Complete metamorphosis and microbiota turnover in insects

Chrisitin Manthey[1], Paul Johnston*[2,3,4], Jens Rolff[1,2,4]

1. Freie Universität Berlin, Institut für Biologie, Evolutionary Biology, Königin-Luise-Strasse 1-3, 14195 Berlin, Germany.

2. Berlin Center for Genomics in Biodiversity Research, Berlin, Germany;

3. Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Berlin, Germany;

4. Berlin-Brandenburg Institute of Advanced Biodiversity Research (BBIB), Berlin, Germany

*Paul Johnston, author for correspondence

*Jens Rolff, author for correspondence

Email: paul.johnston@igb-berlin.de, Jens.rolff@fu-berlin,de

Competing Interest Statement: The authors state that they have no competing interests.

Keywords: holometaboly, beta diversity, evolution of metamorphosis, gut microbiota

Abstract

The insects constitute the majority of animal diversity. Most insects are holometabolous: during complete metamorphosis their bodies are radically re-organized. This re-organization poses a significant challenge to the gut microbiota, as the gut is replaced during pupation, a process that does not occur in hemimetabolous insects. In holometabolous hosts, it offers the opportunity to decouple the gut microbiota between the larval and adult life stages resulting in high beta diversity whilst limiting alpha diversity. Here we studied 18 different herbivorous insect species from 5 orders of holometabolous and 3 orders of hemimetabolous insects. Comparing larval and adult specimens,

we find a much higher beta-diversity and hence microbiota turnover in holometabolous insects compared to hemimetabolous insects. Alpha diversity did not differ between holo-and hemimetabolous insects nor between developmental stages within these groups. Our results support the idea that pupation offers the opportunity to change the gut microbiota and hence facilitates ecological niche shifts. This effect of niche shift facilitation could explain a selective advantage of the evolution of complete metamorphosis, which is a defining trait of the most speciose insect taxon, the holometabola.

Introduction

Insects are the most diverse animal taxon on earth (1, 2) and collectively comprise 50 to 70% of all living species (2, 3). More than 80% of all described insect species are homometabolous—they undergo complete metamorphosis (3) that includes a pupal stage intercalated between the larva and the adult. In the pupa, the insect body is radically remodeled. All larval organs, including the gut, are broken down and reconstructed, resulting in distinct and specialized larval and adult life stages (4–7). Complete metamorphosis is considered a key trait explaining insect diversity (8, 9, but see 10) and only evolved once, the holometabola are a monophyletic group (11). The diversification of the speciose orders of the holometabolous insects coincides with the diversification of the land plants (10, 11).

Exactly why this radical re-organization of the insect body has produced such an astounding radiation is not known (6). One possible explanation could be that intercalating the pupal stage decouples growth and differentiation (6, 12), allowing for efficient and competitive exploitation of ephemeral resources. Another, not mutually exclusive explanation is, that larvae and adults can occupy distinct niches (13), possibly accompanied by a change in the gut microbiota.

51 One of the major internal reconstructions during the pupal stage includes the replacement of the
52 gut epithelium. From the perspective of the microbes in the gut, the epithelial replacement
53 constitutes a dramatic habitat change. The gut microbiota, that can provide nutrition, defense and
54 other services to the insect host, changes in density and community structure, including the
55 elimination of particular microbes during pupation (13, 14). These changes may result from a
56 combination of factors including the drastic anatomical and physiological transformations in the
57 replacement gut, host immune effector induction in the metamorphic gut, bacterial competition for
58 continued occupancy of the pupal gut, and ontogenetic habitat and diet shifts of the host. In the
59 lepidopteran *Galleria mellonella*, the absolute abundance of the microbiota can be reduced by
60 several orders of magnitude during metamorphosis, including the elimination of pathogenic bacteria
61 that would otherwise persist in the adult host (14). In *Galleria*, the host immune system and the
62 symbionts interact with the microbial community in the gut through complete metamorphosis.
63 Observations in other taxa such Lepidoptera (6 and refs therein) as well as in some Coleoptera
64 (15) are consistent with partial host control of the microbiota. The replacement of the gut epithelium
65 potentially offers the insect a unique opportunity to siginficantly alter the gut microbiota, allowing
66 an insect to acquire life stage-specific microbes (13, 14). Such gut microbiota changes during the
67 pupal stage increase the opportunity of niche shifts (16).

68 Only a few studies, however, have investigated changes in the gut microbiota at different stages of
69 host development. The hemimetabolous insect *Pyrrhocoris apterus* (17) hosts a very stable mid-
70 gut community composition with six predominant taxa being consistently abundant throughout
71 development. By contrast in a holometabolous insect, the hymenopteran *Bombus pascuorum,*
72 Parmentier et al. (18) reported different gut microbial communities within larval and adult specimens
73 of a wild nest. The typical core gut bacteria in the adults were absent in the larvae. Hammer,
74 McMillan, & Fierer (19) also found distinct gut microbiota communities in the leaf-chewing larvae
75 and nectar- and pollen-feeding adults in the lepidopteran *Heliconius erato.* Studies of the dipteran
76 *Musca domestica* (20) and the coleopteran *Phalacrognathus muelleri* (21) have found a similar
77 pattern.

78 Here, we investigated whether gut microbiota changes during the adult moult, which includes
79 pupation in the holometabolous insects, differ between hemi- and holometabolous insects.
80 Because of the re-organization of the gut in the pupal stage we expect (a) a significant change in
81 bacterial species composition resulting in much greater betadiversity in holometabolous than in
82 hemimetabolous insects. The diversity of gut microbes can be strongly reduced during pupation
83 (14, 19). (b) We therefore speculated that greater alpha diversity would be observed in the gut
84 microbiota of hemimetabolous insects, given the lack of gut epithelial replacement. Also, as the
85 diversity of the gut microbiota scales positively with size across species Sherill-Mix et al. (22), it is
86 possible that alpha diversity is higher adult than larval insects, especially in hemimetabolous
87 species.

88 To address these questions, we sampled 18 different species from seven major insect orders
89 across the life stages. To reduce geographic variance, we collected all insect species in Central
90 and Northern Europe. To further reduce variance, we sampled only herbivorous insects from
91 terrestrial habitats and excluded social insects. Additionally, we collected a sub-sample of those
92 species from laboratory-reared colonies, consisting of five species from four different insect orders
93 (figure 1) as the gut microbiota may differ between specimens from the field and laboratory (23,
94 24).

95

96 **Results**

97 We obtained 18 insect species covering seven major orders, including three hemimetabolous
98 (Orthoptera, Thysanoptera, Hemiptera) and four holometabolous insect orders (Hymenoptera,
99 Coleoptera, Lepidoptera and Diptera). We sampled larval and adult life stages for all of them. We
100 collected a sub-sample of those 18 species from laboratory-reared colonies that covered five insect
101 species from four different orders, including one hemimetabolous (Thysanoptera) and four
102 holometabolous insect orders (Coleoptera, Lepidoptera and Diptera). (Figure 1)

103 Using 16S rRNA sequencing, we determined the gut microbial compositions per life stage and
104 species and plotted the data. All relative abundance plots can be found in the supplementary
105 material. The data from *Pyrrhocoris apterus* and *Melolontha melolontha*, which originated from two
106 different locations, were pooled as population did not affect alpha diversity (see table 3) nor beta
107 diversity (table 2).

108

109 ***Microbiota turnover at the larval-adult transition***

110 Microbial beta diversity of holometabolous insects was significantly greater than hemimetabolous
111 insects when comparing larval and adult life stages of each species (Two-sample t-test, t = -3.292,
112 df = 14, p = 0.005347, figure 2). With the exception of *Leptinotarsa decemlineata*, all field-collected
113 holometabolous insect species showed significant differences in beta diversity between larval and
114 adult life stages (table 2).Within the hemimetabolous species collected in the field, two species
115 differed significantly in beta diversity: *Chorthippus dorsatus* and *Pyrrhocoris apterus*. The other five
116 Hemimetabola did not differ in beta diversity between life stages. This pattern was consistent in the
117 subset of five laboratory-reared species. To display the differences in beta diversity between life
118 stages for each species, we used Principal Coordinate Analysis (PCoA) ordination (see
119 supplement, figures S1 – S18, PCoA plots per species).

