# Microbiome turnover during offspring development varies with maternal care, but not moult, in a hemimetabolous insect

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**Abstract**

The ecological success of insects often depends on their association with beneficial microbes. However, insect development involves repeated moults, which can have dramatic effects on their microbial communities. Here, we investigated whether and how moulting affects the microbiome of a hemimetabolous insect, and whether maternal care can modulate these effects. We reared European earwig juveniles with or without mothers and used 16S rRNA metabarcoding to analyse the whole microbiome of eggs, recently and old moulted individuals at four developmental stages and the resulting adults. The 218 samples obtained showed that the microbiome diversity changed non-linearly during development and that these changes were associated with bacterial biomarkers. Surprisingly, these changes did not occur during moulting, but rather between the beginning and end of certain developmental stages. We also found that access to maternal care affected the microbiome of both juveniles and adults, even when the last contact with mothers was two months before adulthood. Overall, these results provide new insights into our understanding of the (in)stability of the microbiome in hemimetabolous insects and its independence from moult. More generally, they question the role of microbiome acquisition through maternal care in maintaining family life in species where this behaviour is facultative.

**Keywords**: Microbiome, Horizontal transmission, Vertical transmission, Metamorphosis, Sociality

## Introduction

Insects are the most diverse and abundant animal taxon on Earth, comprising more than half of the animal kingdom (Mora *et al.*, 2011; Berenbaum, 2017; Samways, 2018). One reason for their evolutionary success is their frequent association with a large and complex diversity of beneficial microorganisms (Shapira, 2016; Sudakaran *et al.*, 2017). This is because this association can help their host colonize new ecological niches and mediate insect speciation and adaptation to a variety of environments (Berlanga and Guerrero, 2016; Shapira, 2016). However, maintaining these associations is challenging for insects, as it requires hosts to retain their microbes despite the numerous and successive moults (or ecdysis) that occur between the egg and adult stages (Bright and Bulgheresi, 2010). From a microbial perspective, these moults are risky as they can lead to a partial purge from the host, inducing a microbial bottleneck in which almost all symbionts are lost (McFrederick *et al.*, 2014; Zhukova *et al.*, 2017). Moults can also lead to critical changes in the habitat used by microbes within the host, which in turn can rapidly induce major turnover in their communities (Engel and Moran, 2013). For the hosts, moulting also poses a significant challenge, as they need to retain or (re)acquire the beneficial symbionts (Arce *et al.*, 2012; Wang and Rozen, 2017) while coping with surrounding pathogenic microorganisms susceptible to take their place in the host (Salem *et al.*, 2015; Hammer and Moran, 2019). Therefore, it is necessary for both host insects and beneficial microorganisms to develop strategies to ensure the continuity of their association throughout host development.

To ensure the (re)inoculation and maintenance of beneficial microorganisms throughout development, insects can adopt two non-mutually exclusive strategies. On one hand, they can acquire these microorganisms from direct contact with their

environment and conspecifics, a process called horizontal transmission. This process can be particularly important when individuals live in groups, share a nesting environment and/or when moulting leads to the loss of these microorganisms at each development stage (Raina *et al.*, 2008; Nalepa, 2020; Rose *et al.*, 2023). On the other hand, hosts can acquire beneficial microorganisms from their parents, a process called vertical transmission (Bright and Bulgheresi, 2010; Sachs *et al.*, 2011; Hosokawa and Fukatsu, 2020; Michaud *et al.*, 2020). In insects, vertical transmission was long thought to occur mainly through the transfer of microorganisms directly into the eggs, which the resulting offspring then had to maintain throughout their development. However, many insect parents provide care to their eggs and juveniles after oviposition (Meunier *et al.*, 2022) and recent studies show that this care can also mediate a vertical transmission of microorganisms. For instance, mothers can deposit external secretions containing symbionts on the eggshell, transfer symbionts to their juveniles through mouth-to-mouth contact (trophallaxis) during family life, and juveniles can acquire these symbionts soon after hatching by ingesting their parents' feces (Klass *et al.*, 2008; Lombardo, 2008; Bright and Bulgheresi, 2010; Powell *et al.*, 2014; Zhukova *et al.*, 2017; Onchuru *et al.*, 2018). Access to maternal care can thus ensure the acquisition and reacquisition of beneficial microbes by moulting juveniles, thus possibly strengthening the stability and evolutionary trajectory of symbiotic associations.

While our current understanding of the consequences of moulting and maternal care on the dynamics of the host microbiome during development is mainly based on holometabolous insects, little is known about these consequences on hemimetabolous species (Hammer and Moran, 2019; Girard *et al.*, 2023). The focus on holometabolous species is explained by the fact that their immature stages have a morphology and sometimes an ecology very different from those of adults, which raises obvious

82 questions about the fate of their microbiome during metamorphosis (Johnston *et al.*,
83 2019). In contrast, hemimetabolous insects have immature stages called nymphs that
84 are very similar in morphology and ecology to the adult, and their juveniles undergo
85 only gradual morphological changes through successive moults (Johnston *et al.*,
86 2019). The impact of these gradual changes on the dynamics of the host microbiome
87 has received comparatively much less attention, and a few studies suggest that it may
88 be stable throughout development such as in Blattodea, Orthoptera, and Hemiptera
89 (Sudakaran *et al.*, 2012; Manthey *et al.*, 2022). However, it remains unclear whether
90 this stability is universal across species and, importantly, whether it is due either to a
91 non-purging effect of moulting on microbial communities, the fact that host microbial
92 niches do not change during development and thus select for the same microbial
93 communities, and/or that maternal care ensures the maintenance of the microbial
94 community through vertical transmission.

95 In this study, we investigated whether and how the microbiome of the
96 hemimetabolous European earwig *Forficula auricularia* L. (Order Dermaptera:
97 Forficulidae) changes during juvenile development and tested whether these potential
98 changes were due to moulting events, stage-specific microbial niches and/or offspring
99 access to maternal care. In this species, females oviposit in individual burrows in early
100 winter (Meunier *et al.*, 2012; Tourneur and Meunier, 2020) after which they stop
101 foraging and provide extensive forms of care, including egg grooming (Boos *et al.*,
102 2014; Diehl and Meunier, 2018). About 50 days later, the eggs hatch and the mothers
103 stay with their juveniles for about two more weeks. During this time, they continue to
104 provide care to their nymphs, such as allo-grooming and food provisioning (Lamb,
105 1976; Kölliker, 2007). Mothers leave the nest shortly after the nymphs have moulted
106 for the second time (the first moult occurs at the time of hatching), and the nymphs

then moult three more times before reaching adulthood two months later (Thesing *et al.*, 2015; Tourneur *et al.*, 2020). Whether the offspring microbiome changes during development and whether maternal care influences these changes are unknown in the European earwig. However, the microbiome of the eggshell is known to change over 16 days and to be partly influenced by maternal presence in the maritime earwig *Anisolabis maritima* (Greer *et al.*, 2020).

Here, we reared 20 families of the European earwig from egg to adult stages. Fifteen families were reared with their mothers for the first 15 days after hatching (*i.e.*, the normal duration of family life), while five families were reared without mothers from egg hatching onward. This was possible as post-hatching maternal care is facultative in this species (Thesing *et al.*, 2015). We then used 16S rRNA metabarcoding to analyse the whole microbiome of the eggs, both the freshly and old moulted nymphs of each instar and finally, the resulting adult offspring (males and females) of all these families. Overall, we found that the microbiome of earwig offspring surprisingly changed during development. We also showed that these changes are not due to a purging event during moulting, but rather likely reflect stage-specific microbial niches in the nymphs. Finally, we found that access to maternal care has both short- and long-term effects on the microbiome of offspring.

## Material and methods

### Earwig sampling and laboratory rearing

The eggs, nymphs and adults analysed in this study were the first-generation progeny of 20 females of *F. auricularia* sp "A" (Wirth *et al.*, 1998; González-Miguéns *et al.*, 2020). These 20 females were part of a large field sampling of earwig males and

132 females conducted in an orchard near Valence, France (44°58′38″N, 4°55′43″E) in the
133 summer of 2021. Just after field sampling, these individuals were randomly distributed
134 into plastic containers containing 100 females and 100 males and then maintained
135 under standard laboratory conditions to allow for uncontrolled mating (Koch and
136 Meunier, 2014; Sandrin *et al.*, 2015). In November 2021, each female was isolated to
137 mimic her natural dispersal from the groups and to stimulate oviposition (Kölliker,
138 2007). These females were transferred to individual Petri dishes (55 mm x 12 mm)
139 lined with moist sand (Körner *et al.*, 2018) and maintained in constant darkness at 10°C
140 until oviposition and egg hatching. From isolation until oviposition, females were fed
141 ad libitum with a laboratory-prepared food consisting mainly of carrots, cat food, seeds
142 and pollen (Kramer *et al.*, 2015). Food was renewed each week, but removed from the
143 day of oviposition until the day of egg hatching, as this is when the mothers typically
144 stop their foraging activity (Kölliker, 2007; Van Meyel and Meunier, 2020). From this
145 large pool of females, we haphazardly selected 20 clutches in which the mothers
146 produced 50 eggs (clutch size typically varies from 30 to 60 eggs, Tourneur and
147 Gingras, 1992) for our measurements. The remaining females were used in other
148 experiments not presented here.

