Evidence-based recommendations on storing and handling specimens for analyses of insect microbiota

Tobin J Hammer, Jacob C Dickerson, Noah Fierer

Research on insect microbiota has greatly expanded over the past decade, along with a growing appreciation of the microbial contributions to insect ecology and evolution. Many of these studies use DNA sequencing to characterize the diversity and composition of insect-associated microbial communities. The choice of strategies used for specimen collection, storage, and handling could introduce biases in molecular assessments of insect microbiota, but such potential influences have not been systematically evaluated. Likewise, although it is common practice to surface sterilize insects prior to DNA extraction, it is not known if this time-consuming step has any effect on microbial community analyses. To resolve these methodological unknowns, we conducted an experiment wherein replicate individual insects of four species were stored intact for two months using five different methods—freezing, ethanol, dimethyl sulfoxide (DMSO), cetrimonium bromide (CTAB), and room-temperature storage without preservative—and then subjected to whole-specimen 16S rRNA gene sequencing to assess whether the structure of the insect-associated bacterial communities was impacted by these different storage strategies. Overall, different insect species harbored markedly distinct bacterial communities, a pattern that was highly robust to the method used to store samples. Storage method had little to no effect on assessments of microbiota composition, and the magnitude of the effect differed among the insect species examined. No single method emerged as "best," i.e. one consistently having the highest similarity in community structure to control specimens, which were not stored prior to homogenization and DNA sequencing. We also found that surface sterilization did not change bacterial community structure as compared to unsterilized insects, presumably due to the vastly greater microbial biomass inside the insect body relative to its surface. We therefore recommend that researchers can use any of the methods tested here, and base their choice according to practical considerations such as prior use, cost, and availability in the field, although we still advise that all samples within a study be handled in an identical manner when possible. We also suggest that, in large-scale molecular studies of hundreds of insect specimens, surface sterilization may not be worth the time and effort involved. This information should help researchers design sampling strategies and will facilitate cross-

comparisons and meta-analyses of microbial community data obtained from insect specimens preserved in different ways.

1   **Evidence-based recommendations on storing and handling specimens for analyses of insect**

2   **microbiota**

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16 **Abstract**

18   Research on insect microbiota has greatly expanded over the past decade, along with a

19 growing appreciation of the microbial contributions to insect ecology and evolution. Many of

20 these studies use DNA sequencing to characterize the diversity and composition of insect-

21 associated microbial communities. The choice of strategies used for specimen collection, storage,

22 and handling could introduce biases in molecular assessments of insect microbiota, but such

23 potential influences have not been systematically evaluated. Likewise, although it is common

24 practice to surface sterilize insects prior to DNA extraction, it is not known if this time-

25 consuming step has any effect on microbial community analyses. To resolve these

26 methodological unknowns, we conducted an experiment wherein replicate individual insects of

27 four species were stored intact for two months using five different methods—freezing, ethanol,

28 dimethyl sulfoxide (DMSO), cetrimonium bromide (CTAB), and room-temperature storage

29 without preservative—and then subjected to whole-specimen 16S rRNA gene sequencing to

30 assess whether the structure of the insect-associated bacterial communities was impacted by

31 these different storage strategies. Overall, different insect species harbored markedly distinct

32 bacterial communities, a pattern that was highly robust to the method used to store samples.

33 Storage method had little to no effect on assessments of microbiota composition, and the

34 magnitude of the effect differed among the insect species examined. No single method emerged

35 as "best," i.e. one consistently having the highest similarity in community structure to control

36 specimens, which were not stored prior to homogenization and DNA sequencing. We also found

37 that surface sterilization did not change bacterial community structure as compared to

38 unsterilized insects, presumably due to the vastly greater microbial biomass inside the insect

39 body relative to its surface. We therefore recommend that researchers can use any of the methods

40 tested here, and base their choice according to practical considerations such as prior use, cost,

41 and availability in the field, although we still advise that all samples within a study be handled in

42 an identical manner when possible. We also suggest that, in large-scale molecular studies of

43 hundreds of insect specimens, surface sterilization may not be worth the time and effort

44 involved. This information should help researchers design sampling strategies and will facilitate

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46 specimens preserved in different ways.

47

48 **Introduction**

49

50   Many insects are associated with microbial symbionts that play critical roles in their

51 ecological interactions and have shaped their evolutionary history (Dillon & Dillon, 2004; Engel

52 & Moran, 2013; Douglas, 2015). The structure and function of insect microbiota are increasingly

53 studied using molecular methods such as 16S rRNA gene sequencing, but these methods are

54 often directly adopted from environmental or human microbiota studies and have rarely been

55 evaluated for their efficacy and accuracy when used with insect specimens. The growing field of

56 insect-microbial symbiosis would benefit from improved validation of the sample storage and

57 handling strategies used for studying insect microbiota.

58   Sample storage is one of the most important steps to consider when designing and

59 implementing any DNA sequencing-based analysis of insect-associated microbial communities.

