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3   Long title: **Survey of bacteria associated with western corn rootworm life stages reveals no**
4   **difference between insects reared in different soils**

5   Short title: Bacteriome of western corn rootworm life stages in different soils

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32   **DATA AVAILABILITY STATEMENT**

33   All data are publicly available as Bioproject PRJNA422802, in the NCBI Sequence Read
34   Archive (SRA) database.

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ABSTRACT

Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) is a serious pest of maize (*Zea mays* L.) in North America and parts of Europe. With most of its life cycle spent in the soil feeding on maize root tissues, this insect is likely to encounter and interact with a wide range of soil and rhizosphere microbes. Our knowledge of the role of microbes in pest management and plant health remains incomplete. An important component of an effective pest management strategy is to know which microorganisms are present that could play a role in life history or management. For this study, insects were reared in soils from different locations. Insects were sampled at each life stage to determine the possible core bacteriome. Additionally, soil was sampled at each life stage and resulting bacteria were identified to determine the contribution of soil to the rootworm bacteriome, if any. We analyzed the V4 hypervariable region of bacterial 16S rRNA genes with Illumina MiSeq to survey the different species of bacteria associated with the insects and the soils. The bacterial community associated with insects was significantly different from that in the soil. Some differences appear to exist between insects from non-diapausing and diapausing colonies while no significant differences in community composition existed between the insects reared on different soils. Despite differences in the bacteria present in immature stages and in male and female adults, there is a possible core bacteriome of approximately 16 operational taxonomic units (*i.e.*, present across all life stages). This research may give insights into how resistance to Bt develops, improved nutrition in artificial rearing systems, and new management strategies.

Keywords: *Diabrotica virgifera virgifera*, bacteria, maize, development, Coleoptera, rhizosphere, bacteriome.

58 **INTRODUCTION**

59   Several studies have evaluated the microbial communities associated with lepidopteran

60 pests and other insects that attack food crops (1-4). Interestingly, shifts in community

61 composition or absence of bacteria can reduce the effectiveness of widely adopted management

62 tactics such as crop rotation or maize expressing *Bacillus thuringiensis* Berliner (Bt) proteins.

63 However, few studies have been conducted to document microbiomes within beetles attacking

64 crops (5).

65   The western corn rootworm (*Diabrotica virgifera virgifera* LeConte, WCR) is a

66 chrysomelid beetle whose larvae cause damage to maize root systems. While native to North

67 America, this pest was introduced multiple times to Europe over 20 years ago (6) . Most recent

68 estimates indicate this pest causes two billion dollars (USD) in yield loss and control costs

69 worldwide annually (7, 8), and any regions growing maize should monitor for the presence or

70 arrival of this species. Since its discovery as a pest of maize, the primary control tactic has been

71 crop rotation (9). Recently, transgenic maize hybrids expressing insecticidal proteins from Bt

72 have been used to reduce root damage and economic losses. However, both of these control

73 strategies have instances of failure in the United States of America (10-15).

74   Neonate rootworm larvae (WCR and *D. barberi* Smith & Lawrence) burrow through the

75 soil searching for maize root tissues, and then through maize roots while feeding on root tissue.

76 Thus, larvae of these species are exposed to many species of bacteria and fungi in the soil and

77 rhizosphere. The diversity of bacteria encountered is reflected on larval surfaces and digestive

78 tracts. The microbiomes of larvae and later life stages may be assembled from bacterial and

79 fungal species present during larval development in soil.

80   Insect gut microbiomes are known to influence many aspects of insect growth, nutrition,

81   reproduction, Bt resistance, and pathogen resistance (1, 16-22). Gut microbiota have been shown

82   to affect the response of insects to Bt proteins in Lepidoptera (17, 20-22) and in mosquitoes (23),

83   but this has not been investigated for Coleoptera. In the Old World bollworm (*Helicoverpa*

84   *armigera* Hübner) the manipulation of the larval gut microbiota with antibiotics results in

85   reduced susceptibility to a commercial formulation of Bt, as well as the purified δ-endotoxins

86   Cry1Ab and Cry1Ac (20). In general, the use of antibiotics to manipulate lepidopteran gut

87   microbiota resulted in reduced mortality due to Bt proteins. Selection experiments with *H.*

88   *armigera* on transgenic plants were also conducted in addition to manipulation of gut microbiota

89   with antibiotics (22). When antibiotics were included, susceptibility to Bt was not altered with

90   increasing generations of selection. However, selection in the absence of antibiotics (gut

91   microbiota unaltered) resulted in a nearly 30% increase in larval survival by the F3 generation

92   (22). Thus, resistance to Bt by *H. armigera* developed only when gut microbiota were present. In

93   fact, the reduction in susceptibility to Bt with the addition of antibiotics was greater than the

94   reduction of susceptibility to Bt due to three generations of selection when gut microbiota were

95   present. Gut microbiota were also required for susceptibility of the gypsy moth, *Lymantria*

96   *dispar* (L.), to Bt proteins (17).

97   Larval gut tissue of WCR has a diverse microbial community (18, 24). In WCR, a shift in

98   gut microbiota enterotype was associated with increased resistance to soybean defense

99   compounds, which may have contributed to the development of resistance to crop rotation (24).

100   Comparison of gut microbiota between rotation-resistant WCR populations and wild-type WCR

101   populations revealed shifts in the microbial community composition. Manipulation of WCR gut

102   microbiota with antibiotics reduced the resistance to soybean defensive compounds to a level

103   similar to that of wild- type WCR (24).

104   The contribution of gut microbiota to nutrition, physiology, and Bt resistance in WCR is

105   unknown (18). Feeding of larval WCR on maize root tissue was shown to affect root rhizosphere

106   microbiota composition, indicating a complex, multitrophic interaction (19) . Since gut

107   microbiota play a role in Bt susceptibility in lepidopteran pests and a role in crop rotation

108   resistance in WCR, it is reasonable to hypothesize that the microbiota of WCR can affect how

109   larvae respond to Bt toxins expressed in maize. Consequently, a better understanding of which

110   microbes are associated with WCR and how the insects acquire the microbiome is needed. In this

111   study, we focused on the bacteriome. We compared the bacterial composition of WCR grown in

112   two different soils, at each developmental stage, and alongside the soil from which the various

113   life stages were collected and show that WCR larvae can carry particular species across all life

114   stages (*i.e.*, a core bacteriome) regardless of the environment.

115

RESULTS AND DISCUSSION

117   We conducted the first survey of the bacteriome of WCR and the soil they are found in

118   across all life stages. We investigated the effect of soil origin on the insect bacteriome because

119   WCR occurs across a large region in many different soils throughout the United States of America

120   and Europe. Soil was collected from Higginsville, MO, and the soil bacterial background from

121   which insects emerged was compared to autoclaved soil from Columbia, MO. The results show

122   that earlier life stages reared in soils from different locations contained a significantly different

123   assemblage of bacterial species. However, as the insects matured, those differences declined and

124   all life stages of the insects converged to a similar bacteriome.

125   Sequencing of WCR and soil samples resulted in a mean (± SEM) of 66,759 (± 3,895) and

126   72,868 (± 5,308) reads per sample, respectively. To account for the potential influence of

127   differential coverage on downstream analyses, data were randomly subsampled to a uniform depth

128   of 10,000 reads per sample and all subsequent analyses were performed on this rarefied dataset.