149 On the day of egg hatching, the 20 selected clutches were transferred to larger
150 Petri dishes (145 mm x 13 mm) lined with moist sand (Körner *et al.*, 2018) to manipulate
151 maternal presence during offspring development. Of these 20 clutches, five (randomly
152 selected) had their mothers removed to subsequently prevent post-hatching maternal
153 care and any mother-offspring interactions. For the remaining 15 clutches, the nymphs
154 were kept with their mother for 14 days after egg hatching (which is the natural length
155 of family life in this species) and then separated from their mother for the rest of their
156 development. All clutches were maintained under laboratory conditions at 18-20°C

157 under a 12:12 light:dark photoperiod, and received the laboratory-prepared food twice
158 a week (see above).
159
160 **Experimental design and sample collection**
161 Overall, we analysed the microbiome of 218 samples collected throughout earwig
162 development (Figure 1, Table S1). As ecdysis is known to induce a microbial shift in
163 many insect species, we sampled all developmental stages (except eggs and adults)
164 both immediately after the moult (freshly moulted) and several days after the moult (old
165 moulted). Freshly moulted nymphs have a white (compared to dark) colour, which
166 typically lasts for maximum 3h in early instar nymphs and up to 6h in late instar (MC
167 Cheutin, pers. obs.). For the 15 clutches with maternal care, we obtained a total of 12
168 egg samples (Egg), 37 first instar nymphs of which 12 were freshly moulted (later called
169 L1-Y) and 15 were old moulted (L1-O), 30 second instar nymphs of which 15 were
170 freshly moulted (L2-Y) and 15 were old moulted (L2-O), 30 third instar nymphs of which
171 15 were freshly moulted (L3-Y) and 15 were old moulted (L3-O), 30 fourth instar
172 nymphs of which 15 were freshly moulted (L4-Y) and 15 were old moulted (L4-O), and
173 finally 32 adults of which 11 field-sampled mothers (Mother), 10 adult female offspring
174 (Adult-F) and 11 adult male offspring (Adult-M). For the 5 clutches without maternal
175 care, we only used a total of 21 old moulted first instar nymphs, 16 adult females and
176 20 adult males as we were only interested in the short and long-term effect of maternal
177 presence/absence on offspring s (Figure 1, see details in Table S1). Note that the first
178 observable developmental stage of the nymphs is called L1, as the moult that occurs
179 at hatching is called L0 in this species (Tourneur *et al.*, 2020). Moreover, the sex of an
180 individual can only be determined in adults, where the shape of the forceps is straight

181 in females and curved in males. Each sample was collected individually and
182 immediately transferred to Eppendorf tubes at -80°C until DNA extraction.
183

184 **Genomic extraction and 16S amplification**

185 Total genomic DNA was extracted using the NucleoMag® Tissue extraction kit
186 (Macherey-Nagel™, Düren, Germany) and the V3-V4 region of the 16S rDNA gene
187 was amplified with the prokaryotic primers 343F (5′- ACGGRAGGCAGCAG – 3′) and
188 784R (5′- TACCAGGGTATCTAATC – 3′) (Muyzer *et al.*, 1993) coupled with platform-
189 specific Illumina linkers. We performed PCR reactions using the Taq Polymerase
190 Phusion® High-Fidelity PCR Master Mix with GC buffer and prepared them according
191 to the manufacturer's instructions (Qiagen, Hilder, Germany). PCR amplification steps
192 involved an initial denaturing step for 30 sec at 98°C, followed by 22 amplification
193 cycles (denaturation for 10 sec at 98°C; annealing for 30 sec at 61.5°C; extension for
194 30 sec at 72°C), and ended by a final extension step of 10 min at 72°C. Electrophoresis
195 migration was run to check the probe specificity and amplification success. Extraction
196 and amplification steps involved several blank controls to confirm that samples were
197 not contaminated by environmental microorganisms. Samples were amplified in
198 duplicates and equally pooled for a final product of 30 µL further sequenced with
199 2x250bp Illumina MiSeq technology at the Bio-Environnement platform (University of
200 Perpignan, France). As the blanks were all negative, we did not send them for
201 sequencing.
202

203 **Bioinformatic process**

204 The obtained libraries were trimmed and filtered using the quality profiles from the
205 DADA2 algorithm v1.24.0 (Callahan *et al.*, 2016), cleaned for errors, dereplicated and

206 inferred towards Amplicon Sequence Variants (ASVs) (Glassman and Martiny, 2018).
207 Chimeras were removed and taxonomy assignment was performed on a count table
208 where forward and reverse ASVs were merged and pooled, using the SILVA reference
209 database (release 138) (Quast *et al.*, 2012). The multiple alignment of the sequences
210 was provided with the MAFFT program (Katoh, 2002) and we inferred the phylogenetic
211 tree with the FastTree 2 tool (Price *et al.*, 2010) and Phangorn package v2.8.1 (Schliep,
212 2011). The table was then transformed into a phyloseq object using the phyloseq
213 package v1.40.0 (McMurdie and Holmes, 2013) of which we removed the 529
214 sequences of mitochondrial origin and the 30 800 unknown sequences. As the 2 700
215 Eukarya sequences had no affiliation at Phylum level and were sparsely distributed,
216 they were also removed. Finally, we obtained a final dataset constituted by 4 163 989
217 sequences (3 780 ASVs) ranging from 3 570 to 32 946 sequences/sample.

218

219 **Identification of the core microbiomes**

220 We defined the core microbiome as the set of microbial taxa (*i.e.,* ASVs) that are
221 characteristic of all samples. We obtained this core microbiome using the species
222 abundance distribution (SAD) patterns of each ASVs (Magurran and Henderson,
223 2003). This approach is frequently used in the literature (Fillol *et al.*, 2016; Jeanbille *et*
224 *al.*, 2016; Cheutin *et al.*, 2021; Neu *et al.*, 2021) and allows us to distinguish between
225 core and satellite ASVs, while avoiding the use of subjective and arbitrary occurrence
226 and abundance thresholds (Magurran and Henderson, 2003). We first calculated an
227 index of dispersion (*i.e.,* the variance to mean ratio, VMR) for each ASV within each
228 developmental stage. We then tested whether these indices followed a Poisson
229 distribution, falling between the 2.5 and 97.5% confidence limits of the $Chi^2$ distribution
230 (Krebs, 1999). ASVs with index values outside these confidence limits were considered

231 as part of the core microbiome, while the others were considered as satellite ASVs.
232 This process provided us with stage-dependent core microbiomes for the eggs, each
233 instar nymph stages, adult offspring and mothers, and we finally merged all these cores
234 to obtain a global core microbiome (Figure S1).

235

236 **Functional predictions of the core microbiomes**

237 We used the PICRUST2 algorithm (Douglas *et al.*, 2020) to predict the potential
238 functions associated with each of the core microbiomes generated above. In brief, the
239 tool inserts each ASV sequence into a reference tree (EPA-ng, Barbera *et al.*, 2019)
240 using a hidden-state prediction (HSP, castor, Louca and Doebeli, 2018) and infers a
241 KEGG ortholog function (later named KOs) based on the functional profiles obtained
242 with the nearest-sequenced taxon (MinPath, Ye and Doak, 2009). The functional table
243 counts with KOs obtained for each sample are count-normalized for the ASVs copy
244 counts and multiplied by the gene content prediction resulting from the HSP algorithm.
245 Finally, a table with three levels of KEGG pathways is constructed according to the
246 MetaCyc database v27.1 (Caspi *et al.*, 2020).

247

248 **Evolution of bacterial diversity during offspring development**

249 To test how the microbiomes of offspring changed throughout host development, we
250 calculated the alpha and beta diversities of each sample using qualitative and
251 quantitative indices for both taxonomic and phylogenetic diversity. Regarding alpha
252 diversity, which was only calculated for ASVs (not for functions), we normalized all
253 samples at the minimum sampling size of sequences per individual and checked their
254 accuracy with rarefaction curves (Cameron *et al.*, 2021; Figure S2). For alpha diversity

255 proxies, we used direct observed richness for the qualitative taxonomic richness and
256 the Shannon entropy for its quantitative equivalent. We calculated their phylogenetic
257 equivalents with the Faith and the Allen's indices (Chao *et al.*, 2010). For beta diversity,
258 we calculated the relative abundance of each ASV and KOs by sample, and we
259 calculated the Jaccard distance between samples with ASVs (and KOs)
260 presence/absence and its quantitative equivalent Bray-Curtis. For ASVs, we also used
261 their phylogenetic equivalents with the Unifrac metrics for qualitative (unweighted) and
262 quantitative (weighted) distances (Chao *et al.*, 2010; Yang *et al.*, 2021).

263 To identify which microbes and/or potential functions, if any, are specific to each
264 developmental stage, we assessed the differential abundance of ASVs and KOs with
265 a negative binomial Wald test using the DESeq2 package (Love *et al.*, 2014). In this
266 model, we entered the developmental stage, which includes all stages with young- and
267 old-nymphs. Results are presented under heatmaps using the package *pheatmap*
268 v1.0.12 where ASVs are merged by Genera and KOs are merged by KEGG pathway
269 names (third level).

