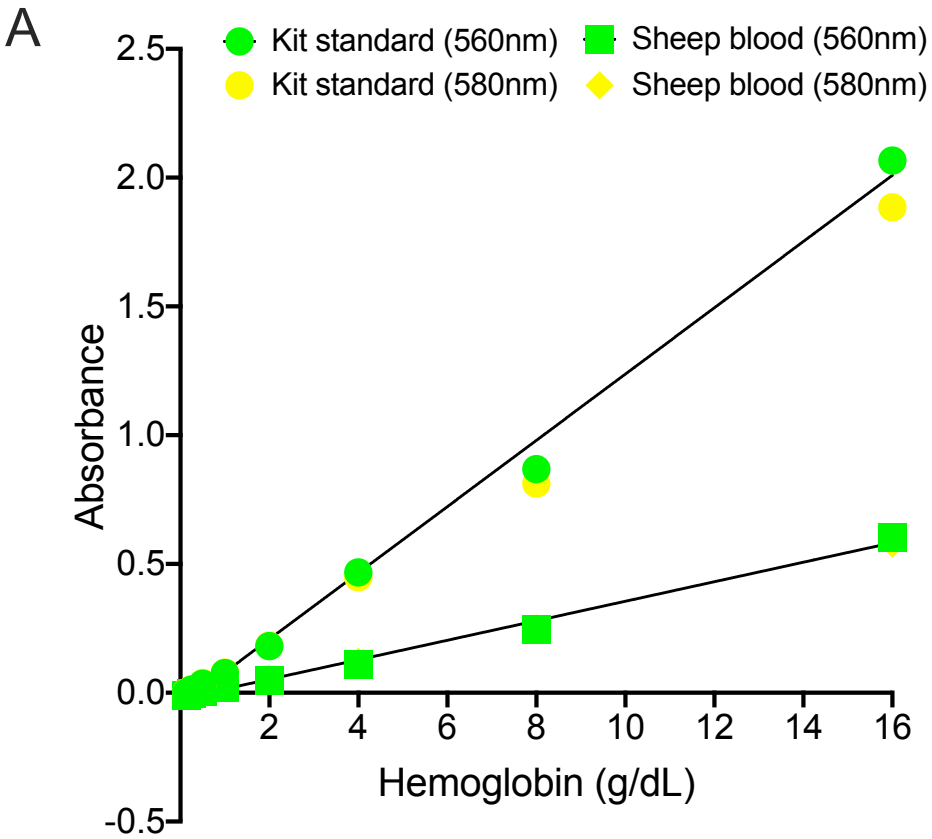
1115

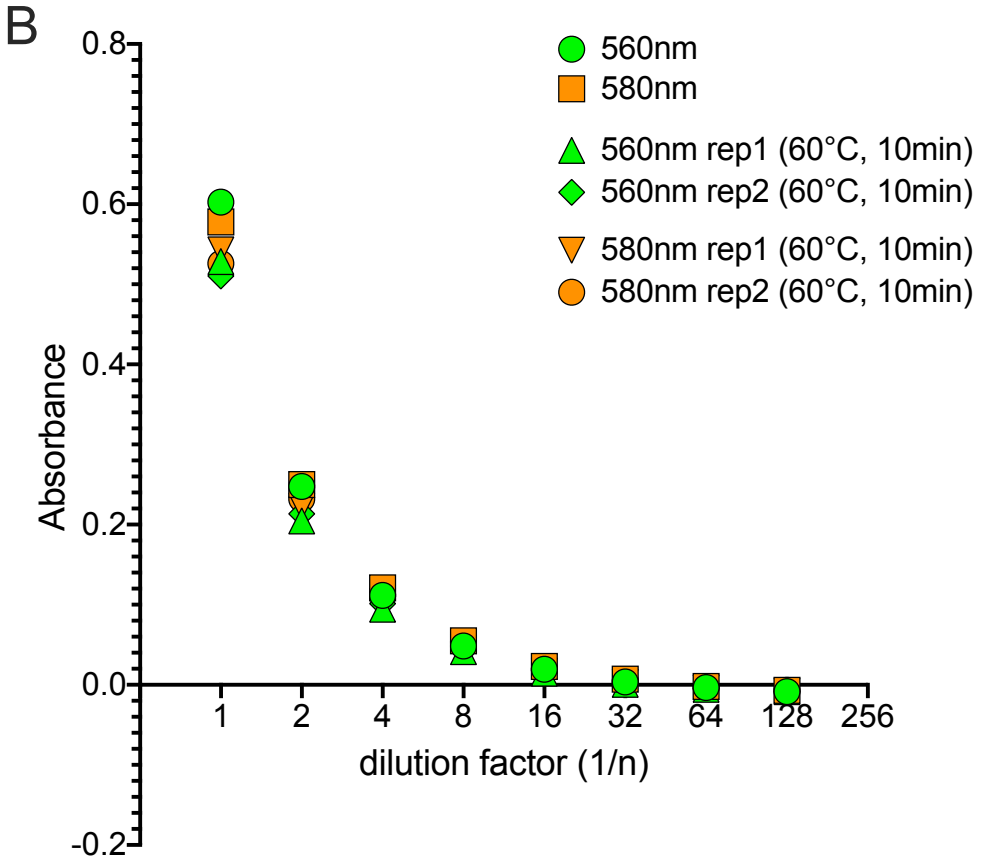
1116

Gene	Log_2Fold 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

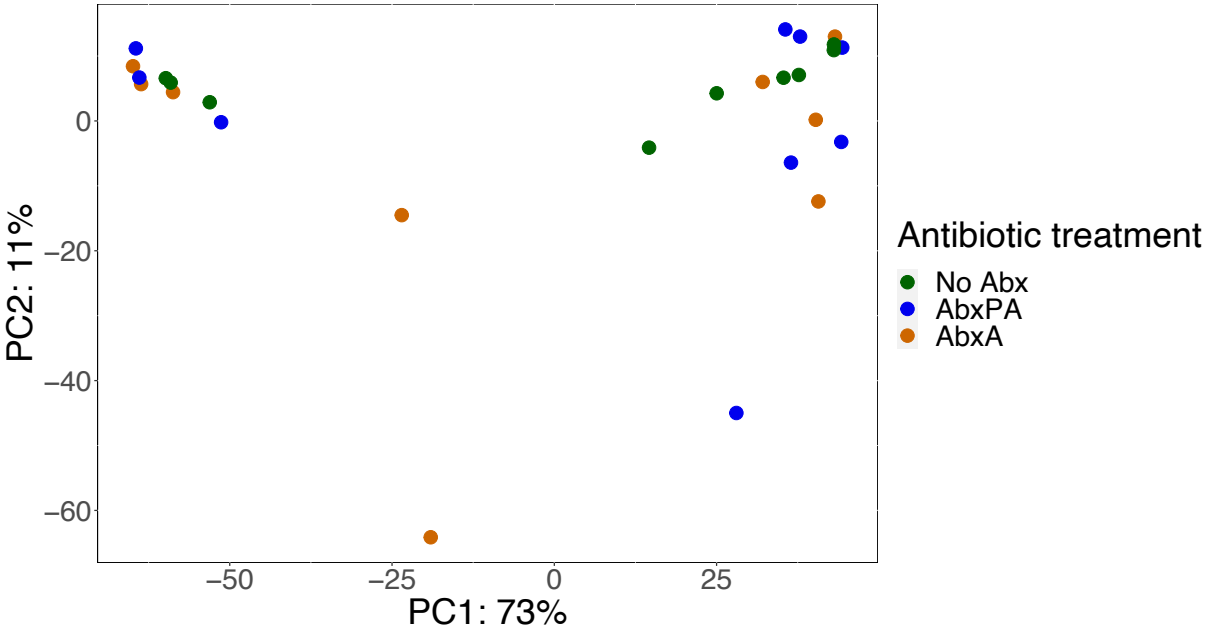
Table S2. DEGs between sugar-fed *Ae. aegypti* without antibiotics or antibiotic treated as pupae and adults. A negative Log_2fold-change refers to downregulation in AbxPA vs No Abx, while a positive Log_2fold-change refers to upregulation in AbxPA vs No Abx.