60 When insect specimens are collected in the field, it is often not possible to extract DNA

61 immediately after insect collection, and so specimens must be preserved in some way that

62   minimizes the impact to microbial community structure. Samples are often irreplaceable, and if

63   they are preserved improperly then they will remain useless no matter what technological

64   advances in DNA extraction, amplification, or sequencing arise in the future (Cary & Fierer,

65   2014).

66   While some studies have shown that storage methodology can affect DNA recovery and

67   amplification success from insects and/or their symbionts (Post, Flook & Millest, 1993; Fukatsu,

68   1999; Mandrioli, Borsatti & Mola, 2006; Moreau et al., 2013), we know of no study that has

69   directly tested how different storage methods impact molecular assessments of insect-associated

70   microbial community structure. Previous studies of insect microbiota have used a range of

71   storage methods including, but not limited to, freezing (Jones, Sanchez & Fierer, 2013; Hammer,

72   McMillan & Fierer, 2014), ethanol (Koch et al., 2013; Estes et al., 2013), RNAlater (Campbell et

73   al., 2004; Sanders et al., 2014), and combinations thereof. We do not know how these different

74   storage strategies may influence assessments of microbial community structure and whether

75   some storage strategies are better suited for some insect groups than others. Furthermore, it

76   remains poorly understood whether and to what degree any potential biases associated with

77   sampling handling strategies may influence conclusions from meta-analyses (Colman, Toolson

78   & Takacs-Vesbach, 2012) or individual studies surveying microbiota of insects stored in

79   different ways.

80   Storage methods could impact insect microbiota through differential preservation of some

81   microbial cell types (e.g., gram-positive versus gram-negative bacteria), differential penetration

82   into insect tissues, or due to the preservative itself contaminating the specimen. Studies

83   comparing the most commonly used storage methods on non-insect samples (e.g., soil, plant

84   leaves, and human feces) have generally found that effects on assessments of bacterial

85   community structure are nonexistent, or negligible in magnitude relative to biological factors that

86   are of interest such as interindividual or interspecific differences in microbiota (Lauber et al.,

87   2010; Rubin et al., 2013; U'Ren et al., 2014; Dominianni et al., 2014; Franzosa et al., 2014).

88   A distinct, but related issue in processing insect specimens for molecular analysis of their

89   microbiota is determining whether the specimens require surface sterilization prior to DNA

90   extraction. This approach typically involves soaking specimens in dilute bleach and is often

91   employed to remove any surface-associated microbes or contaminants from handling or storage

92   reagents so that downstream analyses will just capture internal symbionts, such as those in the

93   gut, bacteriomes, or reproductive structures. Despite the widespread use of surface sterilization

94   (e.g., Rosengaus et al., 2011; Hammer, McMillan & Fierer, 2014; Sanders et al., 2014) and the

95   significant amount of time it adds when extracting DNA from hundreds of specimens, its

96   potential impact on molecular assessments of insect microbiota has not yet been evaluated. If the

97   vast majority of microbial biomass is found inside insects, surface sterilization might have little

98   effect on the whole-insect microbiota. Alternatively, if the procedure is too aggressive, surface

99   sterilization could decrease the abundance of internal microbiota and introduce biases in the

100   assessment of microbial community composition.

101   In this study, we asked: i) Do storage and surface sterilization methods affect insect-

102   associated microbial community structure, and if so, are these effects dependent on the insect

103   species examined?, and ii) How do potential methodological biases compare in magnitude

104   relative to the (presumably large) inter-order differences in microbiota? To address these

105   questions, we conducted an experiment on replicate individuals from four morphologically and

106   phylogenetically disparate insect species collected from the same locality. Specimens were

107   stored for two months using five different treatments before DNA extraction for 16S rRNA-

108   based community profiling. Our goal was to develop and validate a set of "best practices" for the

109   storage and handling of samples prior to DNA-based investigations of insect microbiota.

110

111   **Materials and Methods**

112

113   During the summer of 2013, we collected adult specimens of four insect species in

114   Boulder, Colorado, USA: the cabbage white butterfly *Pieris rapae* (Lepidoptera: Pieridae), the

115   speckle-winged grasshopper *Arphia conspersa* (Orthoptera: Acrididae), the Mexican bean beetle

116   *Epilachna varivestis* (Coleoptera: Coccinellidae), and the honey bee *Apis mellifera*

117   (Hymenoptera: Apidae). We chose to collect the specimens as they would typically be collected

118   by entomologists in order to assess the potential value of previously collected insect specimens

119   for molecular microbial studies, and to evaluate the role of surface sterilization in removing

120   potential contaminants introduced from human skin or collection equipment when samples are

121   not collected or handled aseptically. For example, we used standard nets as well as manual

122   collection (without gloves), and sacrificed insects in jars containing ethyl acetate (Willows-

123   Munro & Schoeman, 2014). Insect bodies were stored intact, except butterflies from which we

124   first removed the wings.