129   Annotated to the taxonomic level of class, the WCR samples were dominated by

130   *Alphaproteobacteria* and *Gammaproteobacteria*, with lower and inconsistent relative abundance

131   of *Actinobacteria*, *Cytophaga*, *Sphingobacteria*, *Betaproteobacteria*, and in the case of surface-

132   sterilized eggs, *Flavobacteriia* and *Deltaproteobacteria* (Fig 1A). Soil samples demonstrated a

133   seemingly more complex composition comprising a greater number of classes and a more even

134   distribution (Fig 1B).

135   Microbial richness and diversity are often correlated with the health of an ecosystem, be

136   it environmental or host-associated. Richness simply denotes the overall number of detected

137   phylotypes in a sample, whereas Shannon and Simpson diversity indices integrate both the

138   richness and evenness of the distribution of phylotypes in a sample. The underlying assumption

139   is that increased numbers of different taxa and more even distributions of those taxa are

140   representative of ecosystems fostering cross-feeding and syntrophic relationships among

141   microbes. In contrast, low richness or asymmetrical distributions might represent an environment

142   with high selective pressures or the presence of dominant taxa in a competitive environment.

143   Analyses of richness and diversity of bacterial communities in WCR and in the soil in

144   which they were maintained revealed several interesting trends. To first determine whether the

145   site of soil origin influenced richness, Shannon diversity index, or Simpson diversity index of

146   WCR bacteria, a two-way ANOVA was performed with soil site (*i.e.*, Columbia or Higginsville)

147   and insect life-stage as fixed variables. Significant main effects of WCR life-stage were detected

148 for richness ($p < 0.001$, F = 8.14), Shannon index ($p = 0.011$, F = 3.48), and Simpson index ($p < 0.001$, F = 5.78). No differences were detected between soil sites for richness, Shannon index, or Simpson index of WCR-associated bacteria ($p = 0.338$, 0.072, and 0.244, respectively). Of note however, similar testing of the soil communities from each site revealed significant site-dependent differences in richness, Shannon index, and Simpson index ($p < 0.001$ for all three metrics, F = 38.52, 197.64, and 25.04, respectively). No life-stage-dependent differences in bacterial richness were detected between the two soil sites, although diversity within soil did significantly vary among life-stages ($p = 0.030$, F = 2.88 and $p < 0.001$, F = 5.53 for Shannon and Simpson indices, respectively).

Collectively, we interpret these data as evidence that the environment has a limited effect on the relative uniformity and richness of the WCR bacteriome. This hypothesis is supported by the nearly log-fold difference in richness between soil and rootworm samples. The fact that no soil-dependent differences were detected in the bacteriome of rootworms themselves, despite the stark differences in the bacterial richness of their respective environments, stands in contrast to the life-stage-dependent differences in richness observed only in the rootworms and not in the soil samples.

Considering WCR samples from the two soils collectively, there was a general trend toward increasing richness in each successive life-stage from egg to pupa followed by a precipitous decline during the pupal molt to adulthood (Fig 2A). Pairwise comparisons of richness between life-stages detected significantly decreased richness of phylotypes in adult WCR relative to several earlier life-stages. Interestingly, an inverse trend was observed in the richness of bacteria in soil samples across life-stages (Fig 2B). In contrast, diversity as assessed via the Simpson index, was higher in sterilized eggs relative to other life-stages while diversity in

171   adult rootworms was much lower (S1A Fig**)**, likely reflecting the increasingly skewed bacterial

172   community structure as the rootworms mature. No life-stage-dependent differences were

173   detected in the diversity of the soil bacterial community (S1B Fig).

174   In order to provide a more comprehensive comparison of the bacterial communities

175   present in each sample, incorporating not just the number but also the identities of shared and

176   unique taxa, principal coordinate analysis (PCoA) and permutational multivariate analyses of

177   variance (PERMANOVA) were performed to visualize and statistically test for differences in

178   community structure, respectively. With both methods, the similarity of any given pair of

179   samples can be determined several different ways. To ensure that any differences detected were

180   robust and to determine the nature of detected differences, we compared samples using both the

181   Bray-Curtis and Jaccard similarity indices. While the Jaccard index is relatively unweighted and

182   determines sample similarity based on the shared presence or absence of taxa, the Bray-Curtis

183   index is weighted to also incorporate the relative abundance of any shared taxa.

184   Regardless of the index used, robust compositional differences were detected among all

185   groups with the exception of the WCR samples reared in soil from different sites, again

186   suggesting selection for a specific bacterial community within the rootworms. Specifically,

187   testing for differences using the Bray-Curtis distances detected significant compositional

188   differences between all pairwise comparisons except between WCR samples reared in different

189   soil (Table 1). Accordingly, PCoA demonstrated a clear separation of soil and WCR samples

190   along PC1 (38.1% of the total variation in the dataset), complete separation of soil communities

191   from the two soil sites along PC2, and partial overlap between WCR communities (Fig 3).

192   Testing based on the Jaccard index found significant differences between all pairwise

193   comparisons. Ordination resulted in a similar pattern and the F value generated from the

194   comparison of WCR reared on soil from the two sites was extremely low relative to the other

195   comparisons, despite having the highest total number of samples included in the comparison

196   (Table 2). Collectively, these data complement the analyses of richness and diversity in

197   supporting the hypothesis that WCR select for a limited subset of host-associated bacteria,

198   largely irrespective of their environment.

199   Annotated to the level of operational taxonomic unit (*i.e.*, the best taxonomic resolution

200   afforded by the 16S rRNA amplicons), the bacterial composition of the adult WCR was

201   incredibly sparse. Of the 474 operational taxonomic units (OTUs) detected in anywhere from one

202   of 18 (5.6%) to 15 of 18 (83.3%) of the adult rootworms, the mean relative abundance was

203   uniformly below 0.3% (S2 Fig). Conversely, the 13 OTUs detected in 16 or greater of the 18

204   adult rootworms were present at a mean relative abundance of greater than 1.5%. Notably, 95.4%

205   of the bacterial DNA recovered from adult rootworms was annotated to three OTUs: *Wolbachia*

206   sp. (85.5 ± 24.0% in 18 of 18 adults), unclassified family *Enterobacteriaceae* (6.2 ± 13.0% in 16

207   of 18 adults), and *Acinetobacter* sp. (4.7 ± 11.6% in 17 of 18 adults).

208   To determine whether inherent differences exist in the bacteriome of WCR based on

209   genetic background, insects from a colony of wild-type WCR that undergo diapause and an

210   experimental non-diapausing WCR laboratory colony were reared to each life stage in autoclaved

211   soil from Columbia, MO, as previously mentioned. All life stages and corresponding soil samples

212   were collected and processed to extract and purify DNA. The V4 region of the 16S ribosomal gene

213   was amplified and sequenced to putatively identify bacteria.