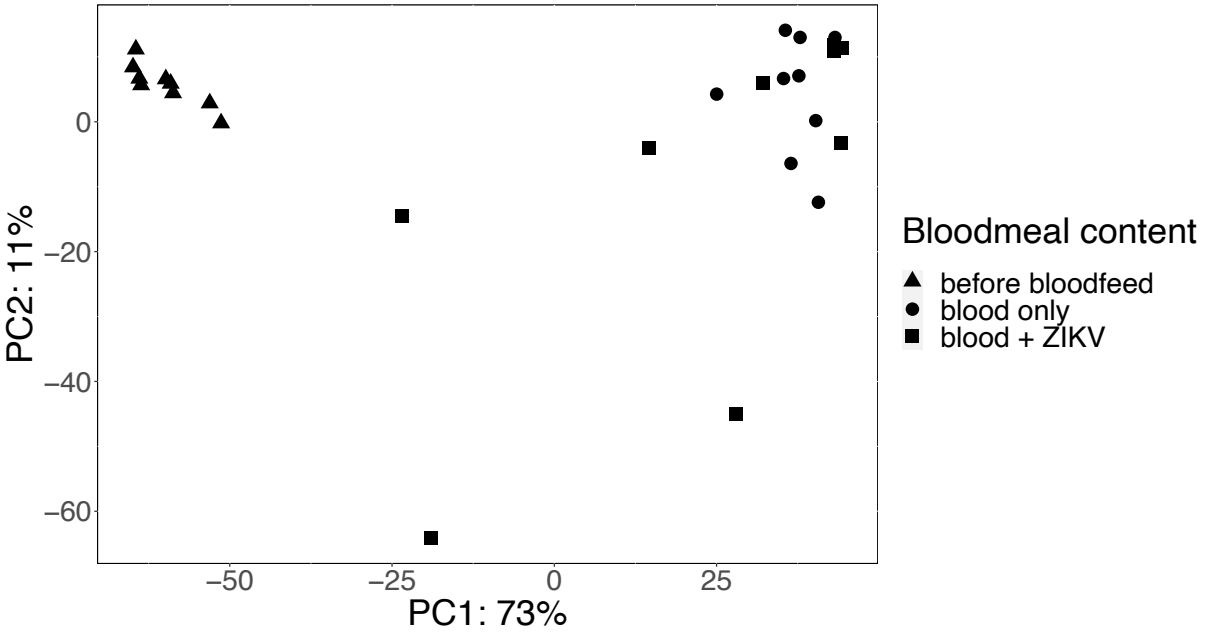

20X
40X
No Abx
gut lumen
fat body
MG epithelium
RBCs
luminal side
PM region
microvilli
BL side
AbxA
gut lumen
fat body
MG epithelium
RBCs
microvilli
PM region
BL side
AbxPA
gut lumen
fat body
MG epithelium
RBCs
luminal side
PM region
BL side


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

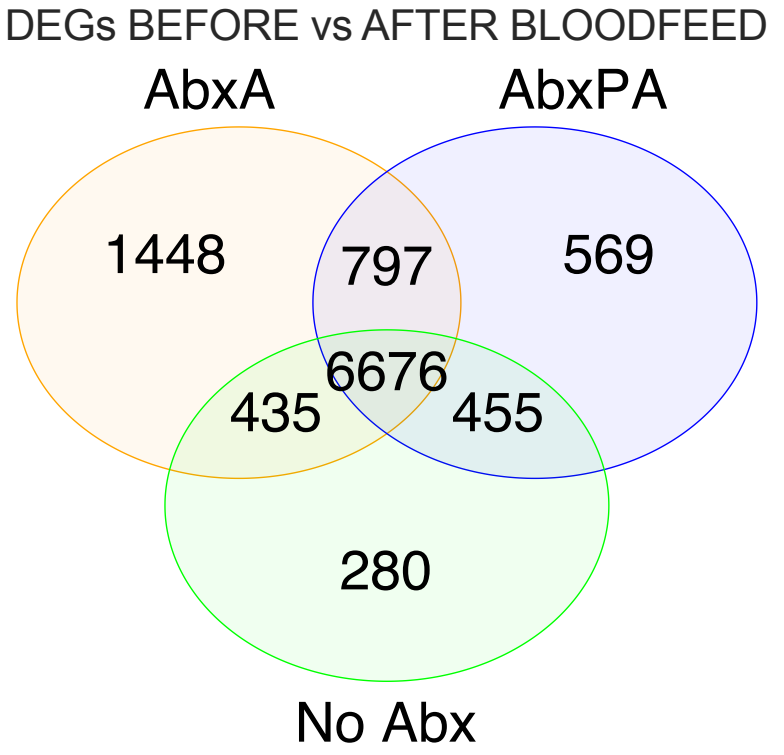

B
560nm
580nm
560nm rep1 (60°C, 10min)
560nm rep2 (60°C, 10min)
580nm rep1 (60°C, 10min)
580nm rep2 (60°C, 10min)
Absorbance
dilution factor (1/n)
0.8
0.6
0.4
0.2
0.0
-0.2
1
2
4
8
16
32
64
128
256