125   By collecting multiple members of the same population at the same time, we aimed to

126   minimize external sources of microbiota variability and thus maximize the potential to detect

127   storage treatment effects. Unlike previous studies on soil or feces (e.g., Lauber et al., 2010;

128   Rubin et al., 2013), a single insect sample cannot be equally divided into sub-samples to test

129   multiple storage treatments; hence, our replicates are individual specimens and we expected

130   some natural variability in microbiota among them. Six individuals were collected for each

131   species-treatment combination, although not all specimens yielded sufficient sequence data for

132   inclusion in downstream analyses. An overview of our experimental design is shown in Figure 1.

133   To test for surface sterilization effects, one set of specimens (chosen randomly from those

134   collected) were surface-sterilized immediately after field collection through a rinse in sterile

135   water (Sigma-Aldrich), a soak in 70% ethanol followed by 10% bleach for 60 s each, and a

136   second rinse in water (similar to Hammer et al., 2014, though without subsequent immersion in

137   liquid N_2). We then thoroughly homogenized the insects with a sterile mortar and pestle, in order

138   to recover DNA from all potential internal symbionts (Rubin et al., 2014). The unsterilized

139   control specimens were homogenized directly after field collection. All homogenized material

140   was frozen at -80°C until DNA extraction.

141   To test for effects of different storage strategies, whole insects were stored (without prior

142   surface sterilization) over eight weeks using one of five different methods detailed below, and

143   then homogenized and frozen at -80°C. These samples were later compared to the controls

144   (specimens homogenized directly after field collection and frozen at -80°C prior to DNA

145   extraction). Although it was not possible to extract microbial DNA from freshly caught insects,

146   we expected the controls to be the most similar to wild insect microbiota, and thus consider them

147   to have the fewest potential biases of any of our treatments. One storage treatment simply

148   involved maintaining dead insects in empty tubes in the laboratory, under ambient conditions

149   (approximately 21°C and 32% humidity). This treatment mimics how insects are often stored in

150   museum collections, and we expected this treatment to have the strongest effect relative to the

151   control.

152   The second storage method involved freezing specimens dry (i.e., no preservative) at -

153   20°C. Freezing is commonly used and should have a lower potential for introducing

154   contaminants than using reagents, but is often impractical in the field and makes sample

155   transport difficult. The final three storage methods—95% ethanol, dimethyl sulfoxide (DMSO)

156   and cetrimonium bromide (CTAB)—are all liquid preservatives that can be used without a

157   freezer. Storage in ethanol is frequently used in insect microbiota studies, and ethanol is

158   relatively inexpensive and easy to acquire in the field, but its flammability often makes it

159   unsuitable for transporting specimens on planes (Moreau et al., 2013)**.** DMSO and CTAB are

160   promising nonflammable chemicals that have also been used to preserve specimens for later

161   DNA sequencing (Nagy, 2010). DMSO and CTAB were salt-saturated and prepared at 20% and

162   2% concentrations, respectively. We note that these storage methods are sometimes used in

163   combination—e.g., ethanol storage at -20°C—but here we evaluate them separately.

164   We extracted total DNA from subsamples of whole insect homogenates—which were

165   further pulverized with an aggressive bead-beating step—using the MoBio Powersoil Kit, from

166   which a ≈300 bp portion (V4 region) of the bacterial 16S rRNA gene was then PCR-amplified,

167   as previously described (Barberán et al., 2014). The barcoded amplicons were pooled and

168   sequenced on the Illumina MiSeq platform (Caporaso et al., 2012). Sequence data were quality-

169   filtered and clustered into operational taxonomic units (OTUs) at the 97% level using the

170   UPARSE pipeline (Edgar, 2013) following (Ramirez et al., 2014). OTU taxonomic affiliations

171   were assigned using the RDP classifier (Wang et al., 2007) against the August 2013 version of

172   the Greengenes database (McDonald et al., 2012), and OTUs identified as mitochondria or

173   chloroplasts were removed. The total proportion of mitochondrial or chloroplast reads for each

174   insect species was: bees, 0.02; grasshoppers, 0.38; beetles, 0.20; butterflies, 0.12. Finally, to

175   standardize sequencing depth across our dataset, we rarefied (randomly subselected) 2000

176   sequence reads from each sample. Sequence data have been deposited and made publicly

177   available on Figshare (http://dx.doi.org/10.6084/m9.figshare.1396464).

178   Analyses were conducted in R v. 2.13.1 (R Core Team, 2013), using the vegan package

179   for multivariate analyses (Oksanen et al., 2013) and ggplot2 (Wickham, 2009) to produce plots.

180   We quantified differences in microbial community structure between samples using the Bray-

181   Curtis dissimilarity metric, after square-root transformation. Associations between microbiota

182   structure and species or storage factors were tested with permutational multivariate ANOVAs

183   (Anderson, 2001). The interaction between species and storage variables was included in all such

184   tests. For multivariate analyses, all R^2 and P values presented below were calculated using

185   PERMANOVA, and a 0.05 significance threshold was used. Nonparametric Mann-Whitney tests

186   were used to assess differences in the relative abundances of particular taxa between sample

187   treatments and controls with analyses focused on those genera that had median relative

188   abundances ≥1% in at least one sample type, applying a false discovery rate correction to

189   account for multiple comparisons.