214   Once the identities of the bacteria were determined, we compared the bacteriomes between

215   the two colonies using PERMANOVA with Bray-Curtis and Jaccard indices. The two indices

216   revealed different patterns. No significant differences were detected between these colonies with

217   the Bray-Curtis index (p=0.10; F=1.90), indicating that insects that do not undergo diapause retain

218   a similar bacteriome as insects that do undergo diapause despite hundreds of generations of

219   laboratory selection. However, PERMANOVA with a Jaccard index revealed significant

220   differences in bacterial communities between insects from a diapausing colony and those from a

221   non-diapausing colony (p=0.0001; F=2.90; Fig 4). Insects from both colonies appear to share many

222   dominant taxa while rarer species appear to be isolated to individual colonies.

223   Exploratory studies documenting the bacterial communities in different organisms may

224   lead to new insights as to the role(s) they may fill or even new management tactics. Over 2,200

225   unique operational taxonomic units (OTUs) were putatively identified in soil and insect samples

226   from both colonies and soils. Our study documented more than 1,100 OTUs present throughout

227   the WCR life cycle. Of these OTUs, 16 were found in every life stage of insects regardless of the

228   colony or rearing soil. We speculate that these 16 OTUs comprise the core bacteriome for WCR.

229   Furthermore, some of these bacteria were never found in the soil suggesting vertical transmission

230   (*i.e.*, parent to progeny) of bacteria is the most likely mechanism for at least some of the WCR

231   bacteriome (Table 3).

232   Many OTUs were discovered in the sterilized eggs of insects from the diapausing

233   colony. However, we cannot be certain whether these bacteria were alive inside the egg or dead

234   on the surface of the egg shell. Given the sculpturing of the chorion, it is possible dead bacteria

235   remained on the surface served as a source of non-viable DNA (19, 25). The protocol we used

236   does not discern between live and dead bacteria. If the bacteria were alive, then it is possible the

237   eggs serve as a source of bacteria that colonize the neonatal gut. There is evidence that some of

238   the bacteria are passed from parents to offspring (Table 3), but we cannot be certain without

239   additional studies. Future experiments should extract rRNA and generate cDNA before sequencing

240   the resulting strands. This method would reduce the likelihood of dead bacterial sequences entering

241   the analysis as RNA degrades rapidly while DNA can persist for many years.

242   We infer that some of these bacteria may be endosymbionts of WCR as particular OTUs

243   never appeared outside of insect samples (Table 3). However, we used laboratory colonies to

244   make inferences about wild-type populations. In theory, the differences between wild-type

245   populations and laboratory colonies should be minimal. In reality, we simply do not know. The

246   geographic distribution of this insect encompasses most of the United States of America and

247   parts of Europe. The soils across these regions are also diverse as are the management tactics

248   employed by farmers. It stands to reason that the bacterial communities are different within and

249   between fields. Future studies will need to include more samples, samples from different

250   locations across the Corn Belt and other regions, and wild-type specimens to validate or

251   invalidate the findings of this research.

252   WCR continues to evolve and adapt to the different management tactics that maize

253   growers are implementing now. Future technologies for pest control, including RNA

254   interference, are still years away from field implementation. New tools and knowledge are

255   needed to combat this pest. This study documents the plethora of bacteria encountered by WCR

256   in different soils and identifies a small core bacteriome retained by WCR. Clearly, there is much

257   to learn about the functions of these different bacteria with regards to WCR.

258

259   **MATERIALS AND METHODS**

260   **Insect rearing.** Eggs from non-diapausing and diapausing colonies of WCR were

261   obtained from the Agricultural Research Service of the United States Department of Agriculture

262   (USDA-ARS). The non-diapausing colony was derived from the primary non-diapausing colony

263   held at Brookings, SD (26). The diapausing colony eggs were from the primary diapausing

264   colony (27) also held at Brookings, SD, and remained in cold storage until needed.

265   For the non-diapausing colony maintained in Columbia, MO, adults of both sexes were

266   placed in cages (30×30×30 cm, Megaview Science Co., Ltd., Taichung, Taiwan) with a

267   photoperiod of 14:10 (L:D) h at 25 °C. Adults were supplied with corn leaf tissue, slices of

268   zucchini, an agar gel to serve as a water source, and an artificial diet for adult rearing (Frontier

269   Agricultural Sciences, Newark, DE). Petri dishes with 70 mesh sieved field soil from Columbia,

270   MO, served as an oviposition site for females. The oviposition site was moistened throughout the

271   week and replaced weekly. The eggs in the Petri dish were separated from the soil by washing

272   through a 60 mesh sieve. The eggs were then divided and placed in two plastic containers (15 ×

273   10 cm, GladWare®, The Glad Products Company, Oakland, CA) with 70 mesh sieved Columbia,

274   MO, field soil. The plastic containers were covered with lids and placed on the bottom racks of a

275   Percival incubator set to run at 25 °C.

Seedling Mats

276   **Insects from non-diapausing colony.** Fifteen seedling mats were planted in March

278   2016. Each seedling mat contained approximately 15 g of maize seed (Monsanto Company,

279   variety DKC 61-79), 6 cm of autoclaved growth medium, and 80 ml of tap water in a 15 × 10 cm

280   plastic container. The growth medium consisted of a mixture of field soil:Pro-Mix BX potting

281   medium (Premier Horticulture Inc., Quakertown, PA) at a 2:1 ratio (v/v) prior to being

282   autoclaved. Seedling mats were allowed to germinate, and coleoptiles emerged through the soil

283   surface prior to infestation.

284   Seedling mat containers were placed on the top rack of the same Percival incubator in

285   which eggs were incubated. Data were collected at six time points: 0 d (neonate larvae), 5 d, 10

286 d, 15 d, 22 d, and adult emergence. Three replicates of each time point were used for this survey.

287 Seedling mats in each replicate were randomly assigned a time point with each seedling mat

288 receiving 25 neonate larvae. The 0 d time point did not require insect feeding and so for this

289 treatment, rather than using seedling mats, 10 neonate larvae were collected directly into 1.5ml

290 microcentrifuge tubes (USA Scientific) and then stored at -80 °C (So-Low, Environmental

291 Equipment, Cincinnati, OH).

292   For the adult emergence time point in this survey, we planted new maize seeds into a

293 larger container (33 × 19 cm, Sterilite Corporation, Birmingham, AL) and allowed the maize to

294 grow for one week prior to infestation. The first and smaller seedling mat had plant tissue

295 removed before being inverted onto the second and larger seedling mat containing soil from the

296 same site. After one week, the larger seedling mat was covered with a mesh screen to prevent

297 escape of emerging adults.

298   **Insects from a diapausing colony**. A total of five replications were conducted for this

299 survey. During this survey, two different growth media were used. The first growth medium

300 remained the same as the previous insect survey, while the second growth media was soil

301 collected from a continuous corn field in Higginsville, MO, in July 2016. This soil was not

302 autoclaved and remained enclosed in a metal container until use in October 2016. In addition to

303 the time points listed previously, two types of eggs were sampled: eggs washed from sieved soil,

304 and eggs washed from sieved soil that were then surface sterilized (28).

305   Once the desired time point was reached, the seedling mats were processed in the same

306 manner as (29). For the 5, 10, 15, and 22 d time points, all aboveground plant material was

307 removed from the container. Next, the soil and root tissue were placed into a Berlese funnel with

308 an attached jar. The jar with a moist filter paper at the bottom was used to collect the larvae.

