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

Marie-Charlotte Cheutin[1], Manon Boucicot[1], Joël Meunier[1]

[1] Institut de Recherche sur la Biologie de l'Insecte, UMR 7261, CNRS, University of Tours, Tours, France

Corresponding author: MC Cheutin, mccheutin@gmail.com

J Meunier ORCID #0000-0001-6893-2064

MC Cheutin ORCID #0000-0001-7711-7512

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

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

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

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

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

under a 12:12 light:dark photoperiod, and received the laboratory-prepared food twice a week (see above).

Experimental design and sample collection

Overall, we analysed the microbiome of 218 samples collected throughout earwig development (Figure 1, Table S1).

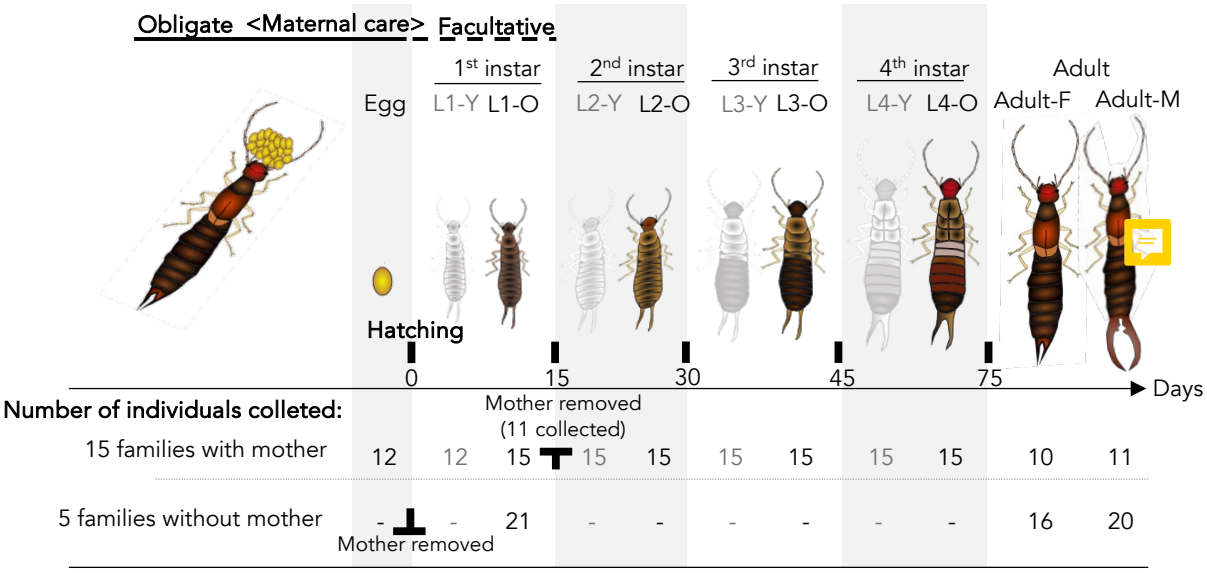


Figure 1: Overview of the experimental design. All developmental stages from eggs to adults were sampled for microbiome analysis before (coloured specimens) and after (white specimens) each moult. Note that the L0 developmental stage was not sampled as it moults during egg hatching (Tourneur *et al.*, 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.

As ecdysis is known to induce a microbial shift in many insect species, we sampled all developmental stages (except eggs and adults) both immediately after the moult (freshly moulted) and several days after the moult (old moulted). Freshly moulted

nymphs have a white (compared to dark) colour, which typically lasts for maximum 3h in early instar nymphs and up to 6h in late instar (MC Cheutin, pers. obs.). For the 15 clutches with maternal care, we obtained a total of 12 egg samples (Egg), 37 first instar nymphs of which 12 were freshly moulted (later called L1-Y) and 15 were old moulted (L1-O), 30 second instar nymphs of which 15 were freshly moulted (L2-Y) and 15 were old moulted (L2-O), 30 third instar nymphs of which 15 were freshly moulted (L3-Y) and 15 were old moulted (L3-O), 30 fourth instar nymphs of which 15 were freshly moulted (L4-Y) and 15 were old moulted (L4-O), and finally 32 adults of which 11 field-sampled mothers (Mother), 10 adult female offspring (Adult-F) and 11 adult male offspring (Adult-M). For the 5 clutches without maternal care, we only used a total of 21 old moulted first instar nymphs, 16 adult females and 20 adult males as we were only interested in the short and long-term effect of maternal presence/absence on offspring s (Figure 1, see details in Table S1). Note that the first observable developmental stage of the nymphs is called L1, as the moult that occurs at hatching is called L0 in this species (Tourneur *et al.*, 2020). Moreover, the sex of an individual can only be determined in adults, where the shape of the forceps is straight in females and curved in males. Each sample was collected individually and immediately transferred to Eppendorf tubes at -80°C until DNA extraction.