190

191   **Results and Discussion**

192

193   *Storage effects*

194   The four insect species we examined harbored distinct bacterial communities (Table S1),

195   and the differences in community structure between insect species were far larger than any

196   differences between storage conditions within individual insect species. In a model including

197   insect species, storage treatment, and the interaction between the two, the effect of species was

198   very strong ($R^2 = 0.866$, $P = 0.001$), as is evident from an unconstrained ordination of all the data

199 where the samples cluster according to insect species they belonged to, and not to the method

200 used to store them (Fig. 2). Overall, we could not reject the null hypothesis of no difference in

201 bacterial community structure among the storage treatments ($R^2 = 0.009$, $P = 0.058$). However,

202 there was a significant interaction, indicating that the strength of a storage effect depended upon

203 the insect species in question ($R^2 = 0.035$, $P = 0.001$).

204 When each insect species was analyzed separately, each of the four insect species

205 exhibited statistically significant effects of storage method on community structure ($R^2 = 0.286 -$

206 0.357, $P = 0.001 - 0.019$), although the magnitude and direction of the effect varied between

207 them (Fig. S1). Thus, storage methods can have effects on insect-associated microbial

208 community structure, but these effects vary by the insect, and are much smaller than species

209 differences. In other words, storing the insect samples in six different ways, including leaving

210 some in empty vials at room temperature for two months, did not hinder our ability to detect

211 species effects, which are often of biological interest (e.g., Colman, Toolson & Takacs-Vesbach,

212 2012; Jones, Sanchez & Fierer, 2013; Yun et al., 2014). Our results parallel previous studies on

213 plant, soil, and human fecal microbiota, which did not find strong effects of commonly used

214 storage methods (Lauber et al., 2010; U'Ren et al., 2014; Dominianni et al., 2014; Franzosa et

215 al., 2014). At least for the storage methods tested here, researchers that want to compare bacterial

216 communities across distinct insect taxa using samples stored under different conditions – e.g.,

217 with sample sets compiled from multiple collectors, or in meta-analyses – may not need to

218 worry, at least when the insect species harbor distinct bacterial communities. However, as our

219 focal insects belong to different taxonomic orders with distinct ecological and physiological

220 attributes, specimen storage may be of more concern when investigators are studying more

221   closely related species. Future research into potential storage biases on microbiota of congeneric

222   insects, for example, would be useful in guiding research at narrower taxonomic scales.

223   Given that we did find a significant effect of storage conditions on our assessments of

224   bacterial community composition within each of the four insect species examined, we

225   recommend that, when possible, the same method be used to store all specimens within a study.

226   In order to help guide researchers in their choice of storage method, we examined whether some

227   storage methods were consistently better than others, i.e. whether some introduced fewer biases

228   in bacterial community analyses compared to the control. To do so, we tested for storage and

229   species effects on bacterial community structure for each method versus the control. Only the

230   room-temperature unpreserved treatment and DMSO had a (marginally) significant main storage

231   effect, although there was an interaction between species and storage effects in each case (Table

232   1, Fig. S2). Further, the storage effect consistently explained only a small proportion of variation

233   in community structure, especially relative to the species effect (Table 1), which was large and

234   easily visible in ordinations regardless of the storage method used (as mentioned above) (Fig.

235   S2). Ranked by mean Bray-Curtis dissimilarity between each storage method and the control,

236   there was no single 'best' method across all four insect species, and often multiple methods were

237   not substantially distinct from one another in the extent to which the bacterial communities

238   differed from those found in control specimens (Fig. 3). While dependent on the question of

239   interest, we can broadly recommend any of the storage methods tested here, although room-

240   temperature storage without preservative should be avoided when possible.

241   As these analyses were conducted on the structure of the entire bacterial community in

242   each specimen, we also wanted to determine whether the different storage treatments altered the

243   relative abundances of individual, dominant bacterial genera, and whether these effects were

244   consistent across insect species. We found that genus-level relative abundances were generally

245   not more similar within- than between-treatments, nor markedly different between storage

246   treatments and the control besides natural interindividual variation (Fig. 4). Furthermore, specific

247   genera that differed significantly between a storage method and the control were mostly

248   concentrated in the room temperature treatment, and none differed consistently across insect

249   species (Table S2). This finding suggests that storage conditions are likely to exert their effects

250   by changing the abundance of resident community members, and not by introducing new

251   'contaminant' taxa.

252   Interestingly, although honey bees had the lowest interindividual heterogeneity of the

253   four insects tested (indicated by Fig. 3 and Fig. 4), which should have increased our ability to

254   detect storage-induced effects, they were overall less affected by storage (excepting the room

255   temperature treatment, where *Lactobacillus* was nearly absent (Fig. 4, Table S2)) than the other

256   insects (Fig. 3). This finding may result from the extremely dense communities inhabiting honey

257   bee guts (possibly reaching ca. 10^7 bacterial colony forming units (CFUs) per bee, (Kwong et al.,

258   2014)), which may buffer them from relatively low-abundance contaminants or minor alterations

259   to resident populations. In contrast, bean beetles, where the apparent storage effects were

260   stronger (Fig. 3), have been reported to contain only 600-1750 bacterial CFUs per beetle (Taylor,

261   1985). Bacterial cell counts have not been conducted in *A. conspersa* and *P. rapae* adults, but we

262   found substantial interindividual variation in the composition of dominant taxa for all three non-

263   bee species, even within control and treatment groups (Fig. 4). In general, variation in both

264   microbial cell numbers and intra-specific variability in bacterial community composition may

265   explain the observed interactions between storage method and insect species. When feasible,

266   quantifying microbial abundances would be a valuable complement, as storage biases might be

267   less of a concern for molecular investigations of insect taxa harboring particularly large numbers

268   of microbes.