120

121 ***Alpha Diversity***

122 We calculated Shannon diversity indices per life stage and species and calculated the difference
123 in alpha diversities between life stages per species. Alpha diversity differences between larval and
124 adult life stages did not differ between holo- and hemimetabolous insects (Two-sample t-test, t = -
125 1.1025, df = 14, p = 0.2889, figure 3). Because Shannon diversity does not allow to control for
126 between-sample variation and sample sizes, we also calculated Hedges' g effect size. Hedges' g
127 did not differ between holo- and hemimetabolous insects (Two-sample t-test, t = -1.3322, df = 14,
128 p = 0.2041, figure 4). The microbial alpha diversity was also not different between holo- versus

hemimetabolous larvae (Two-sample t-test, $t = 0.38317$, $df = 14$, $p = 0.7073$) or adults (Two-sample t-test, $t = -0.18815$, $df = 14$, $p = 0.8535$), respectively (figure 5). Five species did differ significantly in alpha diversity between life stages within the holometabolous insects and showed large effect sizes. Three species showed small and one species negligible effect sizes. Within the hemimetabolous insects, one species differed significantly in alpha diversity between life stages and this and a second species showed large effect sizes. The other Hemimetabola showed negligible (one species), small (three species) and medium effect sizes (one species). See table 4 for more details.

Discussion

We investigated beta and alpha diversity throughout development, comparing 18 insect species from four holo- and three hemimetabolous insect orders. We find a clear pattern: holometabolous insects show a strong microbial turnover between larvae and adults, while this is not found in hemimetabolous insects.

Almost all examined holometabolous insect species showed significantly different gut microbial communities between larval and adult specimens as reflected by the differences in beta diversity. The overall pattern we report is well supported: hemimetabolous insects do not show changes in beta diversity during development. The remaining variation in beta diversity within the holometabolous insects may be partly explained by different ecologies which warrants further investigation. Interestingly, the highest beta diversity in hemimetabolous species was recorded in thrips. They have evolved a neometabolous life-style with two partly quiescent stages between the larva and the adult (7). The stages are also called pupa but their development does not entail the dramatic change in morphology as in holometabolous insects (7).

153 Microbiota turnover seems to be a general pattern within holometabolous, but not hemimetabolous
154 insects, independent of the insects' field and laboratory origin. The microbial composition changes
155 are presumably driven by the intercalated pupal stage in holometabolous insects, which allows a
156 radical remodeling of the hosts' gut, but often is also accompanied by different diet choices of larvae
157 and adults. Prior to pupation a cessation of feeding and purging of the gut contents takes place
158 (14). After that, immune effectors such as lysozyme and AMPs are secreted into the gut (14)
159 followed by anatomical and physiological changes resulting in the replacement of the gut.
160 Competition of the remaining bacteria with possible new colonizers of the adult gut than shapes
161 the adult microbiota. The role of the host immune system is illustrated by a study in *Galleria*
162 *mellonella*, a species where the stage of gut replacement can be precisely determined *in vivo* (26).
163 This study revealed that pupal gut delamination coincides with peak immune gene expression in
164 the gut. This is consistent with other observations in other holometabolous insects with lower
165 temporal resolution (27–29). In contrast, no such effect was observed for the
166 hemimetabolous *Gryllus bimaculatus (26)*. The massive up-regulation of immune genes during gut
167 renewal is therefore a candidate mechanism contributing to the patterns reported in our study. In
168 principal, the insect host can establish a completely new and distinct adult gut microbiome by this
169 reduction of the gut microbiota and a subsequent change in diet of the emerging adult. A different
170 diet will expose the insects to new microbes that can colonize the gut and potentially also facilitate
171 better digestion of the new diet.

172 Alpha diversity did not display a pattern related to holo- vs. hemimetabolous development.
173 Complete metamorphosis results in a reduction of the microbial absolute abundance by orders of
174 magnitude (14 and refs therein). In the light of Hammer *et al*. (19) this could be explained by a
175 recovery of the microbiota upon adult feeding. Another possible explanation for the lack of change
176 in alpha diversity are priority effects, the order and timing of arrivals determining the establishment
177 of a new microbial community. Therefore, the gut microbiota is shaped by a niche modification, in
178 which early arriving species change the types of niches available within the local sites (30).

179 The high turnover though poses the risk of losing beneficial microbes which would result in a cost
180 to both, host and symbiont. Hammer & Moran (13) suggest that holometabolous insects may be
181 less likely to evolve strictly vertically transmitted symbioses than hemimetabolous insects. To
182 overcome this hurdle a number of strategies have evolved to ensure transmission of obligate
183 symbiont between life stages in holometabolous insects. Stoll, et al (31) showed vertical
184 transmission of microbes via bacteriocytes in ant species. The relative number of bacteria-filled
185 bacteriocytes increased strongly during complete metamorphosis. Maire et al. (32) also showed a
186 transmission of microbes via bacteriocytes in weevils by maintaining and relocating bacteriocytes
187 during gut renewal in the pupa. Other specialized structures to transmit symbionts in insects are
188 antennal glands (33) or crypts (34).

189 It has been suggested that microbiota turnover would allow insects to occupy different niches
190 throughout development (13), which most likely contributed to the success of holometabolous
191 insects. Our data are consistent with this hypothesis, the clearance of the gut provides the
192 opportunity for a microbiota turnover, an effect not observed in hemimetabolous insects. It seems
193 possible that this observation is directly related to the decoupling hypothesis, which proposes that
194 growth is confined to the larval stage, while most differentiation occurs in the pupa (6, 12). A
195 facilitation of niche shifts by changes in the gut microbiota, if confirmed by experimental studies,
196 could be considered as an important driver of the evolution of complete metamorphosis. How this
197 relates to the diversification of the main insects orders in relation to the diversification of land plants
198 remains to be seen.

199

200 **Materials and Methods**

201

202 ***Insect Sampling and Preparation***

203 Larval and adult specimens of 18 insect species from seven different insect orders, including
204 Orthoptera, Thysanoptera, Hemiptera, Hymenoptera, Coleoptera, Lepidoptera and Diptera, were

205 sampled in Central and Northern Europe between April and October 2018 (see figure 1 and table
206 1). Pupae were additionally sampled for the three hymenopteran species. In total, sixteen species
207 across development were collected in Northern Germany, *Tenebrio molitor* in Croatia
208 and *Neodiprion sertifer* in Finland. Additionally, a sub-sample of those species was sampled,
209 consisting of five species from four different insect orders, which originated from laboratory-reared
210 colonies. Figure 1 gives an overview of all insect species collected in the field and the subset of
211 those species from laboratory-reared colonies. A total of 643 individual insects were sampled.

212 After collection, the insects were stored individually in 50 ml centrifuge tubes (Falcon tubes) with
213 holes for ventilation. In two very small species, *Frankliniella occidentalis* and *Aleyrodes proletella*,
214 individuals were pooled (see table 1). These pools were kept and used as a biological replicate
215 later. After a 24-hour starvation period, the insects were sacrificed, and preserved by freezing (-
216 80°C), except two field-collected species which were preserved in ethanol (95%): *Frankliniella*
217 *occidentalis* and *Neodiprion sertifer*. Hammer, Dickerson, & Fierer (35) compared two different
218 storage methods, freezing and ethanol, amongst others, and found that the storage method did not
219 affect microbiota composition assessments.

220 ***DNA extraction***

221 Samples of the sacrificed insects were processed on ice under sterile conditions. A biological
222 replicate was an individual insect sample, except for samples of three small species: a replicate
223 of *Frankliniella occidentalis* was pooled from 30 individuals, a replicate of *Aleyrodes proletella* from
224 40 and a replicate of *Drosophila melanogaster* from 10 individuals. The exact number of biological
225 replicates per species and life stage are shown in table 1. After cutting the legs and wings off (only
226 adults), the samples were placed in 2 ml microcentrifuge tubes (Eppendorf Safe-Lock Tubes). Then
227 samples were bead-ground using TCBeads and C1 solution from the PowerSoil DNA Isolation Kit
228 (Qiagen) three times for 30 sec at 30 Hz in a tissue homogenizer. The insects were not dissected
229 before homogenization in order to process all samples under standardized methods as the Thrips
230 and Whiteflies were too small to dissect guts. Insects were not sterilised prior to homogenisation.