309   Specimens of each age were transferred from the jar to a microcentrifuge tube at least once every

310   three hours throughout a typical work day. This tube was then immediately placed into the -80

311   °C freezer for storage until DNA extraction occurred. A new tube was used for each collection

312   time and sample to prevent additional freezing and thawing. During time points when larvae

313   were sampled, soil was also collected from the bottom of the seedling mat prior to drying.

314   No secondary container was used for the diapausing insect survey, but mesh screens were

315   used to keep the adults from escaping the container. Adult emergence containers were checked

316   daily, and adults from each container on a given day were placed into microcentrifuge tubes. Soil

317   was collected from the soil surface where adults must pass to emerge through the soil.

318   **DNA Extraction and Quantification.** Whole insects (1-8 larvae/treatment; 1-2

319   pupae/treatment; a single adult/treatment) were pooled, and DNA extracted using accepted

320   methods (30). The samples were extracted using PowerFecal® DNA Isolation Kit (MO BIO

321   Laboratories, Inc. Catalog No. 12830-50) following the manufacturer's protocol

322   (https://mobio.com/media/wysiwyg/pdfs/protocols/12830.pdf) with the following modifications:

323   one sterile 0.5 cm diameter stainless steel ball bearing was added to the Dry Bead Tube for each

324   adult and soil sample prior to shaking; shaking time was reduced to 5 minutes for adults and 3

325   minutes for all other samples. DNA quality and concentration was determined for each sample

326   by Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and stored at

327   −80°C.

328   **Library construction and sequencing.** All PCR and sequencing was performed at the

329   University of Missouri DNA Core. DNA concentration was determined fluorometrically (Qubit

330   2.0, Life Technologies) prior to analysis. Based on results of fluorometry, all samples were

331   normalized to a standard concentration for PCR amplification. Bacterial 16S rRNA amplicons

332   were generated via amplification of the V4 hypervariable region of the 16S rRNA gene using

333   single-indexed universal primers (U515F/806R) flanked by Illumina standard adapter sequences

334   and the following parameters: 98 ˚C$^{(3:00)}$+[98˚C$^{(0:15)}$+50˚C$^{(0:30)}$+72˚C$^{(0:30)}$] × 25 cycles +72˚C$^{(7:00)}$.

335   Amplicons were then pooled for sequencing using the Illumina MiSeq platform and V2

336   chemistry with 2×250 bp paired-end reads, as previously described (31).

337   **Informatics analysis.** All informatics analyses were performed as previously described

338   (32), at the University of Missouri Informatics Research Core Facility. Input is typically for

339   2x350 bp reads from one of the two MiSeq machines in the DNA Core. The read pairs are joined

340   into contigs by the program FLASH

341   (http://bioinformatics.oxfordjournals.org/content/27/21/2957.long) (33), and culled if found to be

342   short after trimming for a base quality less than 31, and those that are not joined, or are too long

343   or short after contig formation, leaving those that are 275 to 300 nts. Cutadapt

344   (http://journal.embnet.org/index.php/embnetjournal/article/view/200/479) was used to find and

345   trim the primers from the 5' and the 3' ends, culling those contigs lacking both primers. Contigs

346   with the expected number of errors greater than 0.5 were removed by Usearch

347   (http://drive5.com/index.htm), and the remainder were trimmed to length 248. The contig read

348   ids were modified so that samples could be followed throughout by using the Qiime script

349   split_libraries_fastq.py. All samples were then pooled into one FASTA file and metrics for all

350   samples collated into one table. Contigs were clustered *de novo* into an OTU table using the

351   uparse (http://drive5.com/uparse/) algorithm. *De novo* and reference-based chimera detection and

352   removal was performed using Qiime v1.8 (34) software, and remaining contiguous sequences

353   were assigned to operational taxonomic units (OTUs) via *de novo* OTU clustering and a criterion

354   of 97% nucleotide identity. Annotation of selected OTUs was performed using BLAST (35)

355   against the Silva database (https://www.arb-silva.de/) (36) of 16S rRNA sequences and

356   taxonomy. Principal coordinate analysis and PERMANOVA testing were performed using ¼

357   root-transformed and non-transformed OTU relative abundance data, respectively, using Past

358   3.16 (https://folk.uio.no/ohammer/past/) (37). Richness, Shannon diversity index, and Simpson

359   diversity metrics were determined in Past 3.16 using Qiime-generated otu_biom.table files.

360   **Statistical analysis.** Differences in raw and binned OTU richness were tested via

361   ANOVA using SigmaPlot 12.3 (Systat Software Inc., San Jose, CA); p values less than 0.05

362   were considered significant. Differences in the overall composition of the different regions were

363   tested via two- and one-way PERMANOVA of ranked Bray-Curtis or Jaccard distances using

364   the open access Past 3.16 software package (38), downloaded on April 2, 2016.

365

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COMPETING INTERESTS

The authors declare no competing interests.

FINANCIAL DISCLOSURE

AUTHOR CONTRIBUTIONS

B.E.H., L.N.M., T.A.C., and K.S.S. secured funding for the project; D.C.L., L.N.M., T.A.C., B.E.H. and K.S.S. conceived and designed the experiments; D.C.L., L.N.M., M.L.G., and A.C.E. performed the experiments; A.C.E., D.C.L. and K.S.S. analyzed the data; D.C.L., L.N.M., A.C. E. and K.S.S. contributed reagents/materials/analysis tools; and D.C.L., L.N.M., M.L.G, A.C.E., D.L.F., T.A.C., B.E.H., and K.S.S. wrote the paper. All authors read and approved the final version.

TABLES

Table 1. Results of PERMANOVA testing for differences in β-diversity between western corn rootworm (WCR) and soil samples collected from two different sites, based on the Bray-Curtis distance. *p* values and F values are shown in the upper right and lower left portions of the table, respectively.

F values / *p* values		Soil origin		WCR from "X" soil	
		Columbia	Higginsville	Columbia	Higginsville
Soil origin	Columbia		0.0001	0.0001	0.0001
	Higginsville	27.62		0.0001	0.0001
WCR from "X" soil	Columbia	57.08	104.5		0.1498
	Higginsville	38.43	119.7	1.657	

400 **Table 2**. Results of PERMANOVA testing for differences in β-diversity between western corn
401 rootworm (WCR) and soil samples collected from two different sites, based on the Jaccard
402 distance. *p* values and F values are shown in the upper right and lower left portions of the table,
403 respectively.

p values / F values		Soil Origin		WCR from "X" soil	
		Columbia	Higginsville	Columbia	Higginsville
Soil Origin	Columbia		0.0001	0.0001	0.0001
	Higginsville	24.93		0.0001	0.0001
WCR from "X" soil	Columbia	19.62	23.66		0.0001
	Higginsville	18.56	18.6	3.972	

404

405 **Table 3.** Unique operational taxonomic units (OTUs) found in all insect samples regardless of
406 soil origin.