269

270   *Surface Sterilization Effects*

271   A second aim of our study was to determine whether surface sterilization affected insect-

272   associated microbiota. As compared with control specimens (which were not surface sterilized),

273   there was no effect of surface sterilization on insect-associated microbial community structure

274   ($R^2 = 0.004$, $P = 0.344$), and no significant interaction between species and sterilization ($R^2 =$

275   0.022, $P = 0.072$). Again, samples clustered strongly by species ($R^2 = 0.846$, $P = 0.001$),

276   regardless of whether or not they were surface sterilized (Fig. S3), although there was a slight

277   differentiation between treatments within individual species (excepting honey bees). Likewise,

278   the relative abundances of individual genera were generally similar between sterilized and

279   control specimens (Fig. 4).

280   While sterilization is usually intended to remove surface contaminants (including those

281   derived from handling the specimen) or microbes typically present on the cuticle, we do not

282   know if these surface-associated microbes are normally abundant enough to be detected in

283   molecular surveys of whole-insect microbiota. In our study, where all insects were collected

284   without gloves, we found that bacteria known to be common on human hands (including

285   *Staphylococcus*, *Corynebacterium*, and *Propionibacterium*; Fierer et al., 2008) were extremely

286   rare or absent from nearly all of our samples. Only *Staphylococcus* and *Corynebacterium* were

287   detected and only in control *P. rapae* insects (at median proportions of 0.0005 and 0.001,

288   respectively). In light of the general lack of a strong surface sterilization effect at the community

289   level, in tandem with the lack of abundant human-derived contaminants even in control

290 specimens, we argue that surface sterilization may not be worth the time and effort required.

291 Presumably, the high microbial biomass inside many insects (such as in the gut or

292 bacteriocytes)—relative to microbial colonizers or contaminants on the insect cuticle—

293 overwhelms any potential effect of sterilizing the insect surface prior to microbiota analyses.

294 Although our sterilization protocol was similar to or more aggressive than protocols used in

295 previous studies (e.g., Jones, Sanchez & Fierer, 2013; Hammer, McMillan & Fierer, 2014;

296 Sanders et al., 2014), it remains possible that modifications such as a longer soak duration could

297 lead to detectable effects on overall microbiota. However, as we noted earlier, this may have the

298 unintended effect of sterilizing the internal communities that are typically of interest.

299 We recommend omitting surface sterilization from insect microbiota studies, and suggest

300 that any of the storage methods tested here – with the possible exception of room temperature

301 storage without preservative – can be safely used for up to two months if the researcher is not

302 seeking subtle biological patterns that could be obscured by minor storage effects. Each method

303 resolved species differences, and produced reasonably consistent estimates of community

304 structure. Methods may thus be chosen based on practical considerations, such as price,

305 availability, ease of preparation, and travel logistics. For example, CTAB may be a good choice

306 for overseas fieldwork when electricity or dry ice is not available, as it can be maintained at

307 room temperature and is nonflammable. We note, however, that when planning insect collections

308 for microbiota analysis, it is still advisable to standardize methods across all of the specimens.

309 In some cases, researchers are unable to choose how insects are stored in advance, but

310 this should not necessarily limit the utility of those samples for microbial analyses. For example,

311 samples may have already been collected and stored by entomologists for non-microbial

312 purposes (such as morphological analyses). Our data indicate that standard entomological

313 collection methods, where specimens are not handled aseptically, are sufficient for capturing *in*

314 *situ* community structure and biological patterns, and that surface sterilization does not appear to

315 be necessary for microbial studies. Although we do not know how different storage approaches

316 may influence microbial analyses beyond the two month window examined here, we suggest that

317 if storage effects are weak or nonexistent after two months, it is unlikely that they will pose a

318 problem after longer periods of time. However, until longer-term storage effects are explicitly

319 tested, we recommend extracting DNA from specimens within two months of storage when

320 possible.

321 While further investigations into storage effects on insect microbiota would be useful—

322 particularly tests that include combinations of various methods—we anticipate that these

323 findings will allow existing insect samples to be used for microbial DNA sequencing, enable

324 comparative studies that include specimens collected in different ways, and help guide the design

325 and standardization of methods in the rapidly growing field of insect-microbe symbiosis.