231 Hammer et al. (35) found no effect on the bacterial communities of not surface sterilised insect
232 species (butterfly, grasshopper, bee and beetle) compared to control specimens that were surface
233 sterilised. Samples clustered by species independent of surface sterilisation and relative
234 abundances of bacterial genera were similar between sterilised and non-sterilised specimens. Also,
235 surface contaminants derived from handling the specimens were extremely rare in non-sterilised
236 and surface sterilised specimens. As it remains possible that surface sterilisation could affect
237 internal bacterial communities, Hammer et al. (35) recommend omitting surface sterilisation from
238 insect microbiota studies.

239 Total DNA was extracted from 60 µl of tissue homogenate using the PowerSoil DNA extraction kit
240 (Qiagen) under sterile conditions. Tissue homogenates were pretreated with 10 µl Proteinase K
241 and 500 µl Power soil bead solution at 56 °C overnight. Subsequent DNA isolation was continued
242 as indicated in the manufacturer‘s instruction.

243 Negative extraction controls were included to detect and filter contamination. The negative controls
244 consisted of mock samples, which contained no insect tissue.

245 ***Primers and PCR amplification of the 16S rRNA gene fragment***

246 PCR amplification of the 16S rRNA gene fragments was performed with MyTaqTM HS DNA
247 Polymerase and the forward and reverse 515f-806r primer sequence pairs, targeting the V3 – V4
248 region of the 16S rRNA gene (36). The PCR reaction was conducted using 1 µl sample in a total
249 volume of 25 µl. The PCR amplification program was as follows: 94 °C for 1 min, 95 °C for 15 sec,
250 50 °C for 15 sec, two cycles of 72 °C for 45 sec and 2 min, followed by a final extension step to 4
251 °C. A volume of 5 µl of the PCR product was run on a 1.5% agarose gel stained with Sybr Gold at
252 160 V for 40 min.

253 PCR products were purified with CleanNGS CNGS-0050 (GC biotech B.V., Leidse Schouw 2,
254 2408 AE Alpen aan den Rijn, Netherlands) and dual indices and Illumina sequencing adapters
255 were attached by limited-cycle PCR amplification (initial denaturation at 95°C for 2 min followed by
256 eight cycles of denaturation at 95°C for 20 s, annealing at 52°C for 30 s, extension at 72°C for 30

257 s, and a final extension cycle at 72°C for 3 min). The enzymes used were Herculase II Fusion DNA
258 Polymerase (Agilent Technologies Sales & Services GmbH & Co. Hewlett-Packard-Str. 831, 76337
259 Waldbronn, Germany). PCR products were quantified with Quant-iT™ PicoGreen™ dsDNA Assay
260 Kit (Life Technologies GmbH Thermo Fisher Scientific,Frankfurter Straße 129b, 64319 Darmstadt,
261 Germany), measured with Optima Fluostar (BMG LABTECH GmbH, Allmendgrün 8,77799
262 Ortenberg, Germany).

263 ***Sequencing of bacterial community***

264 Amplicon libraries were sequenced for 600 cycles using an Illumina MiSeq (Illumina, San Diego,
265 California, USA) at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv). The
266 resulting 300-bp paired end reads were analysed using a full-stack R (37) pipeline incorporating
267 dada2 (38) and phyloseq (39). Forward reads were trimmed to 240 bp and reverse reads to 160
268 bp. The reads were truncated at the first instance of a quality score less than two and filtered to a
269 maximum amount of estimated errors of two per truncated read. The remaining forward and reverse
270 reads were dereplicated, and error rate estimates were computed. The developed error model was
271 used to infer exact amplicon sequence variants (ASVs) from the amplicon sequencing data. The
272 resulting denoised read pairs were merged. A sequencing table was constructed with the denoised
273 and merged reads and chimaeras were removed. Taxonomy was assigned to the sequence table
274 using the Ribosomal Database Project (40) training set, version 16. Contaminant taxa were
275 identified using prevalence-based filtering from the decontam package (41). Remaining unknown
276 sequences were identified using the Basic Local Alignment Search Tool (BLAST) (42) and
277 taxonomy was assigned using TaxonKit (43). Further remaining unknown sequences (genus level)
278 were renamed with higher taxonomic ranks. Eukaryota were removed, and the resulting sequence
279 variants were agglomerated at the genus level.

280 ***Statistical Analysis***

281 Absolute abundances were plotted for all species and life stages using the R package microbiome
282 (44). Rare bacterial taxa present less than 1% of all taxa per species are not shown in the figures.

283 The sequences were agglomerated at the genus level. Shannon indices were calculated per
284 developmental stage and species using the microbiome package (44). The group means per
285 species were compared with life stage as grouping factor using, according to test assumptions, a
286 Two-sample t-test, Wilcoxon rank-sum test and Welch-test, respectively. See table 4 for more
287 details. To meet the assumption of normally distributed data for the Two-sample t-test, the response
288 variable was transformed before testing for group differences using logarithm transformation
289 for *Chorthippus parallelus* and *Chorthippus dorsatus,* and via reciprocal 1/x^6 transformation for
290 the dataset of *Graphosoma lineatum.* The effect sizes were calculated using the effsize package
291 (45).

292 Bray-Curtis dissimilarity matrices were calculated comparing life stages per species. The data was
293 normalised to proportions to control for read depth prior to ordination. The dissimilarity data was
294 analysed by perMANOVA with life stage as a predictor variable, and a dispersion test was fitted
295 using the vegan package (46). Principle coordinate analysis (PCoA) on Bray-Curtis dissimilarity
296 was used to display the beta diversities per species with life stage as a grouping factor using the
297 phyloseq package (39).

298

299 **Acknowledgments**

300 We are grateful to Olivia Judson and Dino McMahon for comments on the manuscript and to Elisa
301 Bittermann for support in the lab. This research was funded by the DFG (Deutsche
302 Forschungsgemeinschaft)

303

304 **References**

305

1. . M. Berenbaum, “Insect Biodiversity – Millions and Millions” in *Insect Biodiversity*, (John Wiley & Sons, Ltd, 2017), pp. 783–792.

2. C. Mora, D. P. Tittensor, S. Adl, A. G. B. Simpson, B. Worm, How Many Species Are There on Earth and in the Ocean? *PLoS Biology* **9**, e1001127 (2011).

3. D. Grimaldi, M. S. Engel, *Evolution of the Insects* (Cambridge University Press, 2005).

4. . M. J. R. Hall, D. Martín-Vega, Visualization of insect metamorphosis. *Phil. Trans. R. Soc. B* **374**, 20190071 (2019).

5. H. E. Hinton, On the Origin and Function of the Pupal Stage. *Transactions of the Royal Entomological Society of London* **99**, 395–409 (1948).

6. J. Rolff, P. R. Johnston, S. Reynolds, Complete metamorphosis of insects. *Phil. Trans. R. Soc. B* (2019) https:/doi.org/10.1098/rstb.2019.0063.

7. J. W. Truman, The Evolution of Insect Metamorphosis. *Current Biology* **29**, R1252–R1268 (2019).

8. J. L. Rainford, M. Hofreiter, D. B. Nicholson, P. J. Mayhew, Phylogenetic Distribution of Extant Richness Suggests Metamorphosis Is a Key Innovation Driving Diversification in Insects. *PLoS ONE* **9**, e109085 (2014).

9. P. J. Mayhew, Why are there so many insect species? Perspectives from fossils and phylogenies. *Biological Reviews* **82**, 425–454 (2007).

10. F. L. Condamine, M. E. Clapham, G. J. Kergoat, Global patterns of insect diversification: towards a reconciliation of fossil and molecular evidence? *Scientific Reports* **6**, 19208 (2016).

11. B. Misof, *et al.*, Phylogenomics resolves the timing and pattern of insect evolution. *Science* **346**, 763–767 (2014).