270

271 **Impact of maternal presence on offspring bacterial diversity**

272 Finally, we tested whether maternal presence influenced the microbiome of their
273 offspring at both the first developmental stage and the adult stage. To this end, we
274 repeated the analyses described above using the 5 families in which the mothers were
275 removed at egg hatching and the 15 families in which the mothers remained with their
276 nymphs for 15 days after hatching (*i.e.,* until the end of family life). We draw volcano
277 plots with the package *EnhancedVolcano* v1.14.0 to contrast discriminants between
278 the presence or absence of the mother.

279

280 **Statistics**

281 We first tested the effect of developmental stage and moult (fresh versus old moult) on
282 the microbiome diversity of offspring from the 15 families in which nymphs were kept
283 with a mother during family life. For alpha diversity, we conducted four linear mixed
284 models (LMM) in which we entered each of the four microbial alpha diversity indices
285 as a response variable, the sampling time (Egg, L1-Y, L1-O, L2-Y, L2-O, L3-Y, L3-O,
286 L4-Y, L4-O, Adult-F, Adult-M and Mother) as the explanatory variable and the clutch
287 used as a random effect to control for non-independence of the biological samples. We
288 did not use a classical 2-way ANOVA approach because the two levels of moult were
289 not available for each developmental stage (*i.e.,* eggs and adults). We conducted
290 pairwise comparisons between each sampling time using the estimated marginal
291 means of the models, with P values corrected for multiple testing using Tukey methods
292 with the *emmeans* package (Lenth, 2022). For each pairwise comparison, we also
293 calculated $R^2$ using the *MuMIn* package (Bartoń, 2022). For beta diversity, the
294 dissimilarity based on the ASVs and KOs assemblies between each sample was first
295 illustrated in a two-dimensional Principal Coordinates Analyses (PCoA). We then
296 performed Permutational Analyses of Variances (PERMANOVAs) to test the effect of
297 the clutch (as randomized block), the sampling stage and the sex on each distance
298 matrices based on bacterial composition (*i.e.,* Jaccard, Bray-Curtis, weighted and
299 unweighted Unifrac) and on functional predictions (*i.e.*, Jaccard and Bray-Curtis). Post-
300 hoc pairwise tests between stages were performed for each dissimilarity matrix with
301 the package *pairwiseAdonis* v0.4.1 (Martinez Arbizu, 2017). These pairwise
302 comparisons allowed us to address five questions, namely whether (1) microbiome
303 diversity changes between each successive development stage (*i.e.,* Egg *vs* L1-Y, L1-

304 Y *vs* L1-O, L1-O *vs* L2-Y, etc), (2) moulting causes a shift in microbiome diversity
305 between two successive developmental stages (*i.e.,* Egg *vs* L1-Y, L1-O *vs* L2-Y, etc),
306 (3) old nymphs exhibit an instar-specific microbiome diversity (*i.e.,* Egg *vs* L1-O, L1-O
307 *vs* L2-O, L2-O *vs* L3-O, etc), (4) freshly moulted nymphs exhibit an instar-specific
308 microbiome diversity (*i.e.,* L1-Y *vs* L2-Y, L2-Y *vs* L3-Y, etc) and finally, whether (5)
309 adults offspring exhibit a sex-specific microbiome diversity (Adult-M *vs* Adult-F).

310 We tested whether maternal presence had short and/or long-term effects on the
311 microbiome diversity of their offspring using the 15 families with mothers and the 5
312 families without mothers. For alpha diversity, we conducted four LMM in which we
313 entered each of the index values as a response variable, as well as maternal presence
314 (yes or no), the developmental instar (L1-O or adult) and the interaction between these
315 two factors as explanatory variables. When the latter was non-significant, we removed
316 it after model simplification by AIC comparison. We also entered the clutch as a random
317 effect. For beta diversity, we repeated the approach detailed above by testing the effect
318 of the clutch and the presence/absence of a mother in interaction with the stage (L1-O
319 or adult), on both microbial and functional composition.

320 We performed all statistical analyses using R v4.2.0 (R Core Team, 2022). We
321 checked all model assumptions with the *DHARMa* package (Hartig, 2022). We verified
322 variance homoscedasticity between groups by comparing the distance dispersion
323 within group with the *betadisper* function (all $P > 0.05$).

324

325 ## Results

326

327 **Microbiome changes during offspring development (with mothers)**

328 *Description of the core microbiome*

329 In the pool of the 218 earwig samples, we detected a total of 915 ASVs core (24.21%
330 of the initial ASVs diversity), which encompasses 97.67% of the complete sequence
331 dataset (Figure S1, Table S2). This core microbiome consisted mainly of
332 Proteobacteria (52.5%), Firmicutes (26.8%), Bacteroidota (13.9%) and
333 Actinobacteriota (6.6%). They were distributed among 66 bacterial families, of which
334 Lactobacillaceae (Phylum Firmicutes - 23.5%) and Enterobacteriaceae (Phylum
335 Proteobacteria - 15.6%) were the most abundant. The abundance of the other bacterial
336 families depended on the developmental stage of the host (Figure 2). For instance, the
337 families Nocardiaceae (Phylum Actinobacteriota) and Comamonadaceae (Phylum
338 Proteobacteria) dominated only the egg stage (27.6% and 13.2% respectively) and the
339 newly hatched nymphs N1-Y (39.2% and 16.9% respectively). On a finer scale, 50
340 ASVs were present at all developmental stages, a number that increased to 142 ASVs
341 when eggs were excluded (Figure S3A). The 50 common ASVs covered 29 genera,
342 represented mainly by *Chryseobacterium, Sphingobacterium* (Phylum Bacteroidota),
343 *Smaragdicoccus* (Phylum Actinobacterioa), *Kosakonia, Stenotrophomonas*,
344 *Paracoccus, Pseudomonas* and *Acinetobacter* (Phylum Proteobacteria) (Figure S3B).
345 Only a few ASVs were stage specific. For instance, 2 ASVs (ASV66|*Pseudomonas* and
346 ASV482|*Sphingobacterium*) were specific to the eggs, 52 to the nymphs and 26 to the
347 adults (Figure S3A).

348

349 *Changes in alpha and beta diversity during offspring development*

350 The alpha diversity of the earwig gut microbiome changed during the development of
351 the offspring (Figure 3A; Figure S4; Shannon index, Likelihood Ratio $\chi^2_{11} = 59.70$, P =
352 $10^{-8}$ and $P < 10^{-10}$ for all other alpha diversity indices). Markedly, the nature of these

353 changes did not follow a linear or systematic pattern. Alpha diversity did not change
354 between eggs and freshly moulted 1st instar nymphs (pairwise comparisons using log-
355 rank test contrasts for Shannon index; Egg *vs* L1-Y, P = 0.921), nor did it change during
356 the entire 1st instar (L1-Y *vs* L1-O, P = 0.621). However, there was a gradual increase
357 in alpha diversity during this time, as it was greater at the end of the 1st instar than in
358 the eggs (Egg *vs* L1-O, P = 0.052). While this alpha diversity remained at a high level
359 just after the moult into 2nd instar (L1-O *vs* L2-Y, P = 1.000), it then dropped to the
360 lowest level at the end of the 2nd instar (L2-Y *vs* L2-O, *P* = 0.029). This level remained
361 low just after the moult into 3rd instar (L2-O *vs* L3-Y, P = 0.998), but then increased to
362 its highest level at the end of the 3rd instar (L3-Y *vs* L3-O, P = 0.010). Alpha diversity
363 decreased slightly just after the moult into 4th instar (L3-O *vs* L4-Y, P = 0.086, but other
364 indices are significant for this contrast) to finally remain stable until the end of the 4th
365 instar (L4-Y *vs* L4-O, P = 0.702) and in the following adult females (L4-O *vs* Adult-F, P
366 = 0.496). By contrast, it decreased slightly between the end of the 4th instar and the
367 following adult males (L4-O *vs* Adult-M, P = 0.055, but other indices are significant for
368 this contrast).

369 Overall, moult was not associated with changes in offspring alpha diversity,
370 except for the final moult from 4th instar to adult males. In old nymphs, alpha diversity
371 was lower in 2nd compared to 3rd and 4th instars, whereas values were intermediate
372 in 1st instars and adults (Figure 3A). In contrast, in newly moulted nymphs, alpha
373 diversity was lower in 3rd instar compared to 2nd instar, while values were intermediate
374 in 1st and 4th instar (Figure 3A). Finally, in adult offspring, alpha diversity was similar
375 in males and females (Figure 3A, Figures S3, Supplemental File).

376 As with alpha diversity, the beta diversity of the earwig gut microbiome changed
377 as the offspring developed (PERMANOVA performed on all beta diversity metrics,

378 Stage effect $0.074 < R^2 < 0.103$, all $P = 0.001$) (Table 1). Here, however, the beta
379 diversity of each collection time was different from all other collection times for at least
380 one metric in 61 of the 65 pairwise comparisons ($p < 0.05$). Two major microbial
381 turnovers occurred after hatching and during the passage from the 2nd to the 3rd instar
382 nymphs. The PCoA performed on taxonomical distance matrices (*i.e.,* Bray-Curtis and
383 Jaccard) showed a marked separation on the first axis with both microbial assemblies
384 of old 2nd instar nymphs (L2-O) and the young 3rd instar nymphs (L3-Y) on one side,
385 and all the microbial assemblies associated with the other stages on the other side
386 (Figure 3B, Figure S5A). The PCoA based on Unifrac distances shows an additional
387 separation with eggs and newly hatched nymphs (L1-Y) on one side and the other
388 stages on the other side (Figure S5B-C) showing a strong phylogenetic turnover. The
389 only four comparisons that did not present any beta diversity difference were before
390 and after the three first moults, i.e. between eggs and newly hatched nymphs (L1-Y),
391 between old 1st instar nymphs (L1-O) and young 2nd instar nymphs (L2-Y), and
392 between old 2nd instar nymphs (L2-O) and young 3rd instar nymphs (L3-Y), as well as
393 the comparison between adult males and females (Supplemental File).