326

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328

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331

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333

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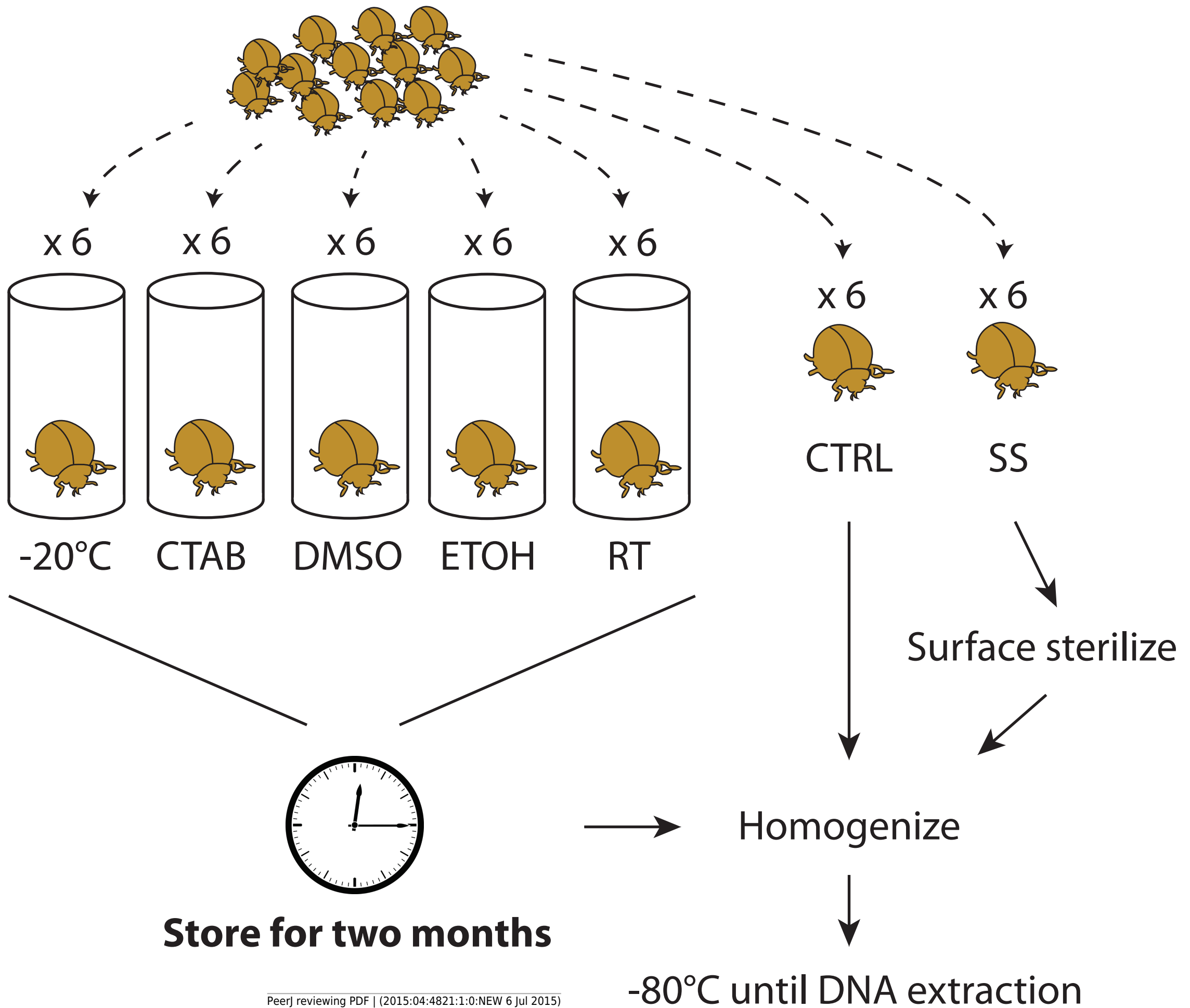
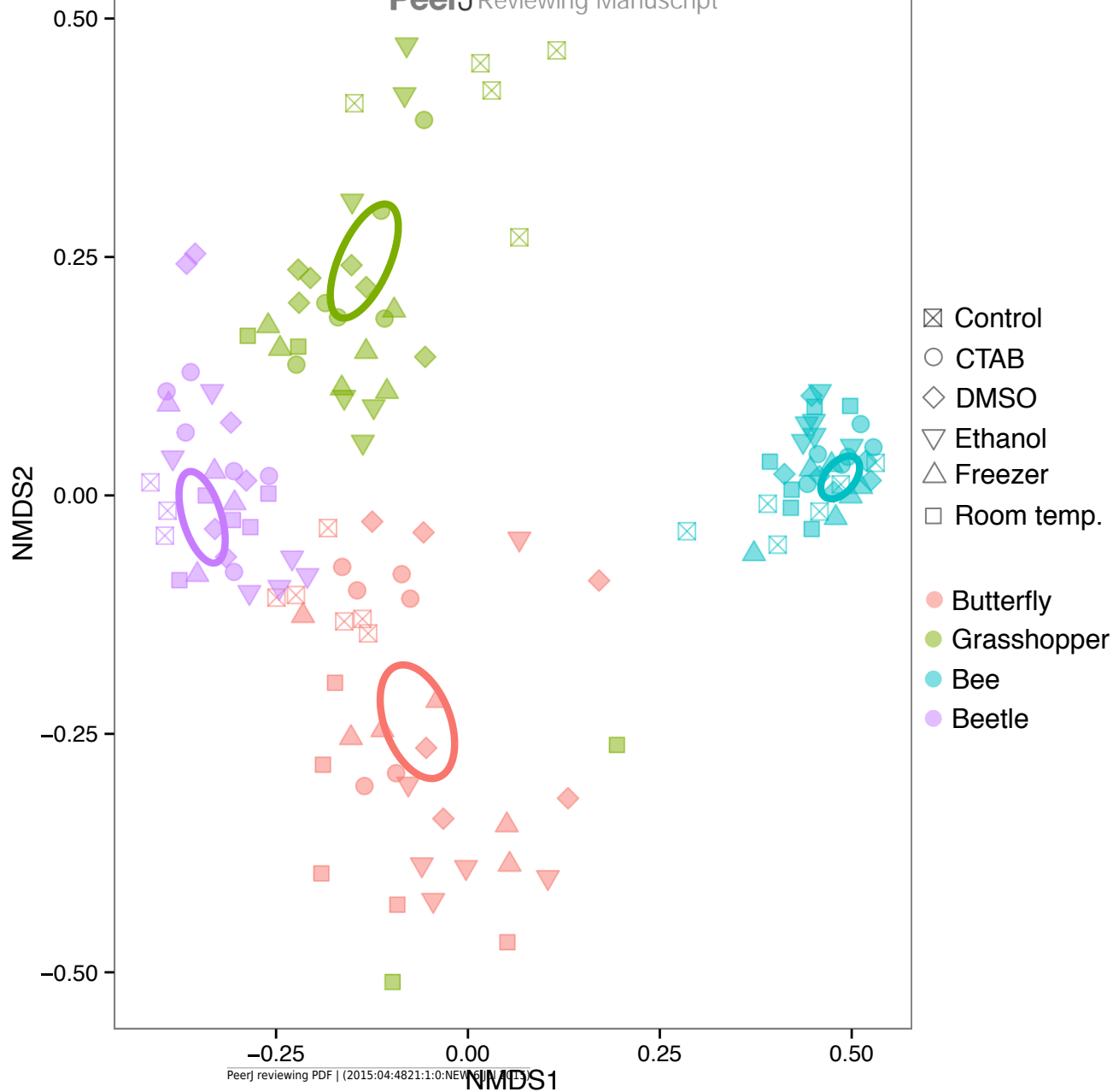
Comparison of storage methods