12. J. D. Arendt, Adaptive Intrinsic Growth Rates: An Integration Across Taxa. *The Quarterly Review of Biology* **72**, 29 (1997).

13. T. J. Hammer, N. A. Moran, Links between metamorphosis and symbiosis in holometabolous insects. *Phil. Trans. R. Soc. B* **374**, 20190068 (2019).

14. P. R. Johnston, J. Rolff, Host and Symbiont Jointly Control Gut Microbiota during Complete Metamorphosis. *PLOS Pathogens* **11**, e1005246 (2015).

15. J. T. Critchlow, A. Norris, A. T. Tate, The legacy of larval infection on immunological dynamics over metamorphosis. *Phil. Trans. R. Soc. B* **374**, 20190066 (2019).

16. P. Engel, N. A. Moran, The gut microbiota of insects – diversity in structure and function. *FEMS Microbiology Reviews* **37**, 699–735 (2013).

17. S. Sudakaran, H. Salem, C. Kost, M. Kaltenpoth, Geographical and ecological stability of the symbiotic mid-gut microbiota in European firebugs, *Pyrrhocoris apterus* (Hemiptera, Pyrrhocoridae). *Molecular Ecology* **21**, 6134–6151 (2012).

18. A. Parmentier, *et al.*, A different gut microbial community between larvae and adults of a wild bumblebee nest (Bombus pascuorum). *Insect Science* **25**, 66–74 (2018).

19. T. J. Hammer, W. O. McMillan, N. Fierer, Metamorphosis of a Butterfly-Associated Bacterial Community. *PLoS ONE* **9**, e86995 (2014).

20. N. de Jonge, *et al.*, Housefly (Musca domestica L.) associated microbiota across different life stages. *Scientific Reports* **10**, 7842 (2020).

21. M. Wang, X. Xiang, X. Wan, Divergence in Gut Bacterial Community Among Life Stages of the Rainbow Stag Beetle Phalacrognathus muelleri (Coleptera: Lucanidae). *Insects* **11**, 719 (2020).

22. S. Sherrill-Mix, *et al*., Allometry and ecology of the bilaterian gut microbiome. MBio **9**, e00319-18 (2018).

23. V. G. Martinson, J. Carpinteyro-Ponce, N. A. Moran, T. A. Markow, A Distinctive and Host-Restricted Gut Microbiota in Populations of a Cactophilic Drosophila Species. *Applied and Environmental Microbiology* **83** (2017).

24. H. Staudacher, *et al.*, Variability of Bacterial Communities in the Moth Heliothis virescens Indicates Transient Association with the Host. *PLOS ONE* **11**, e0154514 (2016).

25. T. Alabi, J. P. Michaud, L. Arnaud, E. Haubruge, A comparative study of cannibalism and predation in seven species of flour beetle. *Ecological Entomology* **33**, 716–726 (2008).

26. P. R. Johnston, V. Paris, J. Rolff, Immune gene regulation in the gut during metamorphosis in a holo- versus a hemimetabolous insect. *Philosophical Transactions of the Royal Society B: Biological Sciences* **374**, 20190073 (2019).

27. T. Mai, *et al.*, 20-hydroxyecdysone positively regulates the transcription of the antimicrobial peptide, lebocin, via BmEts and BmBR-C Z4 in the midgut of Bombyx mori during metamorphosis. *Developmental & Comparative Immunology* **74**, 10–18 (2017).

28. V. W. Russell, P. E. Dunn, Lysozyme in the midgut of Manduca sexta during metamorphosis. *Archives of Insect Biochemistry and Physiology* **17**, 67–80 (1991).

29. Q. Xu, *et al.*, Transcriptional Profiling of Midgut Immunity Response and Degeneration in the Wandering Silkworm, Bombyx mori. *PLoS ONE* **7**, e43769 (2012).

30. T. Fukami, Historical Contingency in Community Assembly: Integrating Niches, Species Pools, and Priority Effects. *Annu. Rev. Ecol. Evol. Syst.* **46**, 1–23 (2015).

31. S. Stoll, H. Feldhaar, M. Fraunholz, R. Gross, Bacteriocyte dynamics during development of a holometabolous insect, the carpenter ant Camponotus floridanus. *BMC Microbiology* (2012).

32. J. Maire, *et al.*, Spatial and morphological reorganization of endosymbiosis during metamorphosis accommodates adult metabolic requirements in a weevil. *PNAS* **117**, 19347–19358 (2020).

33. M. Kaltenpoth, E. Yildirim, M. F. Gürbüz, G. Herzner, E. Strohm, Refining the Roots of the Beewolf-Streptomyces Symbiosis: Antennal Symbionts in the Rare Genus Philanthinus (Hymenoptera, Crabronidae). *Appl. Environ. Microbiol.* **78**, 822–827 (2012).

34.Y. Kikuchi, T. Hosokawa, T. Fukatsu, An ancient but promiscuous host–symbiont association between Burkholderia gut symbionts and their heteropteran hosts. *The ISME Journal* **5**, 446–460 (2011).

35. T. J. Hammer, J. C. Dickerson, N. Fierer, Evidence-based recommendations on storing and handling specimens for analyses of insect microbiota. *PeerJ* **3**, e1190 (2015).

36. L. R. Thompson, *et al.*, A communal catalogue reveals Earth's multiscale microbial diversity. *Nature* **551**, 457–463 (2017).

37. R Core Team, *R: A language and environment for statistical computing.* (R Foundation for Statistical Computing, 2020).

38. B. J. Callahan, *et al.*, DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* **13**, 581–583 (2016).

39. P. Mcmurdie, S. Holmes, *Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data* (2013).

40. J. R. Cole, *et al.*, Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucl. Acids Res.* **42**, D633–D642 (2014).

41. N. M. Davis, D. M. Proctor, S. P. Holmes, D. A. Relman, B. J. Callahan, Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* **6**, 226 (2018).

42. S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, Basic Local Alignment Search Tool. *J. Mol. Biol.*, 403–410 (1990).