394

395 **Stage-specific microbes in the offspring microbiome**

396 A total of 231 ASVs that belonged to 62 bacterial genera were stage-specific (Figure
397 4). The hierarchical clustering analysis based on the Z-score of these discriminants
398 showed a structure that does not strictly follow the chronology of the developmental
399 stages of the offspring, but rather clustered individuals at the end of one instar to
400 individuals at the beginning of the following instar. In particular, they grouped the eggs
401 with newly hatched nymphs (L1-Y), the old 1st (L1-O) with the young 2nd instar nymphs

402 (L2-Y), and the old 2nd instar (L2-O) with the young 3rd (L3-Y), and the old 4th instar
403 nymphs (L4-O) with adults (Figure 4).

404

405 **Short- and long-term effects of maternal care on offspring microbiome**

406 The presence of the mother after hatching increased the alpha-diversity of their
407 offspring microbiome at both 1st instar and adult stages, but only when the phylogeny
408 was taken into account (Figure 5A; Faith: $X^2$ = 9.73, P = 0.002 and Hill1 : $X^2$ = 3.97, P
409 = 0.046). By contrast, it affected neither richness nor entropy (Observed richness: $X^2$
410 = 1.86, P = 0.172 and Shannon: $X^2$ = 1.21, P = 0.271) (Figure S6). In terms of beta-
411 diversity, maternal presence also affected the structure of the bacterial communities
412 (Bray-Curtis: $R^2$ = 0.028, P = 0.001) (Figure 5B, C; Figure S7; Table 1). Note that the
413 PERMANOVA applied to Unweighted Unifrac distances calculated on bacterial
414 assemblies might be affected by a non-homogeneous dispersion of the data as
415 individuals that lived with their mother appeared to be more dispersed (*betadisper*, F
416 = 7.7, P = 0.008). However, the ordination in Figure S7 supports a clustering, for both
417 N1 and adult stages, between the samples that lived with their mother or not.

418 The presence of a mother and the developmental stage were discriminated by
419 19 ASVs belonging to 12 genera in the microbiomes of the offspring. Among these
420 genera, *Rurimicrobium*, *Rhodoferax* and *Raoutella* were strongly discriminant in
421 individuals that had a mother during early life (Log2Fold change > |20|) while no
422 sequence belonging to these genera was present in the orphaned nymphs (Figure 5D).

423

424 **Predictions of potential functions of the different microbiomes**

425 In terms of function, the 915 ASVs of the global core microbiome were predicted to be
426 associated with 4 952 KOs involved in 226 different KEGG pathways. Most of these
427 pathways are related to metabolic routes such as lipid, carbohydrate or xenobiotics
428 biodegradation and biosynthesis of secondary metabolites (Supplemental File). These
429 functional predictions changed throughout the development of the host (Stage effect
430 $0.089 < R^2 < 0.092$, all $P = 0.001$) (Table 1). They followed the same patterns of
431 microbial assemblies, with a clear distinction between the eggs and newly hatched
432 nymphs, the old 2nd and young 3rd instar nymphs and the rest of all stages (Figure 3C,
433 Figure S8 ; see Pairwise results in Supplemental File). Among the 226 different KOs,
434 212 were stage-specific (Figure 6). As for bacteria, the hierarchical clustering of these
435 discriminants showed a structure that does not strictly follow the chronological order of
436 the developmental stages of the offspring (Figure 6).

437 Maternal presence affected the predicted functional structure of the microbiome
438 of offspring (Mother presence $0.035 < R^2 < 0.040$, all $P < 0.009$) (Table 2). This
439 presence discriminated 12 of the 226 KEGG pathways, but only three had a Log2Fold
440 change > |2.5|. These pathways included the Fc gamma R-mediated phagocytosis and
441 GnRH signalling pathway in orphans and the isoflavonoid biosynthesis in offspring that
442 had lived with their mother (Figure 5E).

443

444 ## Discussion

445 The gradual and subtle changes that hemimetabolous juveniles undergo during
446 moulting are often thought to limit profound changes in their microbiota during
447 development (Carrasco and Pérez-Cobas, 2014; Manthey *et al.*, 2022). Our data show
448 that this assumption is not always true, as these changes occur in the European
449 earwig. Using 16S rRNA metabarcoding on 218 samples from egg to adult stages, we

450 found that the microbiome of earwig offspring shows substantial variation throughout
451 their development both in terms of beta diversity (thereafter called “structure”) and
452 alpha diversity (thereafter called “diversity”). Interestingly, these changes did not occur
453 during moulting, but rather between the beginning and end of certain developmental
454 stages. In addition, we found that maternal care partly shapes the microbiome of
455 offspring, even if this behaviour is facultative in the European earwig. Access to
456 maternal care during the first few weeks after hatching affected not only the
457 microbiome of first instar nymphs during family life, but also that of the resulting adults
458 two months after family life has ended.
459
460 **Offspring microbiome varies during development**
461 Our data first reveal that the microbiome diversity (alpha) and structure (beta) of earwig
462 changed during offspring development. This is striking for two main reasons. First,
463 these changes were not linear and only occurred at certain stages of offspring
464 development, which contrasts with the general pattern where microbiome diversity
465 gradually increases with natural growth in offspring body size (Sherrill-Mix *et al.*, 2018).
466 Second, some of these changes occurred in the second and third nymphal instars,
467 even though all instars were fed the same diet and developed in the same laboratory
468 conditions throughout their development.
469 The first marked change in microbiome structure occurred shortly after the
470 nymphs hatched. We showed that both eggs and newly hatched nymphs had a
471 microbiome structure different from that of all other stages. From the egg stage to the
472 freshly moulted 2$^{nd}$ instar nymphs, we observed an increase in diversity due to the
473 gradual colonization of the 1$^{st}$ instar nymphs by new genera, including *Erwinia,*
474 *Raoultella, Flavobacterium* and *Lactobacillus*. These new genera are present in a wide

475 range of insects, where they are often known to perform beneficial functions in
476 juveniles (Malacrinò, 2022). For instance, *Erwinia* is known to reduce the maturation
477 time of the bark beetle *Ips typographus* (Peral-Aranega *et al.*, 2023).

478 The second marked change in microbiome diversity occurred during the
479 development of the 2nd instar nymphs. We found that the diversity drops to its lowest
480 level during the transition from newly moulted to old 2nd instar nymphs and remains
481 low in the newly moulted 3rd instar nymphs (Figure 3A). This fall is reflected by the loss
482 of several genera, including *Smaragdicoccus, Rhodococcus* or *Alkanindiges* (Figure
483 4). The bacterial communities of the newly moulted 3rd instar nymphs are very different
484 from those of any other stage, because of its structural monotony where *Lactobacillus*
485 largely dominates the bacterial composition (Figures 1, 4). Such a decrease in
486 microbiome diversity has been reported in the 3rd instar larvae of the German
487 cockroach *Blattella germanica* (Carrasco and Pérez-Cobas, 2014), where it has been
488 suggested to result from the physiological state of the host at that particular stage
489 (Kirkland *et al.*, 2020). In lower termite workers, the gut flagellates are totally lost prior
490 the ecdysis, probably due to a combination of host starvation and hormonal variation
491 linked to changes in the host social status (Cleveland, 1949; Raina *et al.*, 2008; Nalepa,
492 2017). This may also be the case with earwigs, although our knowledge of the
493 physiological and hormonal peculiarities of each instar is still very limited (Meunier,
494 2024). This decrease may also result from the end of mother-offspring interactions, as
495 we removed mothers from their nymphs 14 days after hatching, which is often shortly
496 after they have reached the 2nd instar (Thesing *et al.*, 2015). This end of family life
497 inherently means that any potential form of coprophagy and trophallaxis between
498 mother and offspring (and thus potential bacterial vertical transmission) ceases, and
499 the juveniles begin to process the food source exclusively on their own. Whether

500 physiological peculiarities of 2nd instar nymphs, suppression of maternal bacterial
501 transfer and/or changes in dietary habits are the drivers of the reported changes in the
502 microbiome diversity remains to be further investigated.