OTUs	Taxonomic Rank	Present in egg soil?[1]	First found in soil[2]
Ruminococcaceae	Family	Yes	Egg
Lachnospiraceae	Family	Yes	Egg
Bacteroidales S24-7	Group	Yes	Egg
Wolbachia (*Delia antiqua*)	Genus	No	Neonate
Tsukamurella sp.	Genus	No	Never
Gordonia sp.	Genus	Yes	Egg
Oscillibacter sp.	Genus	Yes	Egg
Microbacterium sp.	Genus	Yes	Egg
Bacillus megaterium	Species	No	Never
Geobacillus toebii	Species	Yes	Egg
Klebsiella sp. Z1	Species	Yes	Egg
Mycobacterium fortuitum	Species	No	Never
Streptomyces rectiviolaceus	Species	No	Never
Lachnospiraceae NK4A136	Species	Yes	Egg
Pseudomonas sp. FSGRN7	Species	No	Never
Pseudonocardia sp. YIM 68245	Species	No	Never

407 **[1]Were the OTUs found in the soil in which eggs were incubated and neonates emerged?**

408 **[2]If these OTUs were found in insect samples, then which insect life stage were these OTUs**
409 **first detected?**

FIGURE LEGENDS

Fig 1. Stacked bar charts showing relative abundances of bacterial classes detected in corn rootworms at different life stages (A) and in soil from which rootworm samples were collected (B). Horizontal bars below the vertical bars indicate original of soil; black bars = Columbia, MO, white bars = Higginsville, MO.

Fig 2. Main effect of life stage on bacterial richness in western corn rootworm (**A,** $p<0.001$), or the soil from which WCR samples were collected (**B**, $p = 0.040$). Significant pairwise differences are indicated by like letters (Kruskal-Wallis one-way ANOVA on ranks with Dunn's *post hoc*).

Fig 3. Principal coordinate analysis based on Bray-Curtis similarity between bacterial communities detected in western corn rootworm (WCR) at various life stages and soil samples collected from two different sites.

Fig 4. Principal coordinate analysis based on Bray-Curtis similarity between bacterial communities detected in western corn rootworm (WCR) from diapausing and non-diapausing colonies including all life stages, except sterilized ova.

S1 Fig. Main effect of life stage on mean Shannon and Simpson diversity indices in western corn rootworms (**A,** $p < 0.001$), or the soil from which the WCR samples were collected (**B**, $p = 0.040$). Significant pairwise differences indicated like letters (Kruskal-Wallis one-way ANOVA on ranks with Dunn's *post hoc*).

S2 Fig. Principal coordinate analysis based on Jaccard similarity between bacterial communities detected in western corn rootworms (WCR) at various life stages and soil samples collected from two different sites.

S3 Fig. Number and mean relative abundance (above bars) of operational taxonomic units (OTUs) detected at increasing prevalence in adult western corn rootworm samples.

439   **SUPPORTING INFORMATION**

440

441   **REFERENCES**

442   1.   Caccia S, Di Lelio I, La Storia A, Marinelli A, Varricchio P, Franzetti E, et al. Midgut
443   microbiota and host immunocompetence underlie *Bacillus thuringiensis* killing mechanism.
444   Proceedings of the National Academy of Science USA 2016;113(34):9486–91.
445   2.   Dhammi A, van Krestchmar JB, Ponnusamy L, Bacheler JS, Reisig DD, Herbert A, et al.
446   Biology, pest status, microbiome and control of kudzu bug (Hemiptera: Heteroptera:
447   Plataspidae): a new invasive pest in the U.S. International journal of molecular sciences.
448   2016;17(9):1570.
449   3.   Hadapad AB, Prabhakar C, S. , Chandekar SC, Tripathi J, Hire RS. Diversity of bacterial
450   communities in the midgut of Bactrocera cucurbitae (Diptera: Tephritidae) populations and
451   their potential use as attractants. Pest Management Science 2016;72:1222-30.
452   4.   Snyman M, Gupta AK, Bezuindenhout C, Claasens S, van den Berg J. Gut microbiota of
453   Busseola fusca (Lepidoptera: Noctuidae). World Journal of Microbiology and Biotechnology.
454   2016;32:115.
455   5.   Perlatti B, Luiz AL, Prieto EL, Fernandes JB, das Graças Fernandes da Silva MF, Ferreira D,
456   et al. MALDI-TOF MS identification of microbiota associated with pest insect Diabrotica
457   speciosa. Agricultural and Forest Entomology. 2017;19:408-17.
458   6.   Miller N, Estoup A, Toepfer S, Bourguet D, Lapchin L, Derridj S, et al. Multiple
459   transatlantic introductions of the western corn rootworm. Science. 2005;310(5750):992.
460   7.   Metcalf RJ. Foreword. Methods for the Study of Pest Diabrotica. New York, NY USA:
461   Springer-Verlag; 1986. p. vii–xv.
462   8.   Mitchell P. Cost and benefits of controlling pest *Diabrotica* in maize in the United States.
463   24th IWGO Conference; 24-26 Oct. 2011; Freiburg, Germany.
464   9.   Gillette CP. Diabrotica virgifera LeC as a corn root-worm. Journal of Economic
465   Entomology. 1912;5:3.
466   10.   Levine E, Spencer JL, Isard SA, Onstad DW, Gray ME. Adaptation of the western corn
467   rootworm to crop rotation: evolution of a new strain in response to a management practice.
468   American Entomologist. 2002;48:94-107.
469   11.   Gassmann AJ, Petzold-Maxwell JL, Keweshan RS, Dunbar MW. Field-evolved resistance
470   to Bt maize by western corn rootworm. PLoS One. 2011;6(7):e22629.
471   12.   Gassmann AJ, Petzold-Maxwell JL, Clifton EH, Dunbar MW, Hoffmann AM, Ingber DA, et
472   al. Field-evolved resistance by western corn rootworm to multiple Bacillus thuringiensis toxins
473   in transgenic maize. Proceedings of the National Academy of Science USA. 2014;111(14):5141-
474   6.
475   13.   Gassmann AJ, Shrestha RB, Jakka SR, Dunbar MW, Clifton EH, Paolino AR, et al. Evidence
476   of resistance to Cry34/35Ab1 corn by western corn rootworm (Coleoptera: Chrysomelidae):
477   Root injury in the field and larval survival in plant-based bioassays. Journal of Economic
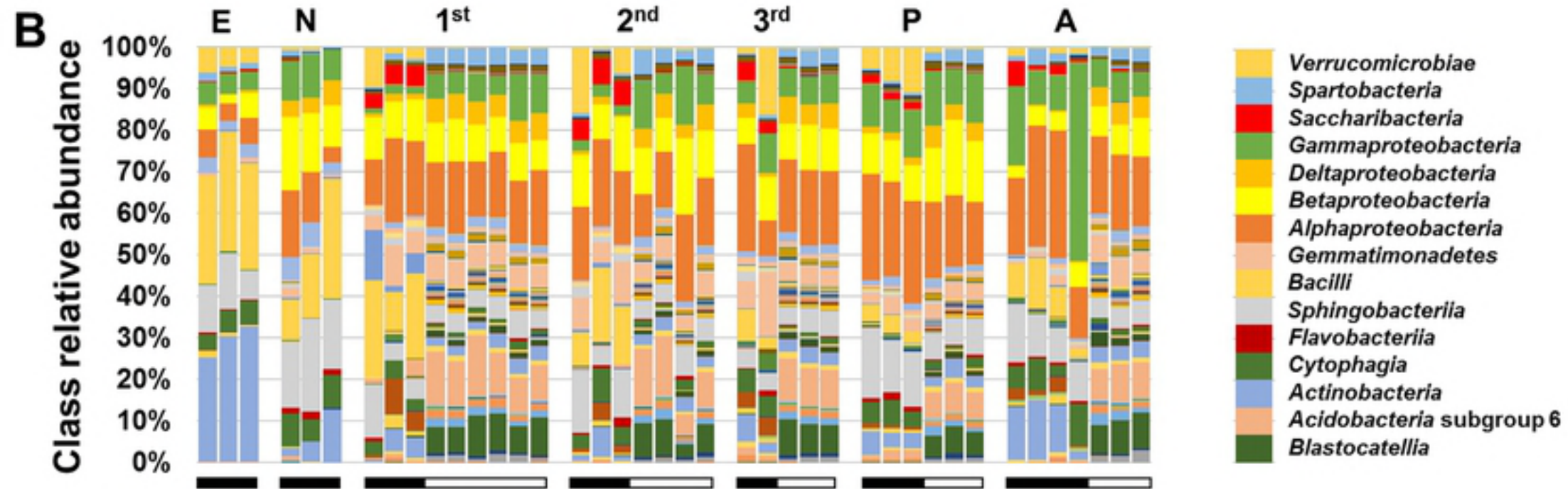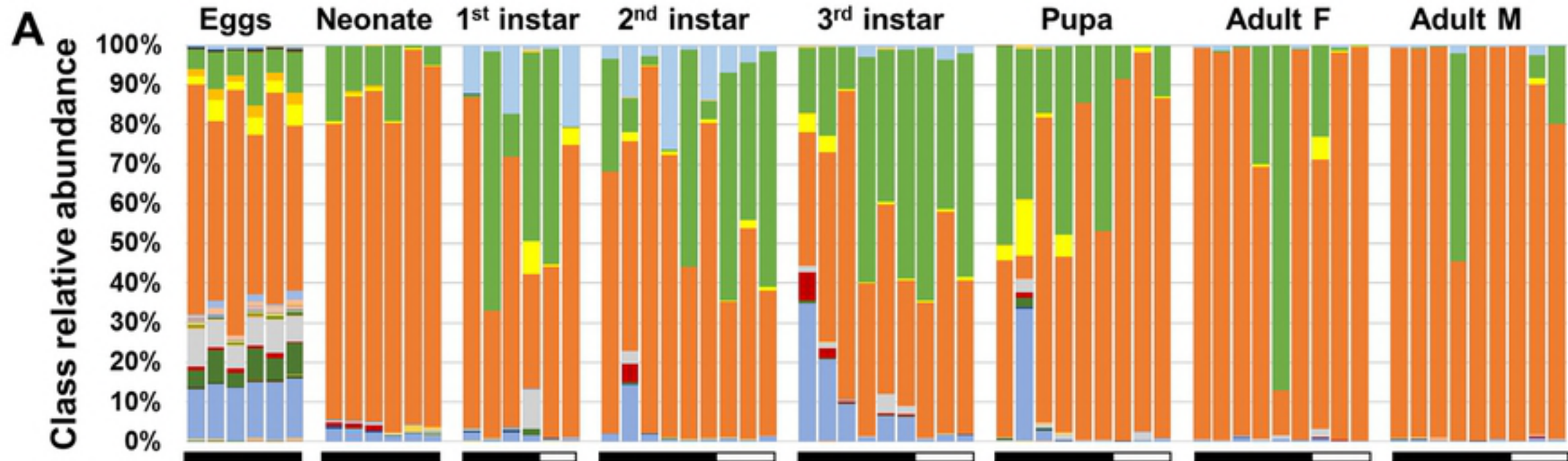478   Entomology. 2016.