43. W. Shen, J. Xiong, TaxonKit: a cross-platform and efficient NCBI taxonomy toolkit. *bioRxiv*, 513523 (2019).

44. L. Lathi, *et al.*, *microbiome R package* (2019).

45. M. Torchiano, *effsize: Efficient Effect Size Computation* (2020).

46. J. Oksanen, *et al.*, *Vegan: community ecology package* (2008).

Figures and Tables

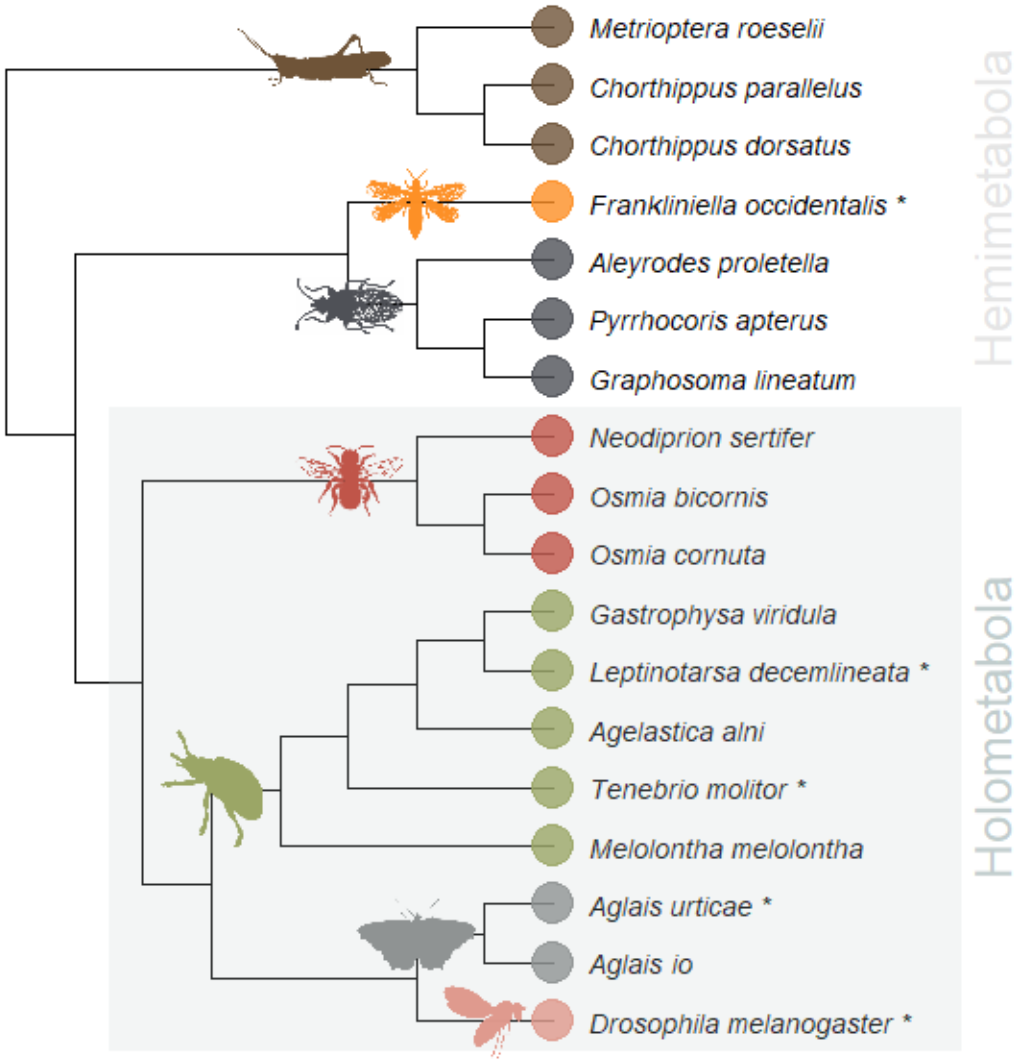

Figure 1: Phylogenetic tree of the 18 field-collected species. The five species marked with a star were additionally sampled from laboratory-reared colonies. The 11 highlighted species represent the monophyletic group of the holometabolous insects. The other seven species are hemimetabolous.

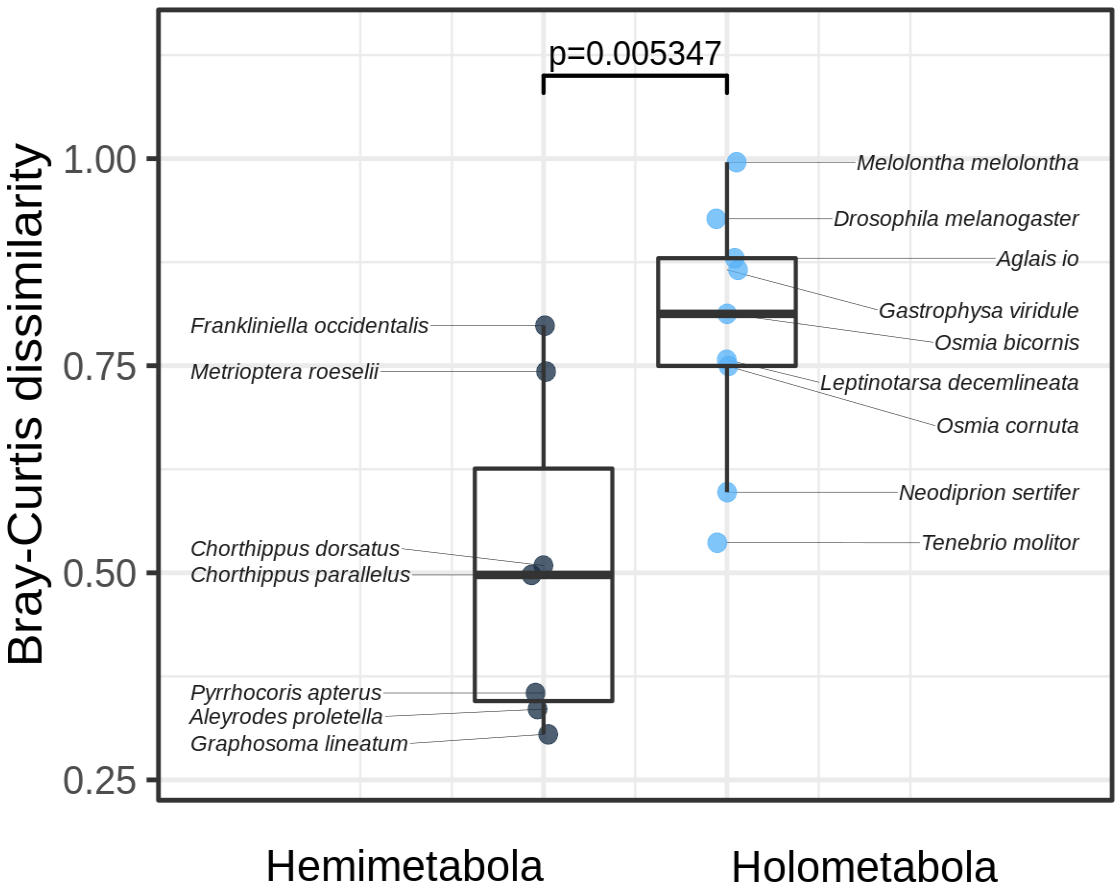

Figure 2: The average beta diversity (Bray-Curtis dissimilarity) in larval and adult gut communities among hemi- versus holometabolous insects (Two-sample t-test, t = -3.292, df = 14, p = 0.005347). Each point represents the beta diversity between life stages of a particular insect.

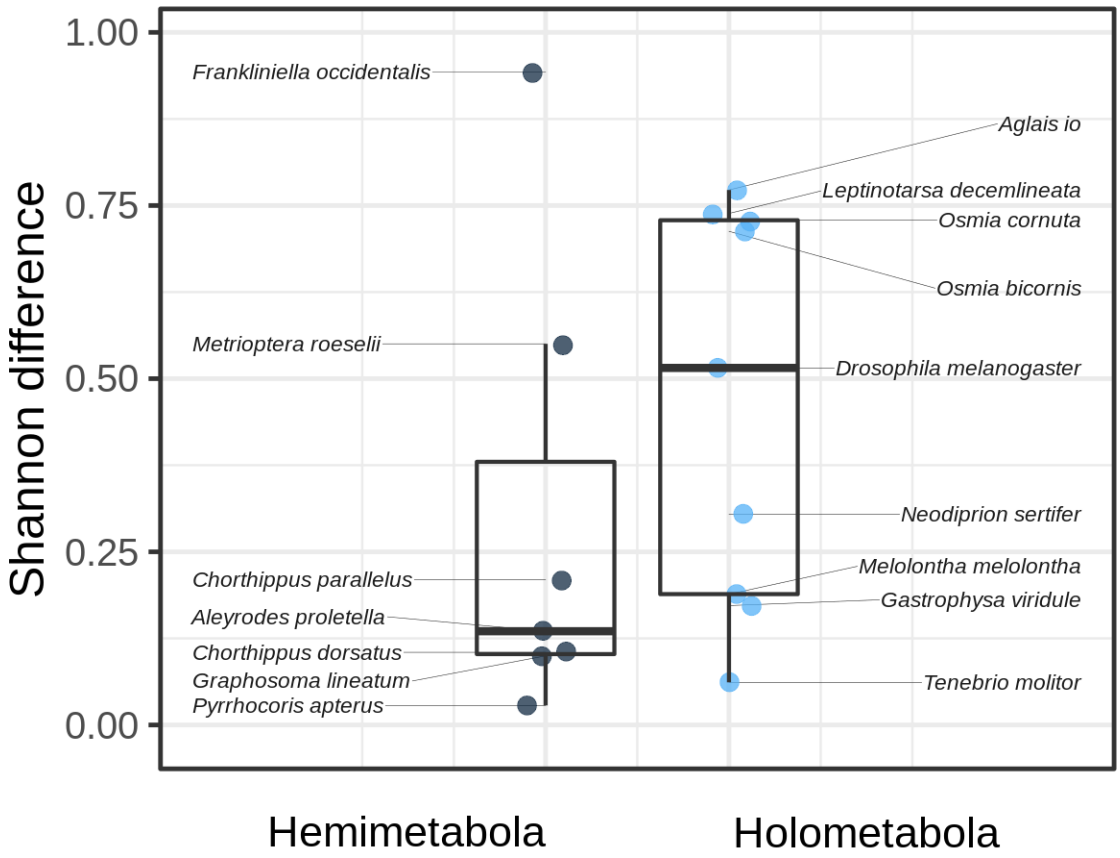

Figure 3: Average alpha diversity among hemi- and holometabolous insects (Two-sample t-test, t = -1.1025, df = 14, p = 0.2889). Each point represents the difference in alpha diversity, measured as Shannon difference, between larval and adult bacterial communities of a particular insect.