503 The last marked change in microbiome diversity was an increase during the
504 development of the 3rd instar nymphs, mostly explained by the colonization of new
505 genera, such as *Nubsella, Massilia, Gemmobacter* or *Chitinophaga.* These genera are
506 common colonizers of insect guts (Da Silva Correia *et al.*, 2018; Guégan *et al.*, 2018;
507 Paddock *et al.*, 2022) but can also be found in the environment (Mayoral-Peña *et al.*,
508 2022). For example, bacteria of the genus *Nubsella* are mutualistic with certain
509 phytophagous insects and are likely to be important for the fitness of walking sticks (Lü
510 *et al.*, 2019; Li *et al.*, 2020). Interestingly, the genus *Chitinophaga* is known to be
511 involved in the degradation of chitin, a major component of the insect skeleton (Glavina
512 Del Rio *et al.*, 2010). Whether their presence is a mutualistic association with earwigs
513 aiding in cuticle digestion during cannibalism or during the reingestion of their own
514 exuviae is an open question and will be developed below.

515

516 **Moulting does not alter microbiome diversity**

517 Moulting did not induce any changes in the microbiome diversity and structure of
518 earwig offspring, except for the moults between the 3rd and 4th instar nymphs. This
519 overall lack of moulting effect is in line with studies carried out in other hemimetabolous
520 insects showing that the gut microbiome remains stable during development (Manthey
521 *et al.*, 2022). For earwigs, we propose several possible explanations. First, nymphs
522 could possess a microbial reservoir that is preserved during moulting. Such a reservoir
523 is present, for example, in the bean bug *Riptortus clavatus* to maintain their
524 *Burkholderia* symbionts during moult (Kikuchi and Yumoto, 2013). However, the

525 presence of such a reservoir has never been documented in earwigs. Another
526 possibility is that earwig nymphs re-inoculate themselves with their own bacteria by
527 eating their shed cuticle after moulting, as reported in cockroaches (Mira, 2000). Even
528 if earwig nymphs are regularly observed eating their shed cuticle during laboratory
529 rearing (J Meunier, pers. obs), the role of this behaviour to inoculate a lost microbiome
530 has never been investigated in this species. Finally, earwig nymphs may be able to re-
531 inoculate themselves through their frequent (in)direct contact with their siblings by
532 means of coprophagy and trophallaxis (Falk *et al.*, 2014; Kramer and Meunier, 2016).
533 These social acquisitions allow the persistence and the (re)acquisition of lost bacteria
534 in numerous arthropods, such as termites (Raina *et al.*, 2008; Michaud *et al.*, 2020),
535 cockroaches (Nalepa, 2020), and spiders (Rose *et al.*, 2023). Overall, these data call
536 for future experiments to disentangle which parameter explains the resistance of the
537 earwig microbiome to moulting, and to understand why they are no longer efficient for
538 the moult between the 3rd and 4th instar nymphs.
539
540 **Bacterial functions could play a role throughout juvenile development**
541 The reported changes in the structure of the offspring microbiome during development
542 are associated with changes in the predicted potential functions of their bacteria that
543 could be beneficial to the host. For instance, many of the bacteria overrepresented in
544 eggs and newly hatched nymphs are known to produce and accumulate lipids such as
545 *Rhodococcus, Delftia* and *Pedobacter* (Alvarez *et al.*, 1997; Liu *et al.*, 2016; Franks *et*
546 *al.*, 2021). As completing the transition from eggs to nymphs is a highly energetic
547 process, our data suggest that earwig embryos may not only obtain this energy from
548 egg lipid reserves, but also from these bacteria (Ziegler and Vanantwerpen, 2006;
549 Diether and Willing, 2019). Moreover, this would be consistent with the predictions of

550 PICRUST2, which show a strong positive correlation in lipogenesis processes such as
551 adipocytokine signalling pathway and unsaturated fatty acid biosynthesis (Figure 6).
552 The bacterial loss observed in the third nymph instar was also found in terms of
553 predicted functions, as all pathways except cell developmental pathways, amino acids
554 and sugar metabolism were underrepresented in this developmental stage compared
555 to the subsequent ones. Finally, the acquisition of new genera such as *Nubsella,*
556 *Massilia, Gemmobacter* or *Chitinophaga* during the development of the third nymphal
557 instar came with new predicted functions linked to energy uptake, amino acids and
558 vitamins B biosynthesis, which act as a coenzyme in numerous pathways involved in
559 the fatty acid synthesis, glucogenesis or amino acids synthesis. These vitamins are
560 essential during insect development, but they cannot be synthetized by animals
561 themselves and are often acquired through alimentation or provided by the microbiota
562 (Douglas, 2017; Kinjo *et al.*, 2022). Although these functions may provide insights into
563 our general understanding of the driver of microbiome changes during offspring
564 development, they must be considered with caution as the validity of the approach is
565 debatable due to the short length of the amplicons and the lack of genome reference
566 concerning insect-associated microbial communities (Djemiel *et al.*, 2022). Future
567 studies are thus needed to confirm these potential functions in earwigs, for example
568 using transcriptomic analyses.
569

570 **Maternal care shapes the microbiomes structure of offspring**

571 In animals, maternal care is often considered to be an important mediator of microbial
572 transmission from parents to offspring (Bright and Bulgheresi, 2010; Sachs *et al.*, 2011;
573 Hosokawa and Fukatsu, 2020). Our data suggests that the European earwig is no
574 exception. Access to maternal care not only shapes the microbiome structure,

575 phylogenetic diversity and functions of 1st instar nymphs, but also those of the resulting
576 adults, even though none of these adults had any contact with their mothers in the
577 previous two months. We found that the microbiome of nymphs with maternal care
578 contained a higher diversity of phylogenetically distant bacteria compared to their
579 orphaned counterparts. Not surprisingly, the bacteria found in these nymphs were also
580 found in the microbiome of their mothers (including *Raoultella*, *Rurimicrobium* and
581 *Sphingobacterium*). This is likely due to direct or indirect maternal transmission during
582 post-hatching family life. However, even if maternal transmission contributes to the
583 shape of the microbiome, the non-significant effect of maternal care on alpha diversity
584 in terms of taxonomic richness, Shannon entropy or Allen index, combined with the
585 small, albeit significant effect on microbial structure, suggests that maternal presence
586 is not the main route of bacterial acquisition in this species. Indeed, we also found an
587 overabundance of some ASVs in the orphan offspring, such as sequences related to
588 *Comamonas*, *Lactobacillus* or *Serratia*. Since these bacteria are often generalists,
589 associated with laboratory rearing conditions (Malacrinò, 2022), and common in the
590 mothers tested, they are likely to come from the rearing environment. However, their
591 overabundance in orphaned nymphs suggests that they were outcompeted by
592 maternally transmitted bacteria in non-orphaned nymphs. In addition to these
593 differences, we found one major discriminant (potential) function in the tended nymphs,
594 related to isoflavonoid biosynthesis, and two in the orphan nymphs, related to the
595 endocrine and immune systems. These predicted functions are consistent with
596 previous phenotypic studies showing that orphaned nymphs develop faster to adults,
597 produce larger adults with longer male appendages, but contradict with other studies
598 showing that orphaning has limited long-term effects on the basal immunity of the
599 nymphs and resulting adults (Meunier and Kölliker, 2012; Thesing *et al.*, 2015;

600 Vogelweith *et al.*, 2017; Körner *et al.*, 2020). Here again, these predicted functions
601 need to be taken with caution. Whether all or some of these offspring phenotypes are
602 indeed due to maternally derived bacteria remains to be further explored, *e.g.* with the
603 use of gnotobiotic lineages.
604

605 **Conclusion**

606 Overall, we showed that the European earwig microbiome changes multiple times
607 during offspring development. Interestingly, these changes were independent of
608 moulting. The fact that moulting did not induce any purge or shift in the bacterial
609 communities of the nymphs calls for future studies to test whether this is due to the
610 presence of a bacterial reservoir, moult consumption and/or social interactions with
611 siblings. Our data suggest that the predicted functions of some components of these
612 microbiomes are relevant to the developmental stage at which they occur, such as
613 lipogenesis or steroid synthesis in early stages, and nutrient and vitamin synthesis in
614 late stages. However, future studies are required to confirm the functional role of the
615 microbiome changes in this species. Finally, we showed that maternal care is an
616 important short- and long-term determinant of the offspring microbiome. Given that
617 earwig nymphs do not require maternal care to develop and survive (Kölliker, 2007;
618 Thesing *et al.*, 2015; Kramer and Meunier, 2016) and that nymph can also develop in
619 absence of any social interactions (Van Meyel and Meunier, 2022), our results call for
620 future studies to investigate the role of these socially-acquired bacteria (and other
621 potential members of their microbiota such as fungi, viruses and other microorganisms)
622 in the biology of the European earwig and, more generally, in the early evolution and
623 maintenance of facultative family life in insects (Archie and Tung, 2015; Körner *et al.*,
624 2023).

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## Author Contributions

All authors contributed to the study conception. The manuscript was written by M-C Cheutin and J. Meunier; Analyses were carried by M-C Cheutin and J. Meunier; Molecular analyses, sampling and animal rearing were performed by M-C Cheutin and M. Boucicot. All authors read and approved the final version of the manuscript.

## Data Availability

The data set and scripts are available on Zenodo with the DOI https://zenodo.org/doi/10.5281/zenodo.10776543. Libraries for each sample are deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession no. PRJNA1066258.

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## Conflict of interest

We declare that our work does not have any conflict of interest.

## Ethics approval statements

Our investigation complies with the current European Directive 2010/63/EU that does not acquire ethical approval on invertebrates. All animals were handled with care until necessary sacrifices.