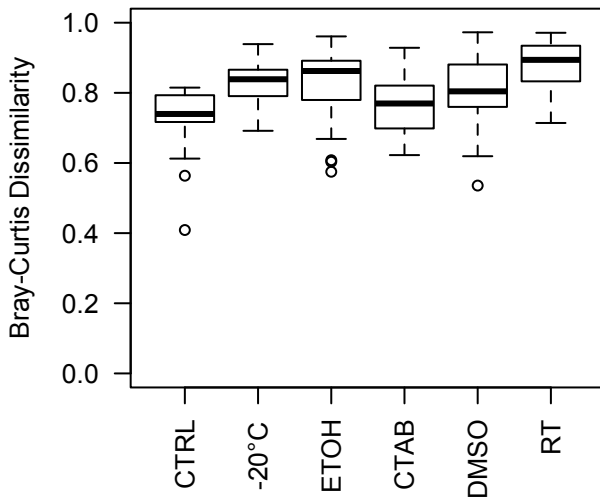
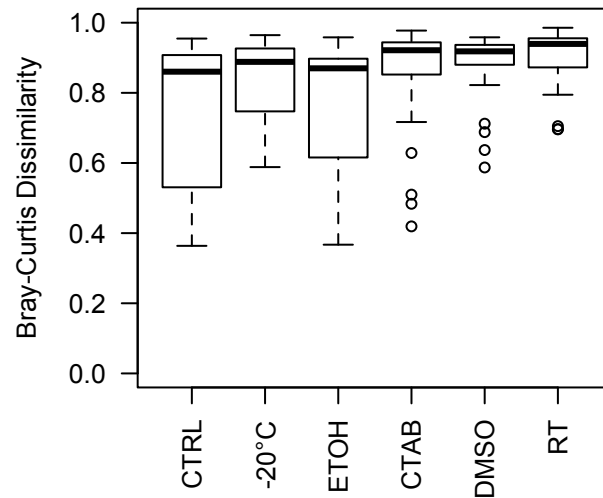
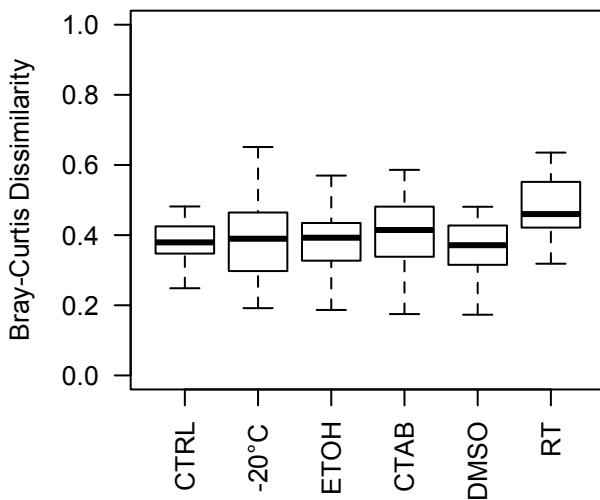
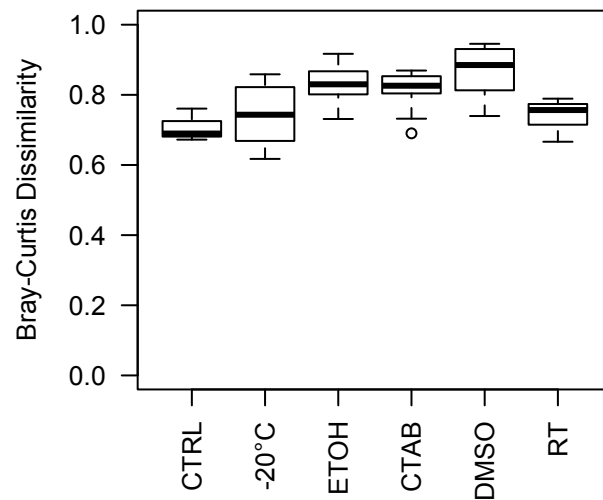
Results from pairwise PERMANOVA tests conducted for samples from each storage method with the controls. Methods are ranked by increasing percentage of variance explained by the storage term. Ordination plots that correspond to these tests are shown in Fig. S2. Significant effects ($P < 0.05$) are indicated by asterisks.