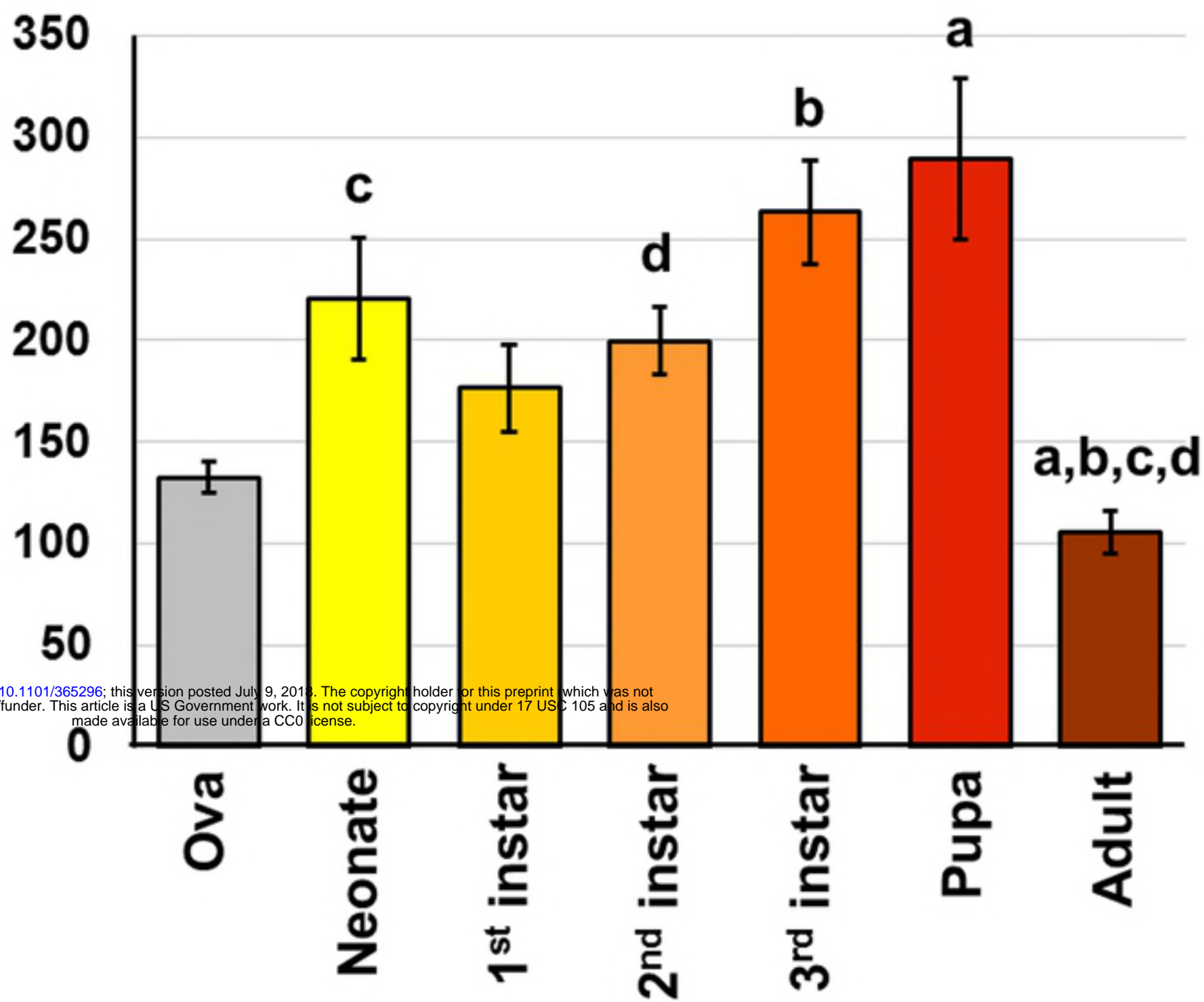Bacteriome of western corn rootworm life stages in different soils