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945

946 **Table 1:** Effect of developmental stage (from eggs to adults) on microbiome alpha and
947 beta diversity at the bacterial (white) and functional (grey) levels. Significant p-values
948 (P <.005) are indicated in bold.
949

| | Alpha diversity | | | | Beta diversity | | | |
|---|---|---|---|---|---|---|---|---|
| | **Index** | **$R^2$** | **$Chi^2$** | **P** | **Distance** | **$R^2$** | **F** | **P** |
| Bacteria (ASVs) | Obs. richness | .436 | 87.23 | **<.001** | Jaccard | .074 | 1.94 | **<.001** |
| | Shannon | .335 | 59.00 | **<.001** | Bray-Curtis | .082 | 2.50 | **<.001** |
| | Faith | .398 | 71.13 | **<.001** | Un. Unifrac | .065 | 2.73 | **<.001** |
| | Allen | .335 | 78.57 | **<.001** | W. Unifrac | .103 | 4.82 | **<.001** |
| Functions (KOs) | - | - | - | - | Jaccard | .092 | 3.42 | **<.001** |
| | - | - | - | - | Bray-Curtis | .089 | 3.54 | **<.001** |

950

951 **Table 2:** Effect of access to (A) maternal care during family life and (B) offspring age

952 on microbiome alpha and beta diversity at bacterial (white) and functional (grey) levels.

953 Significant p-values (P <.005) are indicated in bold.

954

| | Alpha diversity | | | | Beta diversity | | | |
|---|---|---|---|---|---|---|---|---|
| | Index | $R^2$ | $Chi^2$ | P | Distance | $R^2$ | F | P |
| **(A) Maternal care** | | | | | | | | |
| Bacteria (ASVs) | Obs. richness | .134 | 1.86 | .172 | Jaccard | .023 | 2.50 | **<.001** |
| | Shannon | .087 | .980 | .323 | Bray-Curtis | .028 | 3.28 | **<.001** |
| | Faith | .234 | 8.15 | **.004** | Un. Unifrac | .074 | 9.79 | **<.001** |
| | Allen | .095 | 2.80 | .093 | W. Unifrac | .037 | 5.13 | **.003** |
| Functions (KOs) | - | - | - | - | Jaccard | .035 | 4.13 | **.009** |
| | - | - | - | - | Bray-Curtis | .040 | 4.82 | **<.001** |
| **(B) Nymphs vs Adults** | | | | | | | | |
| Bacteria (ASVs) | Obs. richness | .134 | 6.31 | **.012** | Jaccard | .052 | 5.68 | **<.001** |
| | Shannon | .137 | 6.05 | **.014** | Bray-Curtis | .077 | 9.07 | **<.001** |
| | Faith | .209 | 4.15 | **.042** | Un. Unifrac | .108 | 14.16 | **<.001** |
| | Allen | .181 | 12.52 | **<.001** | W. Unifrac | .178 | 24.82 | **<.001** |
| Functions (KOs) | - | - | - | - | Jaccard | .083 | 9.72 | **<.001** |
| | - | - | - | - | Bray-Curtis | .084 | 10.06 | **<.001** |

955

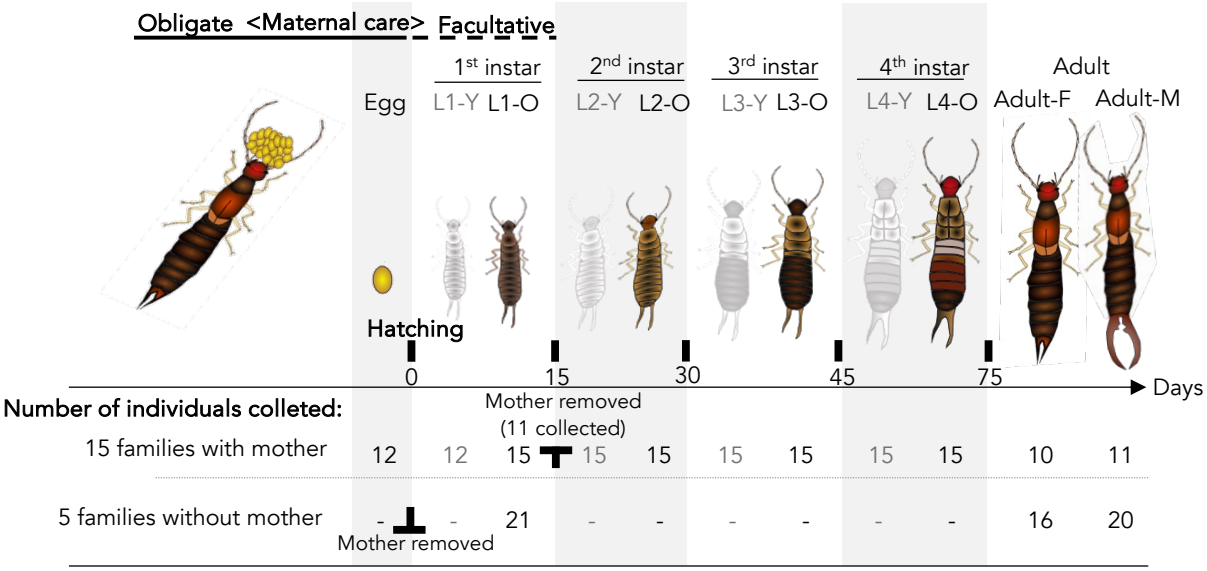


**Figure 1: Overview of the experimental design.** All developmental stages from eggs to adults were sampled for microbiome analysis before (coloured specimens) and after (white specimens) each moult. Note that the L0 developmental stage was not sampled as it moults during egg hatching (Tourneur *et al.*, 2020). Although it is facultative, post-hatching maternal care usually continues until the nymphs reach the 2nd instar. We collected a total of 150 samples across all developmental stages in families with post-hatching maternal care, and 58 samples from the 1st instar and adults in families without maternal care.

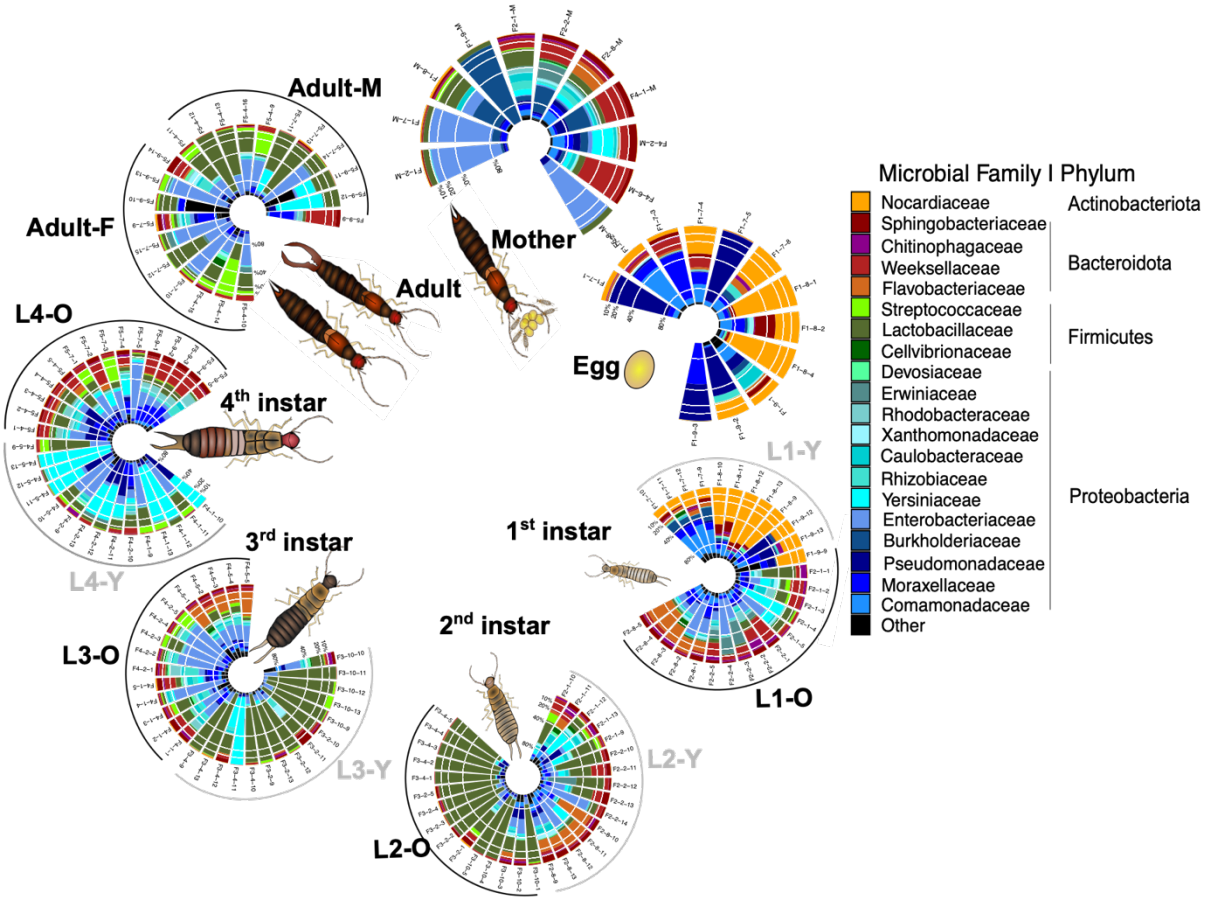


**Figure 2: Composition of the European earwig core microbiome**. **A)** Individual core microbiome at family scale, ordered by relative importance, grouped, and coloured by bacterial family. Specimens are ordered according to their developmental stage with freshly moulted young nymphs (grey) on one side of the circle, and old nymphs (black) on the other side.