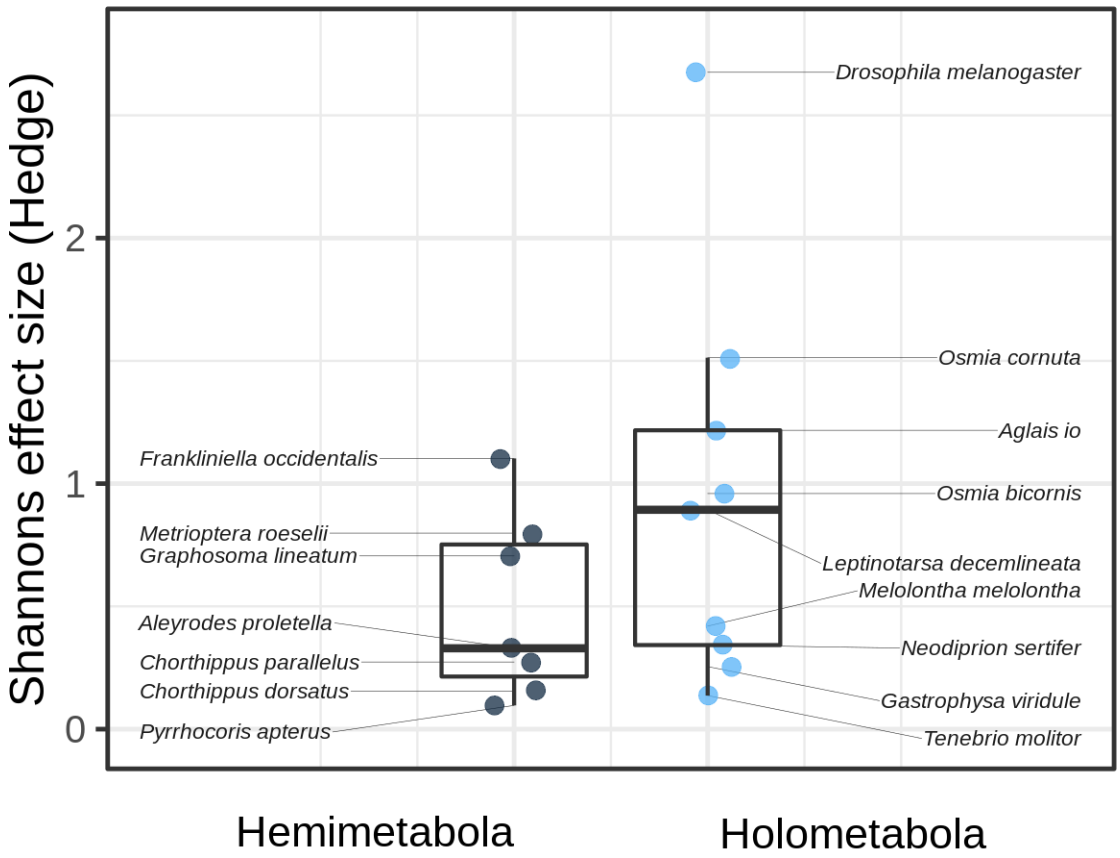

Figure 4: Average Shannons effect size between larval and adult gut communities among hemi- and holometabolous insects (Two-sample t-test, t = -1.3322, df = 14, p = 0.2041). Each point represents Shannons effect size, measured as Hedges‘ g, between life stages of a particular insect.

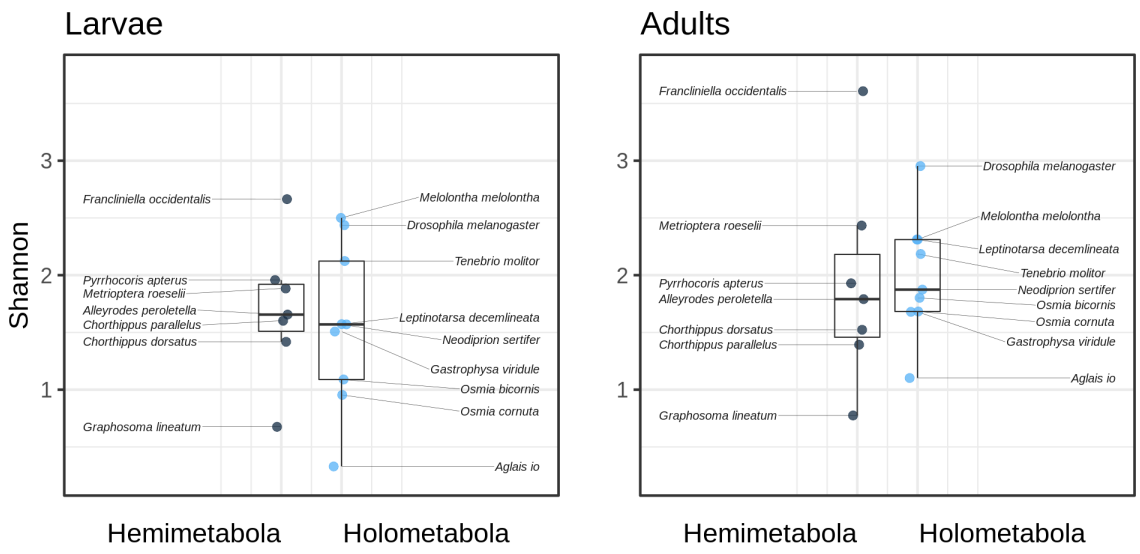

Figure 5: Average larval alpha diversity between hemi- and holometabolous insects (Two-sample t-test, t = 0.38317, df = 14, p = 0.7073, left), and of adults (Two-sample t-test, t = -0.18815, df = 14, p = 0.8535, right) . Each point represents the alpha diversity, measured as Shannon difference, of Larvae (left) or Adults (right).

*Table 1: Locations from which the insects where collected. Note: [*1] 1 replicate equals 40 individuals, [*2] 1 replicate equals 10 individuals, [*3] 1 replicate equals 30 individuals*

Order	Field – species	Location	Lon	Lat	Date	N_{Larvae}	N_{Pupae}	N_{Adults}
Orthoptera	*Chorthippus parallelus*	Wernigerode, Germany	10.776667	51.835833	June 2018	16	na	9
	Chorthippus dorsatus	Vienenburg, Germay	10.565278	51.950833	June 2018	10	na	16
	Metrioptera roeselii	Oker, Wernigerode, Germany	10.360944	52.523844	June 2018	2	na	7
Hemiptera	*Pyrrhocoris apterus*	Treptower Park, Berlin, Germany	13.47	52.490278	Apr – May 2018	11	na	12
		Zoology, Freie Universität Berlin	13.306653	52.45414	Apr – June 2018	22	na	14
	Graphosoma lineatum	Nature reserve Drömling, Germany	11.131111	52.4825	May – June 2018	21	na	2
	Alleyrodes peroletella	Estedt, Germany	11.3585	52.5759	Aug 2018	10 [*1]	na	10 [*1]
Thysanoptera	*Francliniella occidentalis*	Thaer-Institute of Agricultural and Horticultural Sciences, Humboldt-Universität Berlin, Germany	13.299165	52.468107	Sept 2018	10 [*2]	na	10 [*2]
Coleoptera	*Agelastica alni*	Wernitz, Germany	11.231667	52.487778	May – July 2018	20	na	10
		Watersport centre Schmoeckwitz, Humboldt-Universität Berlin, Germany	13.649167	52.377778	May – July 2018	10	na	12
	Gastrophysa viridule	Treptower Park, Berlin, Germany	13.47	52.490278	July 2018	12	na	11
	Melolontha melolontha	Wernitz, Germany	11.231667	52.487778	May – June 2018	10	na	10
		Magdeburg, Germany	11.616667	52.133333	May – June 2018	10	na	10
	Tenebrio molitor	Letovanić, Croatia	16.17127968	45.485598	July 2018	12	na	14
	Leptinotarsa decemlineata	Magdeburg, Germany	11.65232	52.13183	June – Aug 2018	23	na	7
Hymenoptera	*Osmia cornuta*	Berlin, Germany	13.322082	52.486689	Apr – May 2018	18	11	12
	Osmia bicornis	Berlin, Germany	13.470234	52.482677	Apr – June 2018	12	5	5
	Neodiprion sertifer	Finnish Forest Research Institute, Vantaa Research Centre, Asikkala, Finland	26.083056	60.75	July 2018	26	7	13
Lepidoptera	*Aglais urticae*	Wernigerode, Germany	13.361961	52.401887	June – Aug 2018	10	na	10
	Aglais io	Nature conservation station Marienfelde, Germany	13.361961	52.401887	July – Oct 2018	13	na	10
Diptera	*Drosophila melanogaster*	Berlin, Germany	13.470234	52.482677	July 2018	10 [*3]	na	10 [*3]