479   14.   Zukoff SN, Ostlie KR, Potter B, Meihls LN, Zukoff AL, French L, et al. Multiple assays
480   Iidicate varying levels of cross resistance in Cry3Bb1-selected field populations of the western
481   corn rootworm to mCry3A, eCry3.1Ab, and Cry34/35Ab1. Journal of Economic Entomology.
482   2016;109:1387–98.
483   15.   Ludwick, D.C. , Meihls LN, Ostlie KR, Potter BD, French L, Hibbard BE. Minnesota field
484   population of western corn rootworm (Coleoptera: Chrysomelidae) shows incomplete
485   resistance to Cry34/35Ab1 and Cry3Bb1. Journal of Applied Entomology. 2017;141:28-40.
486   16.   Douglas AE. Nutritional interactions in insect-microbial symbioses: aphids and their
487   symbiotic bacteria Buchnera. Annual Review of Entomology. 1998;43:17-37.
488   17.   Broderick NA, Raffa KF, Handelsman J. Midgut bacteria required for *Bacillus
489   thuringiensis* insecticidal activity. Proceedings of the National Academy of Sciences USA.
490   2006;103:15196-9.
491   18.   Dematheis F, Kurtz B, Vidal S, Smalla K. Microbial communities associated with the larval
492   gut and eggs of the western corn rootworm. PLoS One. 2012;7(e44685):e44685.
493   19.   Dematheis F, Zimmerling U, Flocco C, Kurtz B, Vidal S, Kropf S, et al. Multitrophic
494   interaction in the rhizosphere of maize: Root feeding of western corn rootworm larvae alters
495   the microbial community composition. PLOS One. 2012;5(e37228):e37228.
496   20.   Paramasiva I, Sharma HC, Krishnayya PV. Antibiotics influence the toxicity of the δ-
497   endotoxins of *Bacillus thuringiensis* towards the cotton bollworm, *Helicoverpa armigera*. BMC
498   Microbiology. 2014;14:200.
499   21.   Paramasiva I, Shouche Y, Kulkarni GJ, Krishnayya PV, Akbar SM, Sharma HC. Diversity in
500   gut microflora of *Helicoverpa armigera* populations from different regions in relation to
501   biological activity of *Bacillus thuringiensis* delta-endotoxin Cry1Ac. Archives of Insect
502   Biochememistry and Physiology. 2014;87(4):201-13.
503   22.   Visweshwar R, Sharma HC, Akbar SMD, Sreeramulu K. Elimination of gut microbes with
504   antibiotics confers resistance to *Bacillus thuringiensis* toxin proteins in *Helicoverpa armigera*
505   (Hubner). Applied Biochememistry and Biotechnology. 2015;177:1621-37.
506   23.   Patil CD, Borase HP, Salunke BK, Patil SV. Alteration in *Bacillus thuringiensis* toxicity by
507   curing gut flora: novel approach for mosquito resistance management. Parasitology Research.
508   2013;112(9):3283-8.
509   24.   Chu CC, Spencer JL, Curzi MJ, Zavala JA, Seufferheld MJ. Gut bacteria facilitate
510   adaptation to crop rotation in the western corn rootworm. Proceedings of the National
511   Academy of Science USA. 2013;110(29):11917-22.
512   25.   Krysan J. Introduction: biology, distribution, and identification of pest Diabrotica. In:
513   Krysan JL, Miller TA, editors. Methods for the Study of Pest Diabrotica: Springer-Verlag; 1986. p.
514   1-23.
515   26.   Branson TF. Viability and hatching pattern of eggs of the western corn rootworm
516   exposed to chill periods of different durations. Entomologia Experimentalis et Applicata.
517   1976;19:77-81.
518   27.   Jackson J. Rearing and handling of *Diabrotica virgifera virgifera* and *Diabrotica
519   undecimpunctata howardi*. Methods for the study of pest Diabrotica: Springer-Verlag, New
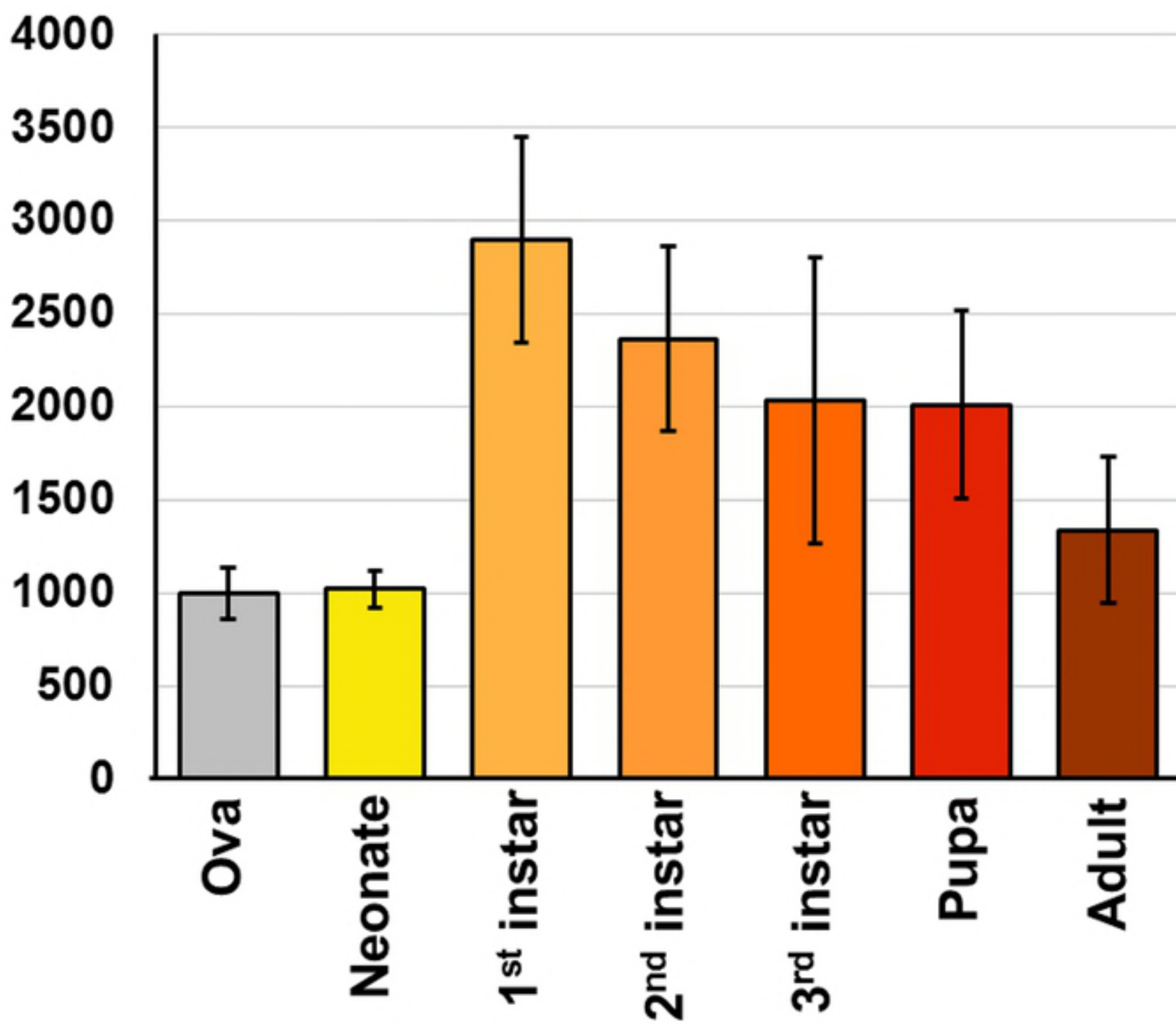520   York, USA; 1986. p. 25–47.