Genomic extraction and 16S amplification

Total genomic DNA was extracted using the NucleoMag® Tissue extraction kit (Macherey-Nagel™, Düren, Germany) and the V3-V4 region of the 16S rDNA gene was amplified with the prokaryotic primers 343F (5′- ACGGRAGGCAGCAG – 3′) and 784R (5′- TACCAGGGTATCTAATC – 3′) (Muyzer *et al.*, 1993) coupled with platform-specific Illumina linkers. We performed PCR reactions using the Taq Polymerase

Phusion® High-Fidelity PCR Master Mix with GC buffer and prepared them according to the manufacturer's instructions (Qiagen, Hilder, Germany). PCR amplification steps involved an initial denaturing step for 30 sec at 98°C, followed by 22 amplification cycles (denaturation for 10 sec at 98°C; annealing for 30 sec at 61.5°C; extension for 30 sec at 72°C), and ended by a final extension step of 10 min at 72°C. Electrophoresis migration was run to check the probe specificity and amplification success. Extraction and amplification steps involved several blank controls to confirm that samples were not contaminated by environmental microorganisms. Samples were amplified in duplicates and equally pooled for a final product of 30 µL further sequenced with 2x250bp Illumina MiSeq technology at the Bio-Environnement platform (University of Perpignan, France). As the blanks were all negative, we did not send them for sequencing.

Bioinformatic process

The obtained libraries were trimmed and filtered using the quality profiles from the DADA2 algorithm v1.24.0 (Callahan *et al.*, 2016), cleaned for errors, dereplicated and inferred towards Amplicon Sequence Variants (ASVs) (Glassman and Martiny, 2018). Chimeras were removed and taxonomy assignment was performed on a count table where forward and reverse ASVs were merged and pooled, using the SILVA reference database (release 138) (Quast *et al.*, 2012). The multiple alignment of the sequences was provided with the MAFFT program (Katoh, 2002) and we inferred the phylogenetic tree with the FastTree 2 tool (Price *et al.*, 2010) and Phangorn package v2.8.1 (Schliep, 2011). The table was then transformed into a phyloseq object using the phyloseq package v1.40.0 (McMurdie and Holmes, 2013) of which we removed the 529 sequences of mitochondrial origin and the 30 800 unknown sequences. As the 2 700

Eukarya sequences had no affiliation at Phylum level and were sparsely distributed, they were also removed. Finally, we obtained a final dataset constituted by 4 163 989 sequences (3 780 ASVs) ranging from 3 570 to 32 946 sequences/sample.

Identification of the core microbiomes

We defined the core microbiome as the set of microbial taxa (*i.e.,* ASVs) that are characteristic of all samples. We obtained this core microbiome using the species abundance distribution (SAD) patterns of each ASVs (Magurran and Henderson, 2003). This approach is frequently used in the literature (Fillol *et al.*, 2016; Jeanbille *et al.*, 2016; Cheutin *et al.*, 2021; Neu *et al.*, 2021) and allows us to distinguish between core and satellite ASVs, while avoiding the use of subjective and arbitrary occurrence and abundance thresholds (Magurran and Henderson, 2003). We first calculated an index of dispersion (*i.e.,* the variance to mean ratio, VMR) for each ASV within each developmental stage. We then tested whether these indices followed a Poisson distribution, falling between the 2.5 and 97.5% confidence limits of the Chi^2 distribution (Krebs, 1999). ASVs with index values outside these confidence limits were considered as part of the core microbiome, while the others were considered as satellite ASVs. This process provided us with stage-dependent core microbiomes for the eggs, each instar nymph stages, adult offspring and mothers, and we finally merged all these cores to obtain a global core microbiome (Figure S1).