Order	Laboratory – reared species	Origin	N_{Larvae}	N_{Pupae}	N_{Adults}
Thysanoptera	*Francliniella occidentalis*	Institute of Biology, Developmental Biology, Martin-Luther-Universität Halle-Wittenberg, Germany	3 [*2]	na	2 [*2]
Coleoptera	*Tenebrio molitor*	Institute of Biology, Applied Zoology / Animal Ecology, Freie Universität Berlin, Germany	14	na	6
	Leptinotarsa decemlineata	Julius Kühn-Institut (JKI) Federal Research Centre for Cultivated Plants, Kleinmachnow, Germany	15	na	9
Lepidoptera	*Aglais urticae*	Commercial supplier, Worldwide Butterflies Ltd., United Kingdom	16	na	6
Diptera	*Drosophila melanogaster*	Institute of Biology, Applied Zoology / Animal Ecology, Freie Universität Berlin, Germany	9 [*3]	na	10 [*3]

Table 2: Results of perMANOVA testing differences in Beta diversity between life stages

Order	Field – species	Factor	Df	Sum Sq	Mean Sq	Pseudo-F	R^2	P	group dispersions
Orthoptera	*Chorthippus parallelus*	*Life stage*	1	0.4454	0.44540	1.8122	0.07304	**0.121**	homogenous
		Residuals	23	5.6528	0.24577		0.92696		
		Total	24	6.0982			1.00000		
	Chorthippus dorsatus	*Life stage*	1	0.7328	0.73280	3.2021	0.12221	**0.019 ***	homogenous
		Residuals	23	5.2635	0.22885		0.87779		
		Total	24	5.9963			1.00000		
	Metrioptera roeselii	*Life stage*	1	0.27721	0.27721	0.99211	0.12414	**0.416**	homogenous
		Residuals	7	1.95587	0.27941		0.87586		
		Total	8	2.23308			1.00000		
Hemiptera	*Pyrrhocoris apterus*	*Life stage*	1	0.5017	0.50169	3.0134	0.04937	**0.002 ****	homogenous
		Population	1	0.3371	0.33713	2.0250	0.03318	0.034 *	
		Residuals	56	9.3232	0.16649		0.91746		
		Total	58	10.1621			1.00000		
	Graphosoma lineatum	*Life stage*	1	0.002420	0.0024199	0.96475	0.04392	**0.404**	homogenous
		Residuals	21	0.052674	0.0025083		0.95608		
		Total	22	0.055094			1.00000		
	Alleyrodes peroletella	*Life stage*	1	0.31711	0.31711	2.0528	0.10237	**0.058**	homogenous
		Residuals	18	2.78048	0.15447		0.89763		
		Total	19	3.09759			1.00000		
Thysanoptera	*Francliniella occidentalis*	*Life stage*	1	0.34693	na	0.8665	0.17805	**0.700**	homogenous
		Residuals	4	1.60158	na		0.82195		
		Total	5	1.94850			1.00000		
Coleoptera	*Agelastica alni*	excluded							
	Gastrophysa viridula	*Life stage*	1	3.0512	3.05117	17.521	0.45485	**0.001 *****	heterogenous
		Residuals	21	3.6569	0.17414		0.54515		
		Total	22	6.7081			1.00000		
	Melolontha melolontha	*Life stage*	1	5.0221	5.0221	19.4543	0.33561	**0.001 *****	heterogenous
		Population	1	0.3905	0.3905	1.5128	0.02610	0.134	
		Residuals	37	9.5515	0.2581		0.63829		
		Total	39	14.9641			1.00000		
	Tenebrio molitor	*Life stage*	1	0.9748	0.97482	3.3482	0.12243	**0.001 *****	homogenous
		Residuals	24	6.9875	0.29114		0.87757		
		Total	25	7.9623			1.00000		
	Leptinotarsa decemlineata	*Life stage*	1	0.4587	0.45866	1.2788	0.04368	**0.228**	homogenous
		Residuals	28	10.0430	0.35868		0.95632		
		Total	29	10.5016			1.00000		
Hymenoptera	*Osmia cornuta*	*Life stage*	1	0.9475	0.94753	2.7374	0.09525	**0.023 ***	homogenous
		Residuals	26	8.9998	0.34614		0.90475		
		Total	27	9.9473			1.00000		
	Osmia bicornis	*Life stage*	1	1.2385	1.23846	4.0532	0.19252	**0.001 *****	heterogenous
		Residuals	17	5.1943	0.30555		0.80748		
		Total	18	6.4328			1.00000		
	Neodiprion sertifer	*Life stage*	1	0.5352	0.53521	2.0298	0.05201	**0.032 ***	heterogenous
		Residuals	37	9.7561	0.26368		0.94799		
		Total	38	10.2913			1.00000		
Lepidoptera	*Aglais urticae*	excluded							
	Aglais io	*Life stage*	1	1.0125	1.01253	2.4208	0.12465	**0.003 ****	homogenous
		Residuals	17	7.1105	0.41827		0.87535		
		Total	18	8.1231			1.00000		
Diptera	*Drosophila melanogaster*	*Life stage*	1	1.22434	1.22434	41.677	0.71028	**0.001 *****	heterogenous
		Residuals	17	0.49941	0.02938		0.28972		
		Total	18	1.72375			1.00000		

Order	Field – species	Factor	Df	Sum Sq	Mean Sq	Pseudo-F	R^2	**P**	group dispersions
Thysanoptera	*Francliniella occidentalis*	*Life stage*	1	0.071812	0.071812	1.5018	0.3336	**0.200**	homogenous
		Residuals	3	0.143449	0.047816		0.6664		
		Total	4	0.215261			1.00000		
Coleoptera	*Tenebrio molitor*	*Life stage*	1	1.0638	1.06380	4.0714	0.18446	**0.003 ****	heterogenous
		Residuals	18	4.7032	0.26129		0.81554		
		Total	19	5.7670			1.00000		
	Leptinotarsa decemlineata	*Life stage*	1	0.11578	0.115776	2.0327	0.08458	**0.072**	homogenous
		Residuals	22	1.25303	0.056956		0.91542		
		Total	23	1.36881			1.00000		
Lepidoptera	*Aglais urticae*	*Life stage*	1	0.9213	0.92129	2.7522	0.12653	**0.014 ***	homogenous
		Residuals	19	6.3602	0.33475		0.87347		
		Total	20	7.2815			1.00000		
Diptera	*Drosophila melanogaster*	*Life stage*	1	1.5345	1.53450	17.001	0.50001	**0.001 *****	heterogenous

Table 3: Analysis of variance table for linear model fits to compare alpha diversities between larval and adult life stages and populations per species. Shown are the species which originated from two different locations.