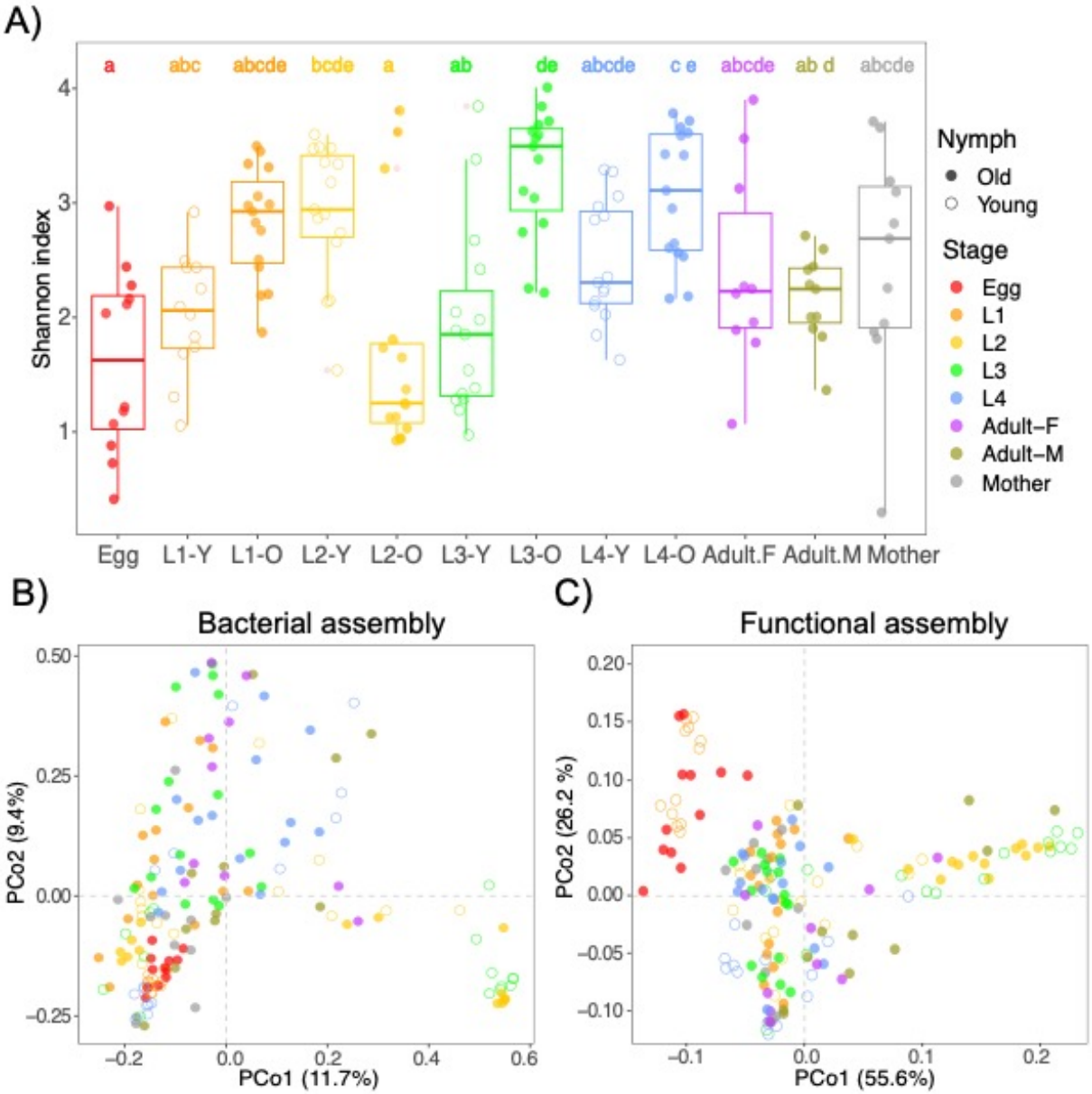


**Figure 3: Microbiome variation during offspring development.** The diversity is compared before and after moults (circle fill), between adult males (Adult-M) and females (Adult-F) and between each established stage (coloured boxes). **A)** Shannon index comparisons for bacterial alpha diversity. Contrasts are provided after pairwise comparisons of the marginal means of the linear model with the Shannon index value as response variable, the sampling stage as explanatory variable, and the clutch as random variable. Letters above the boxes indicate significant grouping contrast for emmeans (LR $\chi^2_{11} = 59.70$, $P = 10^{-8}$; Significant contrasts for emmeans $P \leq 0.05$) (see

981 the Supplemental File and Figure S4 for the estimated marginal means comparisons
982 and the other indices). Boxplots represent the median (middle bar) and the interquartile
983 range (box) with whiskers representing the 1.5-fold the interquartile range. Dots
984 represent the value for an individual (with red dot are outliers). **(B-C)** Principal
985 coordinates analyses (PCoA) plots illustrating Bray-Curtis distances calculated
986 between samples based on their ASVs and their KOs compositions that both vary
987 between nymph stages (respectively, $R^2 = 0.082$, $P = 0.001$ and $R^2 = 0.089$, $P = 0.001$,
988 see the Supplemental File and Figure S5 for the pairwise comparisons between stages
989 and the other indices). Visualization in two dimensional dimensions where dots
990 represent each host microbial composition coloured by its nymph stage and adult sex
991 while the fill represents the moulting event.
992

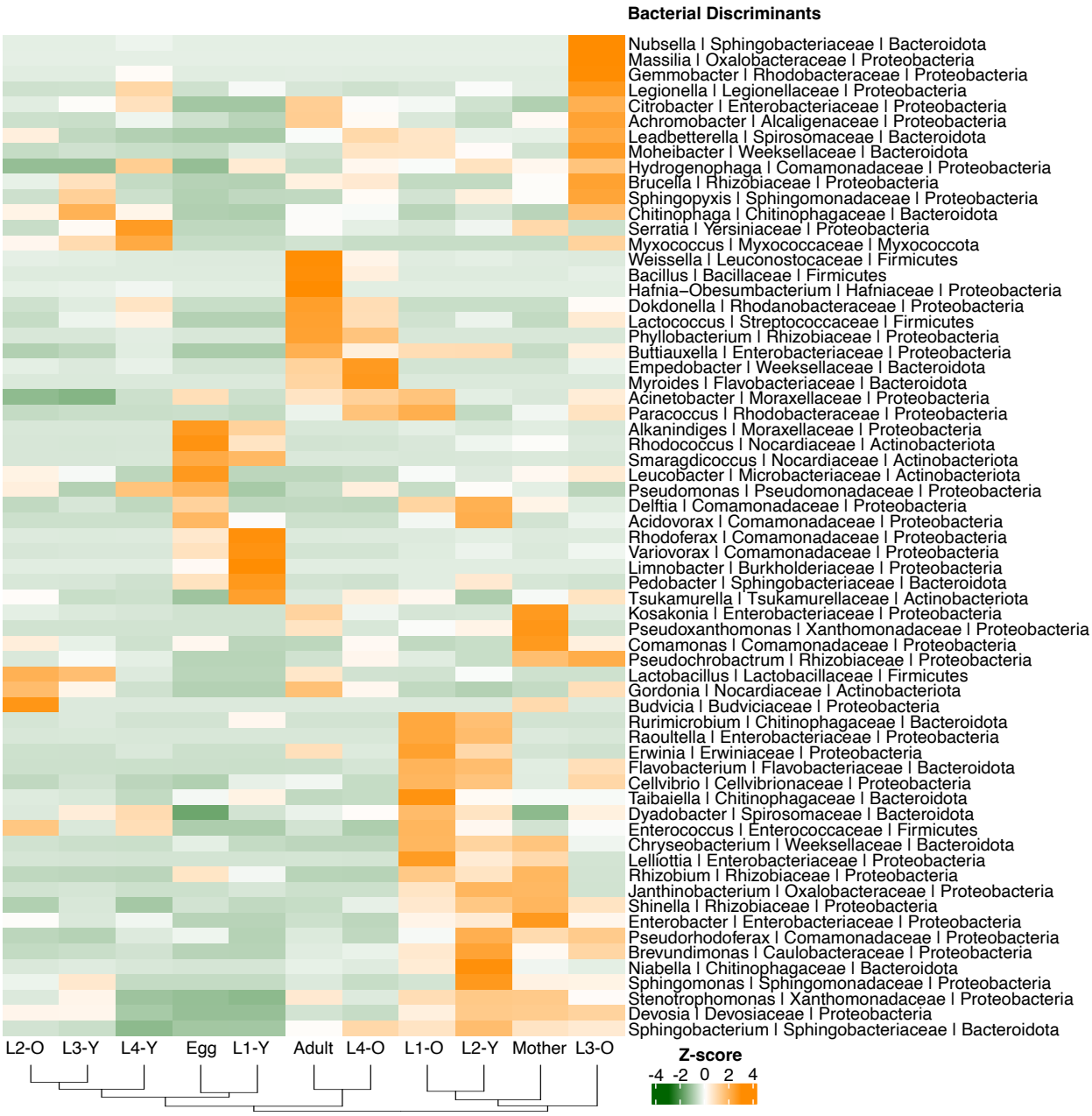


**Figure 4: Heatmaps of hierarchical clustering based on the 62 genera indicators of developmental stages in the European earwig.** Raw represent the Z-score associated to the bacterial genus in each sampling stage.