Functional predictions of the core microbiomes

We used the PICRUST2 algorithm (Douglas *et al.*, 2020) to predict the potential functions associated with each of the core microbiomes generated above. In brief, the

tool inserts each ASV sequence into a reference tree (EPA-ng, Barbera *et al.*, 2019) using a hidden-state prediction (HSP, castor, Louca and Doebeli, 2018) and infers a KEGG ortholog function (later named KOs) based on the functional profiles obtained with the nearest-sequenced taxon (MinPath, Ye and Doak, 2009). The functional table counts with KOs obtained for each sample are count-normalized for the ASVs copy counts and multiplied by the gene content prediction resulting from the HSP algorithm. Finally, a table with three levels of KEGG pathways is constructed according to the MetaCyc database v27.1 (Caspi *et al.*, 2020).

Evolution of bacterial diversity during offspring development

To test how the microbiomes of offspring changed throughout host development, we calculated the alpha and beta diversities of each sample using qualitative and quantitative indices for both taxonomic and phylogenetic diversity. Regarding alpha diversity, which was only calculated for ASVs (not for functions), we normalized all samples at the minimum sampling size of sequences per individual and checked their accuracy with rarefaction curves (Cameron *et al.*, 2021; Figure S2). For alpha diversity proxies, we used direct observed richness for the qualitative taxonomic richness and the Shannon entropy for its quantitative equivalent. We calculated their phylogenetic equivalents with the Faith and the Allen's indices (Chao *et al.*, 2010). For beta diversity, we calculated the relative abundance of each ASV and KOs by sample, and we calculated the Jaccard distance between samples with ASVs (and KOs) presence/absence and its quantitative equivalent Bray-Curtis. For ASVs, we also used their phylogenetic equivalents with the Unifrac metrics for qualitative (unweighted) and quantitative (weighted) distances (Chao *et al.*, 2010; Yang *et al.*, 2021).

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

Impact of maternal presence on offspring bacterial diversity

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

Statistics

We first tested the effect of developmental stage and moult (fresh versus old moult) on the microbiome diversity of offspring from the 15 families in which nymphs were kept with a mother during family life. For alpha diversity, we conducted four linear mixed models (LMM) in which we entered each of the four microbial alpha diversity indices as a response variable, the sampling time (Egg, L1-Y, L1-O, L2-Y, L2-O, L3-Y, L3-O, L4-Y, L4-O, Adult-F, Adult-M and Mother) as the explanatory variable and the clutch

used as a random effect to control for non-independence of the biological samples. We did not use a classical 2-way ANOVA approach because the two levels of moult were not available for each developmental stage (*i.e.,* eggs and adults). We conducted pairwise comparisons between each sampling time using the estimated marginal means of the models, with P values corrected for multiple testing using Tukey methods with the *emmeans* package (Lenth, 2022). For each pairwise comparison, we also calculated R^2 using the *MuMIn* package (Bartoń, 2022). For beta diversity, the dissimilarity based on the ASVs and KOs assemblies between each sample was first illustrated in a two-dimensional Principal Coordinates Analyses (PCoA). We then performed Permutational Analyses of Variances (PERMANOVAs) to test the effect of the clutch (as randomized block), the sampling stage and the sex on each distance matrices based on bacterial composition (*i.e.,* Jaccard, Bray-Curtis, weighted and unweighted Unifrac) and on functional predictions (*i.e*., Jaccard and Bray-Curtis). Post-hoc pairwise tests between stages were performed for each dissimilarity matrix with the package *pairwiseAdonis* v0.4.1 (Martinez Arbizu, 2017). These pairwise comparisons allowed us to address five questions, namely whether (1) microbiome diversity changes between each successive development stage (*i.e.,* Egg *vs* L1-Y, L1-Y *vs* L1-O, L1-O *vs* L2-Y, etc), (2) moulting causes a shift in microbiome diversity between two successive developmental stages (*i.e.,* Egg *vs* L1-Y, L1-O *vs* L2-Y, etc), (3) old nymphs exhibit an instar-specific microbiome diversity (*i.e.,* Egg *vs* L1-O, L1-O *vs* L2-O, L2-O *vs* L3-O, etc), (4) freshly moulted nymphs exhibit an instar-specific microbiome diversity (*i.e.,* L1-Y *vs* L2-Y, L2-Y *vs* L3-Y, etc) and finally, whether (5) adults offspring exhibit a sex-specific microbiome diversity (Adult-M *vs* Adult-F).