Order	Field – species	Factor	Df	Sum Sq	Mean Sq	Pseudo-F	P
Hemiptera	*Pyrrhocoris apterus*	*Life stage*	1	0.0116	0.011604	0.1425	0.7072
		Population	1	0.1827	0.182706	2.2443	**0.1397**
		Residuals	56	4.5589	0.081408		
Coleoptera	*Melolontha melolontha*	*Life stage*	1	0.3567	0.35672	1.7861	0.1896
		Population	1	0.0053	0.00532	0.0266	**0.8713**
		Residuals	37	7.3897	0.19972		

Table 4: Results of t-tests used to compare larval and adult alpha diversities (Shannon). Also listed are the effect sizes (Hedges` g).

		Test parameters						Hedges` g			
Order	Field – species	Statistical test	W	T	Df	P		estimate	Effect size	Lower 95% CI	Upper 95%
Orthoptera	*Chorthippus parallelus*	Two sample t-test	na	-0.726	23	**0.475**		-0.292	**small**	-1.130	0.545
	Chorthippus dorsatus	Two sample t-test	na	0.744	23	**0.465**		0.294	**small**	-0.527	1.115
	Metrioptera roeselii	Two sample t-test	na	1.120	7	**0.300**		0.798	**medium**	-0.933	2.529
Hemiptera	*Pyrrhocoris apterus*	Two sample t-test	na	-0.373	57	**0.710**		-0.097	**negligible**	-0.615	0.422
	Graphosoma lineatum	Two sample t-test	na	-2.313	21	**0.031**	*	-0.976	**large**	-1.900	-0.052
	Alleyrodes peroletella	Two sample t-test	na	0.769	18	**0.452**		0.329	**small**	-0.577	1.235
Thysanoptera	*Francliniella occidentalis*	Two sample t-test	na	1.688	4	**0.167**		1.103	**large**	-0.844	3.049
Coleoptera	*Agelastica alni*	excluded									
	Gastrophysa viridule	Welch-test	na	0.612	13	**0.551**		0.255	**small**	-0.585	1.095
	Melolontha melolontha	Two sample t-test	na	-1.354	38	**0.184**		0.420	**small**	-1.054	0.215
	Tenebrio molitor	Two sample t-test	na	0.359	24	**0.723**		0.137	**negligible**	-0.650	0.924
	Leptinotarsa decemlineata	Two sample t-test	na	2.128	28	**0.042**	*	0.894	**large**	0.003	1.784
Hymenoptera	*Osmia cornuta*	Two sample t-test	na	4.080	26	**3.8E-4**	***	1.513	**large**	0.650	2.375
	Osmia bicornis	Two sample t-test	na	2.162	17	**0.045**	*	0.960	**large**	-0.028	1.947
	Neodiprion sertifer	Two sample t-test	na	1.029	37	**0.310**		0.342	**small**	-0.336	1.021
Lepidoptera	*Aglais urticae*	excluded									
	Aglais io	Wilcoxon	90	na	na	**0.027**	*	1.217	**large**	0.272	2.162
Diptera	*Drosophila melanogaster*	Two sample t-test	na	6.095	17	**1.2E-5**	***	2.675	**large**	1.401	3.949

		Test parameters						Hedges` g			
Order	Field – species	Statistical test	W	T	Df	P		estimate	Effect size	Lower 95% CI	Upper 95%
Thysanoptera	*Francliniella occidentalis*	Two sample t-test	na	-4.096	3	**0.026**	*	-2.719	large	-5.622	0.1833
Coleoptera	*Tenebrio molitor*	Two sample t-test	na	-0.638	18	**0.531**		-0.298	small	-1.285	0.688
	Leptinotarsa decemlineata	Two sample t-test	na	0.234	22	**0.817**		0.095	negligible	-0.749	0.940
Lepidoptera	*Aglais urticae*	Two sample t-test	na	-3.769	20	**0.001**	**	-1.736	large	-2.830	-0.641
Diptera	*Drosophila melanogaster*	Welch-test	na	4.891	12	**3.2E-4**	***	2.068	large	0.921	3.214

Table 5: Shannon indices and evenness

Order	Field – species	Life stage	N	Hs	SD (Hs)	E	SD (E)
Orthoptera	*Chorthippus parallelus*	*Larva*	16	**1.602**	0.775	**0.668**	0.175
		Adult	9	**1.393**	0.671	**0.662**	0.175
	Chorthippus dorsatus	*Larva*	10	**1.418**	0.745	**0.707**	0.169
		Adult	15	**1.523**	0.587	**0.793**	0.155
	Metrioptera roeselii	*Larva*	2	**1.884**	0.515	**0.744**	0.187
		Adult	7	**2.434**	0.627	**0.765**	0.075
Hemiptera	*Pyrrhocoris apterus*	*Larva*	33	**1.957**	0.292	**0.782**	0.082
		Adult	26	**1.929**	0.284	**0.819**	0.059
	Graphosoma lineatum	*Larva*	8	**0.676**	0.024	**0.802**	0.214
		Adult	15	**0.775**	0.166	**0.707**	0.165
	Alleyrodes peroletella	*Larva*	10	**1.656**	0.362	**0.875**	0.125
		Adult	10	**1.791**	0.423	**0.868**	0.052
Thysanoptera	*Francliniella occidentalis*	*Larva*	3	**2.664**	0.892	**0.858**	0.057
		Adult	3	**3.607**	0.373	**0.891**	0.020
Coleoptera	*Agelastica alni*	excluded					
	Gastrophysa viridule	*Larva*	12	**1.508**	0.354	**0.813**	0.076
		Adult	11	**1.680**	0.870	**0.648**	0.250
	Melolontha melolontha	*Larva*	20	**2.501**	0.476	**0.887**	0.040
		Adult	20	**2.312**	0.403	**0.778**	0.087
	Tenebrio molitor	*Larva*	12	**2.123**	0.466	**0.784**	0.089
		Adult	14	**2.185**	0.409	**0.775**	0.099
	Leptinotarsa decemlineata	*Larva*	23	**1.572**	0.761	**0.778**	0.150
		Adult	7	**2.311**	0.944	**0.768**	0.096
Hymenoptera	*Osmia cornuta*	*Larva*	16	**0.954**	0.407	**0.723**	0.149
		Pupa	8	**1.268**	0.611	**0.699**	0.122
		Adult	12	**1.683**	0.540	**0.843**	0.104
	Osmia bicornis	*Larva*	11	**1.089**	0.279	**0.744**	0.126
		Pupa	5	**1.011**	0.335	**0.814**	0.172
		Adult	8	**1.802**	1.054	**0.790**	0.143
	Neodiprion sertifer	*Larva*	26	**1.570**	0.941	**0.712**	0.179
		Pupa	7	**2.123**	0.497	**0.748**	0.086
		Adult	13	**1.874**	0.701	**0.665**	0.131
Lepidoptera	*Aglais urticae*	excluded					
	Aglais io	*Larva*	13	**0.330**	0.463	**0.752**	0.390
		Adult	9	**1.102**	0.782	**0.551**	0.352
Diptera	*Drosophila melanogaster*	*Larva*	10	**2.437**	0.170	**0.908**	0.024
		Adult	9	**2.953**	0.199	**0.900**	0.025

Order	Field – species	Life stage	N	Hs	SD (Hs)	E	SD (E)
Thysanoptera	*Francliniella occidentalis*	*Larva*	3	**2.067**	0.338	**0.633**	0.062
		Adult	2	**0.948**	0.200	**0.462**	0.016
Coleoptera	*Tenebrio molitor*	*Larva*	14	**1.377**	0.793	**0.641**	0.273
		Adult	6	**1.158**	0.363	**0.732**	0.164
	Leptinotarsa decemlineata	*Larva*	15	**1.515**	0.295	**0.768**	0.077
		Adult	9	**1.545**	0.324	**0.751**	0.103
Lepidoptera	*Aglais urticae*	*Larva*	16	**1.585**	0.609	**0.760**	0.220
		Adult	6	**0.541**	0.475	**0.883**	0.163
Diptera	*Drosophila melanogaster*	*Larva*	9	**0.940**	0.212	**0.580**	0.118
		Adult	10	**1.758**	0.479	**0.704**	0.126