521 28.   Pleau MJ, Huesing JE, Head GP, Feir DJ. Development of an artificial diet for the western
522 corn rootworm. Entomologia Experimentalis et Applicata. 2002;105:1-11.
523 29.   Meihls LN, Higdon ML, Siegfried BD, Miller NJ, Sappington TW, Ellersieck MR, et al.
524 Increased survival of western corn rootworm on transgenic corn within three generations of on-
525 plant greenhouse selection. Proceedings of the National Academy of Science USA.
526 2008;105(49):19177–82.
527 30.   Chen H, Rangasamy M, Tan SY, Wang HC, Siegfried BD. Evaluation of Five Methods for
528 Total DNA Extraction from Western Corn Rootworm Beetles. PloS ONE. 2010;5(8).
529 31.   Ericsson AC, Davis JW, Spollen W, Bivens N, Givan S, Hagan CE, et al. Effects of vendor
530 and genetic background on the composition of the fecal microbiota of inbred mice. PLoS One.
531 2015;10(2):e0116704.
532 32.   Hart ML, Meyer A, Johnson PJ, Ericsson AC. Comparative Evaluation of DNA Extraction
533 Methods from Feces of Multiple Host Species for Downstream Next-Generation Sequencing.
534 PLoS One. 2015;10(11):e0143334.
535 33.   Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome
536 assemblies. Bioinformatics 27:2957-2963. Bioinformatics. 2011;27:2957-63.
537 34.   Kuczynski J, Stombaugh J, Walters WA, González A, Caporaso JG, Knight R. Using QIIME
538 to analyze 16S rRNA gene sequences from microbial communities. Current Protocols in
539 Microbiology. 2012;27:1E.5.1-E.5.20.
540 35.   Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST
541 and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research.
542 1997;25:3389-402.
543 36.   DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a
544 chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and
545 Environmental Microbiology. 2006;72(7):5069-72.
546 37.   Hammer Ø, Harper DAT, Ryan PD. PAST: Paleontological statistics software package for
547 education and data analysis. Palaeontol Electronica. 2016;4.
548 38.   Quast C PE, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA
549 ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic
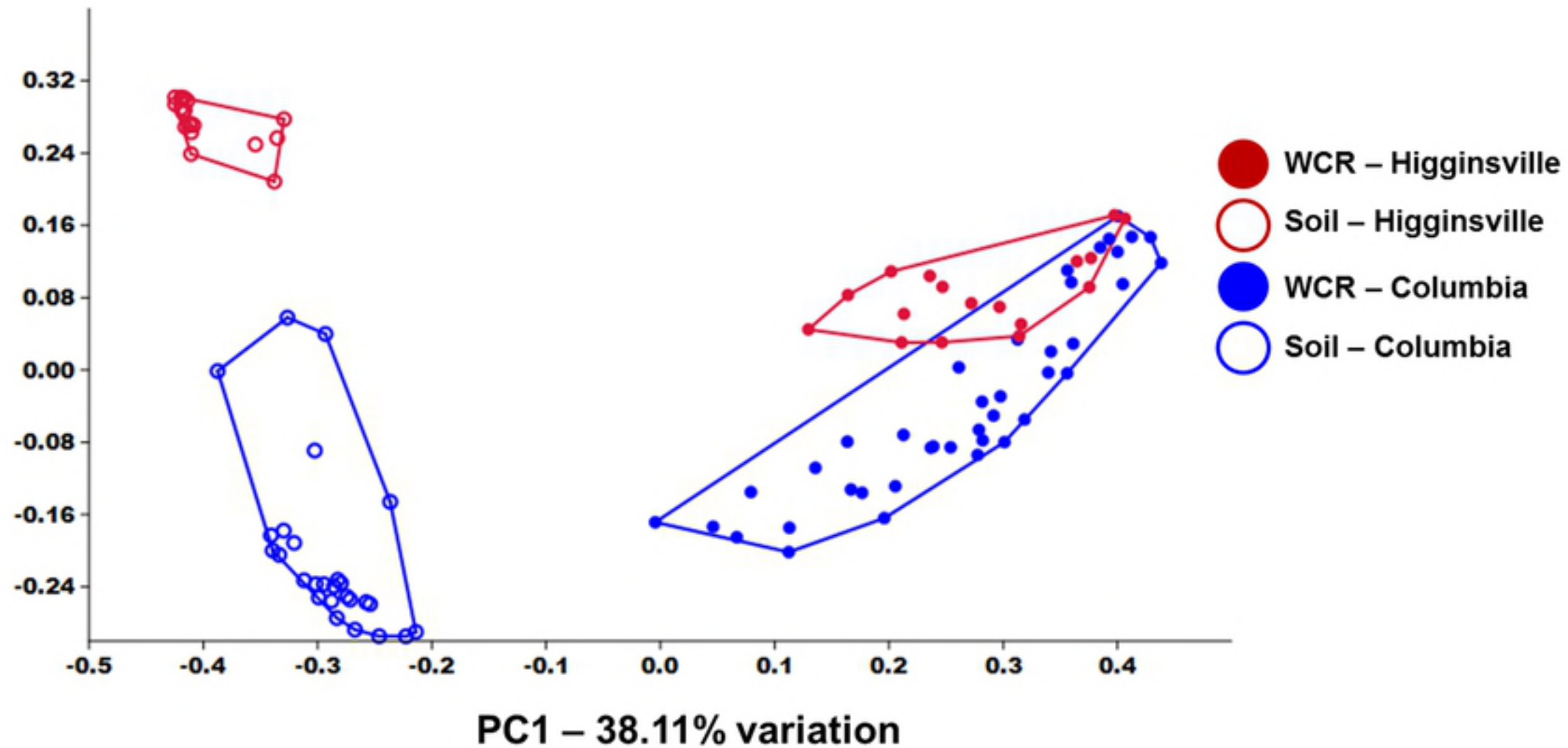550 Acids Research. 2013;41:D590-D6

551

A

Class relative abundance (%) — Eggs, Neonate, 1st instar, 2nd instar, 3rd instar, Pupa, Adult F, Adult M

B

Class relative abundance (%) — E, N, 1st, 2nd, 3rd, P, A

Legend:
- *Verrucomicrobiae*
- *Spartobacteria*
- *Saccharibacteria*
- *Gammaproteobacteria*
- *Deltaproteobacteria*
- *Betaproteobacteria*
- *Alphaproteobacteria*
- *Gemmatimonadetes*
- *Bacilli*
- *Sphingobacteriia*
- *Flavobacteriia*
- *Cytophagia*
- *Actinobacteria*
- *Acidobacteria* subgroup 6
- *Blastocatellia*