We tested whether maternal presence had short and/or long-term effects on the microbiome diversity of their offspring using the 15 families with mothers and the 5

families without mothers. For alpha diversity, we conducted four LMM in which we entered each of the index values as a response variable, as well as maternal presence (yes or no), the developmental instar (L1-O or adult) and the interaction between these two factors as explanatory variables. When the latter was non-significant, we removed it after model simplification by AIC comparison. We also entered the clutch as a random effect. For beta diversity, we repeated the approach detailed above by testing the effect of the clutch and the presence/absence of a mother in interaction with the stage (L1-O or adult), on both microbial and functional composition.

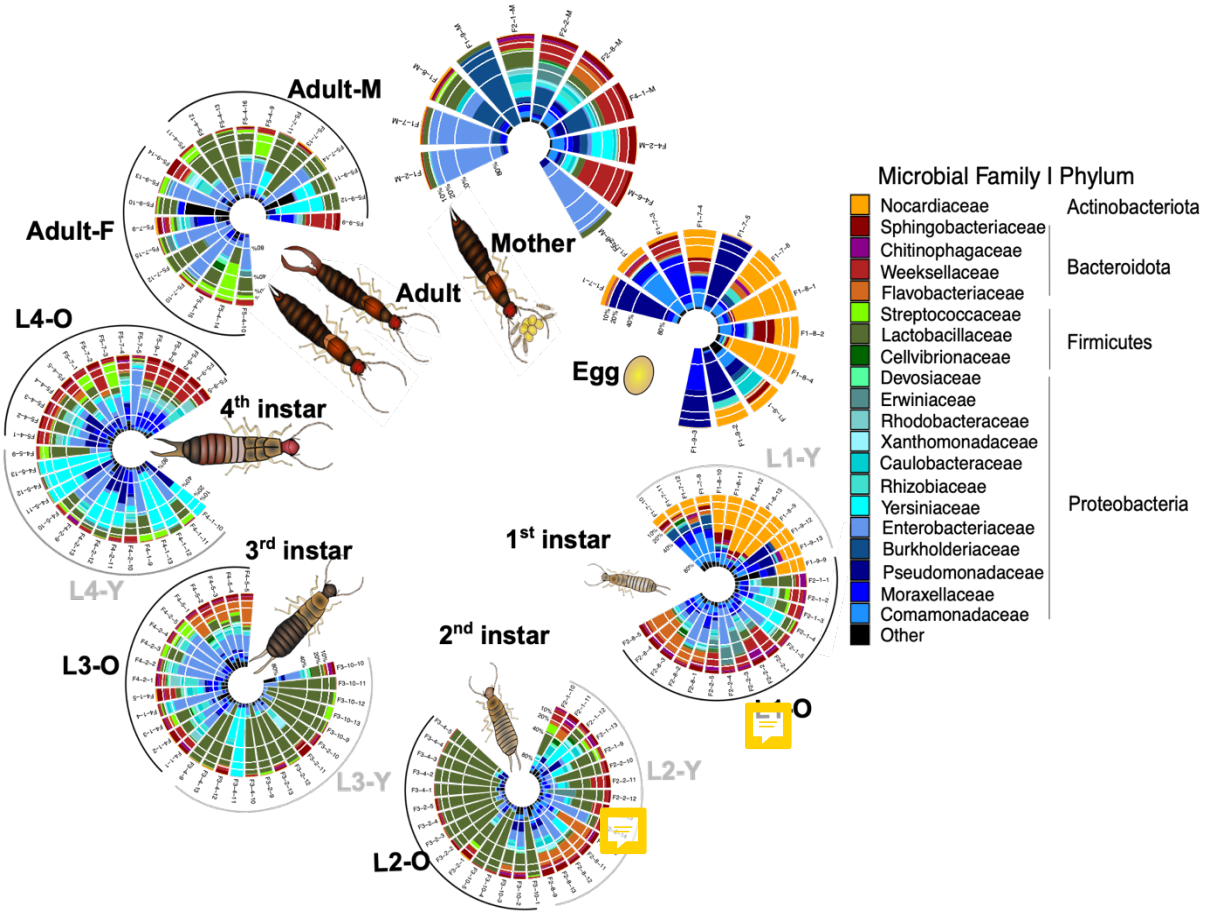
We performed all statistical analyses using R v4.2.0 (R Core Team, 2022). We checked all model assumptions with the *DHARMa* package (Hartig, 2022). We verified variance homoscedasticity between groups by comparing the distance dispersion within group with the *betadisper* function (all $P > 0.05$).