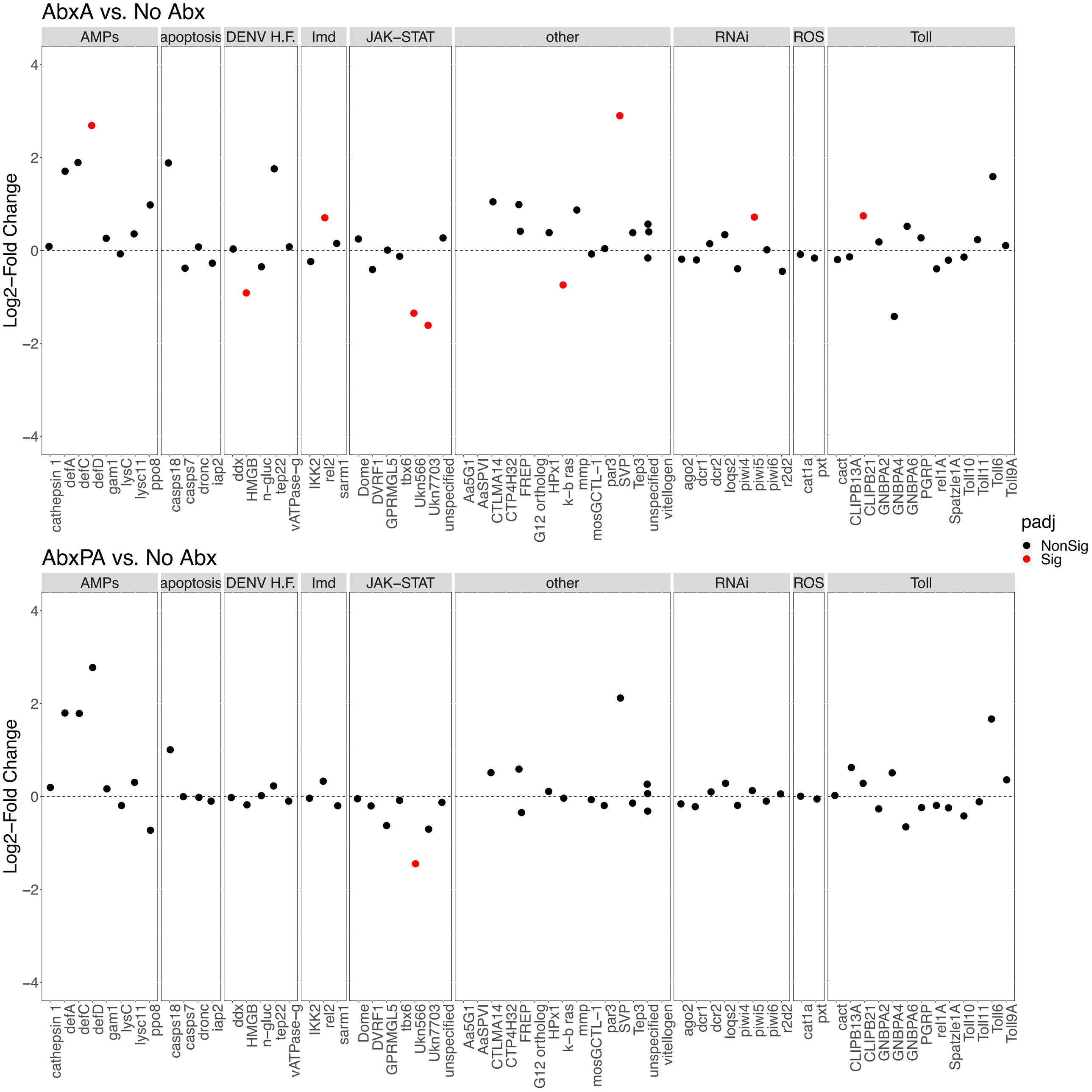
A


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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