Storage method (versus control)	Model Term	R^2	P values
Freezer	Species	0.849	0.001*
	Storage	0.003	0.285
	Species x Storage	0.047	0.002*
CTAB	Species	0.862	0.001*
	Storage	0.005	0.149
	Species x Storage	0.030	0.010*
Ethanol	Species	0.820	0.001*
	Storage	0.007	0.168
	Species x Storage	0.035	0.016*
DMSO	Species	0.839	0.001*
	Storage	0.011	0.040*
	Species x Storage	0.046	0.001*
Room Temperature	Species	0.809	0.001*
	Storage	0.017	0.041*
	Species x Storage	0.049	0.004*

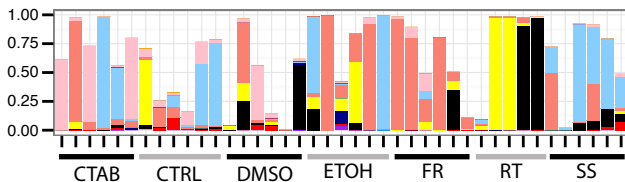
Experimental design

Overview of how insect samples were collected and processed for later whole-specimen bacterial 16S rRNA gene sequencing. Only beetles are shown, but the design was identical for the other three insect species used in the study. CTAB = cetrimonium bromide, DMSO = dimethyl sulfoxide, ETOH = 95% ethanol, RT = room temperature without preservative, CTRL = control (no storage or sterilization prior to homogenization), SS = surface sterilized (sterilization but no storage prior to homogenization).

Ordination of insect microbiota

Nonmetric multidimensional scaling ordination showing how insect-associated bacterial communities cluster by host insect species (colors) versus specimen storage method (symbols). Ellipses represent 95% confidence intervals around species group centroids.

Beta diversity among insect species and storage methods

Boxplots of Bray-Curtis dissimilarities between samples from each storage method and the controls, separated by the four insect species tested. Higher values indicate that the storage method had larger effects on bacterial community structure relative to the control. Note that, due to interindividual variability in the microbiota, there was variation even among the control specimens. CTAB = cetrimonium bromide, DMSO = dimethyl sulfoxide, ETOH = 95% ethanol, RT = room temperature without preservative, CTRL = control.

Composition of bacterial communities in study insects

Relative abundances of dominant genera for each individual specimen analyzed in the study. Seventeen genera are displayed, representing the five most abundant from each insect species (three genera were in the top five of more than one insect species). In cases where there was no genus-level identification based on the Greengenes taxonomy, the lowest-level classification is given. Blank areas show the proportion of the community for each sample that does not belong to these dominant genera. CTAB = cetrimonium bromide, DMSO = dimethyl sulfoxide, ETOH = 95% ethanol, RT = room temperature without preservative, FR = freezer at -20°C, CTRL = control, SS = surface-sterilized.

Butterflies

Grasshoppers

Beetles

Bees

Rhodococcus

Bifidobacterium

Phormidiaceae

Alicyclobacillus

Enterococcus

Lactobacillus

Lactococcus

Neisseriaceae

Kingella

Neisseria

Gammaproteobacteria

Klebsiella

Providencia

Pasteurellales

Acinetobacter

Pseudomonadaceae

Entomoplasmatales