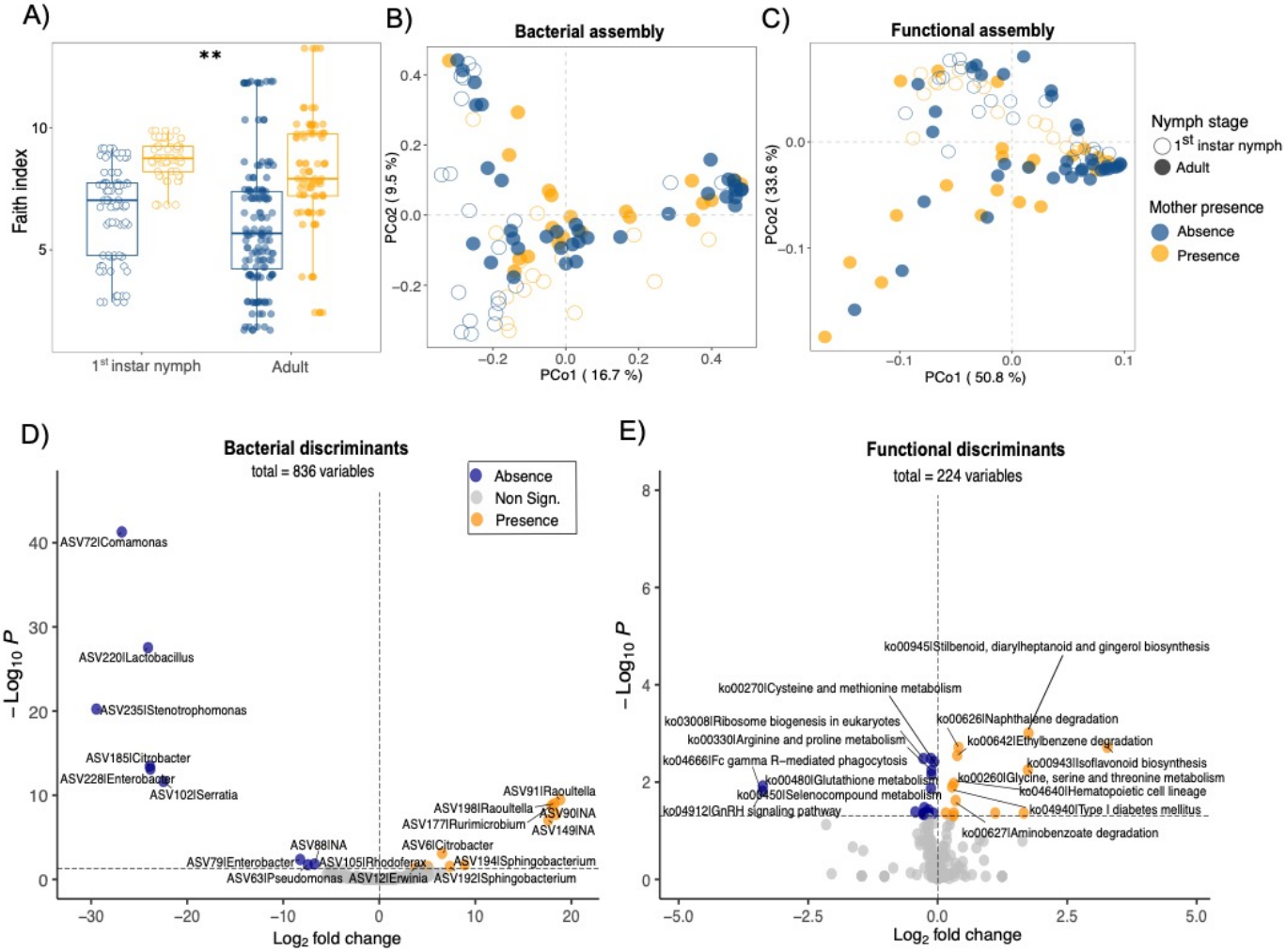


**Figure 5: Effect of maternal presence (orange) or absence (blue) on the microbiome of 1st instar nymphs (empty circles) and adults (fill circles). (A)** Alpha diversity is represented with the phylogenetic diversity index of Faith. **(B, C)** Beta diversity is showed through a two-dimensional PCoA according to the Bray-Curtis distances between microbiome sample pairs for bacterial and functional assemblies. (**C** , **D**) Volcano plots representing the 19 ASVs and the 33 KEGG pathways discriminating the absence (blue) or presence (orange) of mothers during family life. Boxplots represent the median (middle bar) and the interquartile range (box) with whiskers representing the 1.5-fold the interquartile range. ** < 0.01.

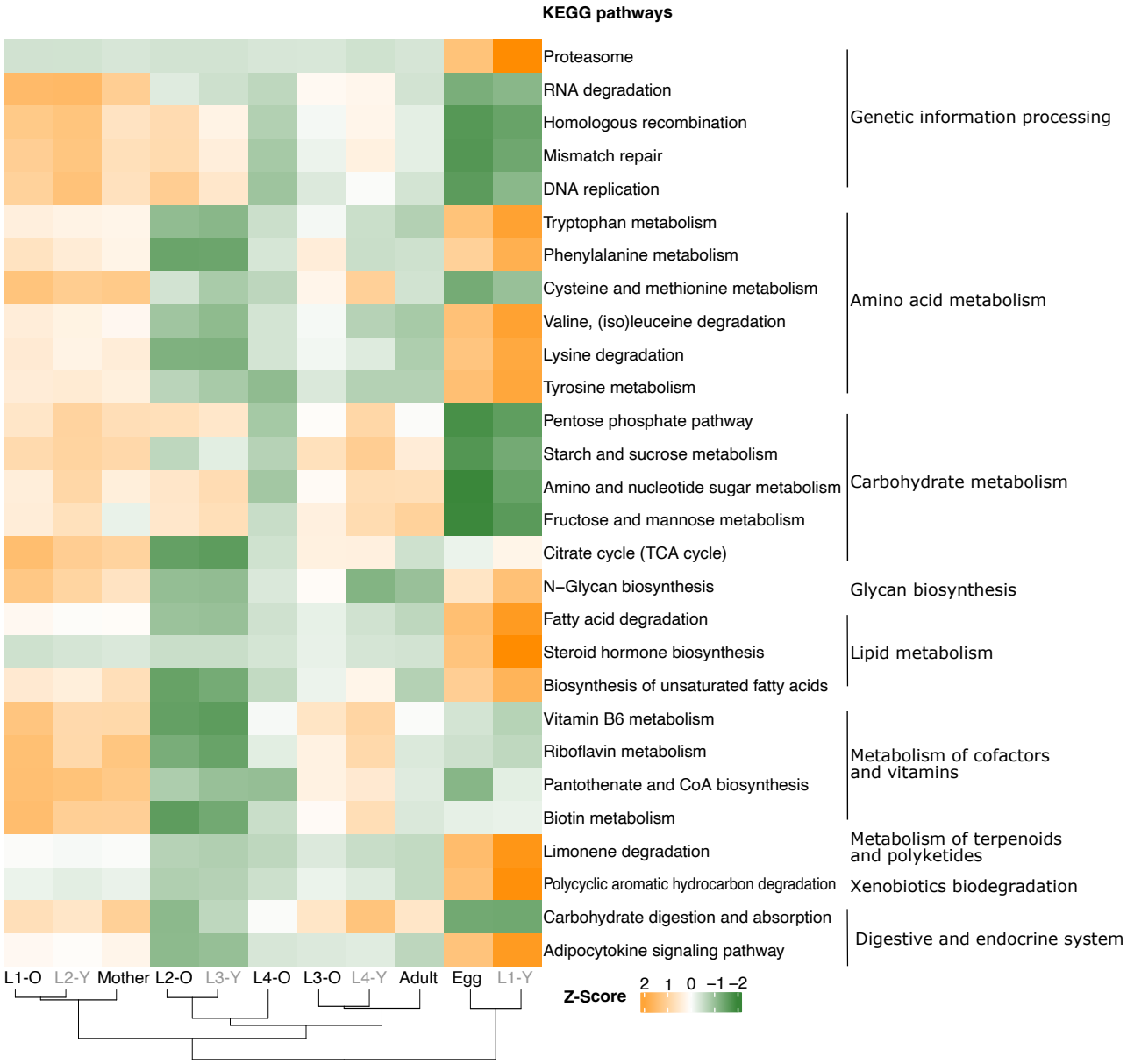


**Figure 6: Heatmaps of hierarchical clustering based on the KEGG pathways indicators of developmental stages in the European earwig.** Raw represent the Z-score associated to the pathway name in each sampling stage (young-nymphs are coloured in grey).