Results

Microbiome changes during offspring development (with mothers)

Description of the core microbiome

In the pool of the 218 earwig samples, we detected a total of 915 ASVs core (24.21% of the initial ASVs diversity), which encompasses 97.67% of the complete sequence dataset (Figure S1, Table S2). This core microbiome consisted mainly of Proteobacteria (52.5%), Firmicutes (26.8%), Bacteroidota (13.9%) and Actinobacteriota (6.6%). They were distributed among 66 bacterial families, of which Lactobacillaceae (Phylum Firmicutes - 23.5%) and Enterobacteriaceae (Phylum Proteobacteria - 15.6%) were the most abundant. The abundance of the other bacterial families depended on the developmental stage of the host (Figure 2).



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

352

353 For instance, the families Nocardiaceae (Phylum Actinobacteriota) and
354 Comamonadaceae (Phylum Proteobacteria) dominated only the egg stage (27.6% and
355 13.2% respectively) and the newly hatched nymphs N1-Y (39.2% and 16.9%
356 respectively). On a finer scale, 50 ASVs were present at all developmental stages, a
357 number that increased to 142 ASVs when eggs were excluded (Figure S3A). The 50
358 common ASVs covered 29 genera, represented mainly by *Chryseobacterium,*
359 *Sphingobacterium* (Phylum Bacteroidota), *Smaragdicoccus* (Phylum Actinobacterioa),

360 *Kosakonia, Stenotrophomonas*, *Paracoccus, Pseudomonas* and *Acinetobacter*
361 (Phylum Proteobacteria) (Figure S3B). Only a few ASVs were stage specific. For
362 instance, 2 ASVs (ASV66|*Pseudomonas* and ASV482|*Sphingobacterium*) were
363 specific to the eggs, 52 to the nymphs and 26 to the adults (Figure S3A).

364

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

366 The alpha diversity of the earwig gut microbiome changed during the development of
367 the offspring (Figure 3A; Figure S4; Shannon index, Likelihood Ratio $\chi^2_{11} = 59.70$, P =
368 10^{-8} and P < 10^{-10} for all other alpha diversity indices). Markedly, the nature of these
369 changes did not follow a linear or systematic pattern. Alpha diversity did not change
370 between eggs and freshly moulted 1st instar nymphs (pairwise comparisons using log-
371 rank test contrasts for Shannon index; Egg *vs* L1-Y, P = 0.921), nor did it change during
372 the entire 1st instar (L1-Y *vs* L1-O, P = 0.621). However, there was a gradual increase
373 in alpha diversity during this time, as it was greater at the end of the 1st instar than in
374 the eggs (Egg *vs* L1-O, P = 0.052). While this alpha diversity remained at a high level
375 just after the moult into 2nd instar (L1-O *vs* L2-Y, P = 1.000), it then dropped to the
376 lowest level at the end of the 2nd instar (L2-Y *vs* L2-O, *P* = 0.029). This level remained
377 low just after the moult into 3rd instar (L2-O *vs* L3-Y, P = 0.998), but then increased to
378 its highest level at the end of the 3rd instar (L3-Y *vs* L3-O, P = 0.010). Alpha diversity
379 decreased slightly just after the moult into 4th instar (L3-O *vs* L4-Y, P = 0.086, but other
380 indices are significant for this contrast) to finally remain stable until the end of the 4th
381 instar (L4-Y *vs* L4-O, P = 0.702) and in the following adult females (L4-O *vs* Adult-F, P
382 = 0.496). By contrast, it decreased slightly between the end of the 4th instar and the
383 following adult males (L4-O *vs* Adult-M, P = 0.055, but other indices are significant for
384 this contrast).

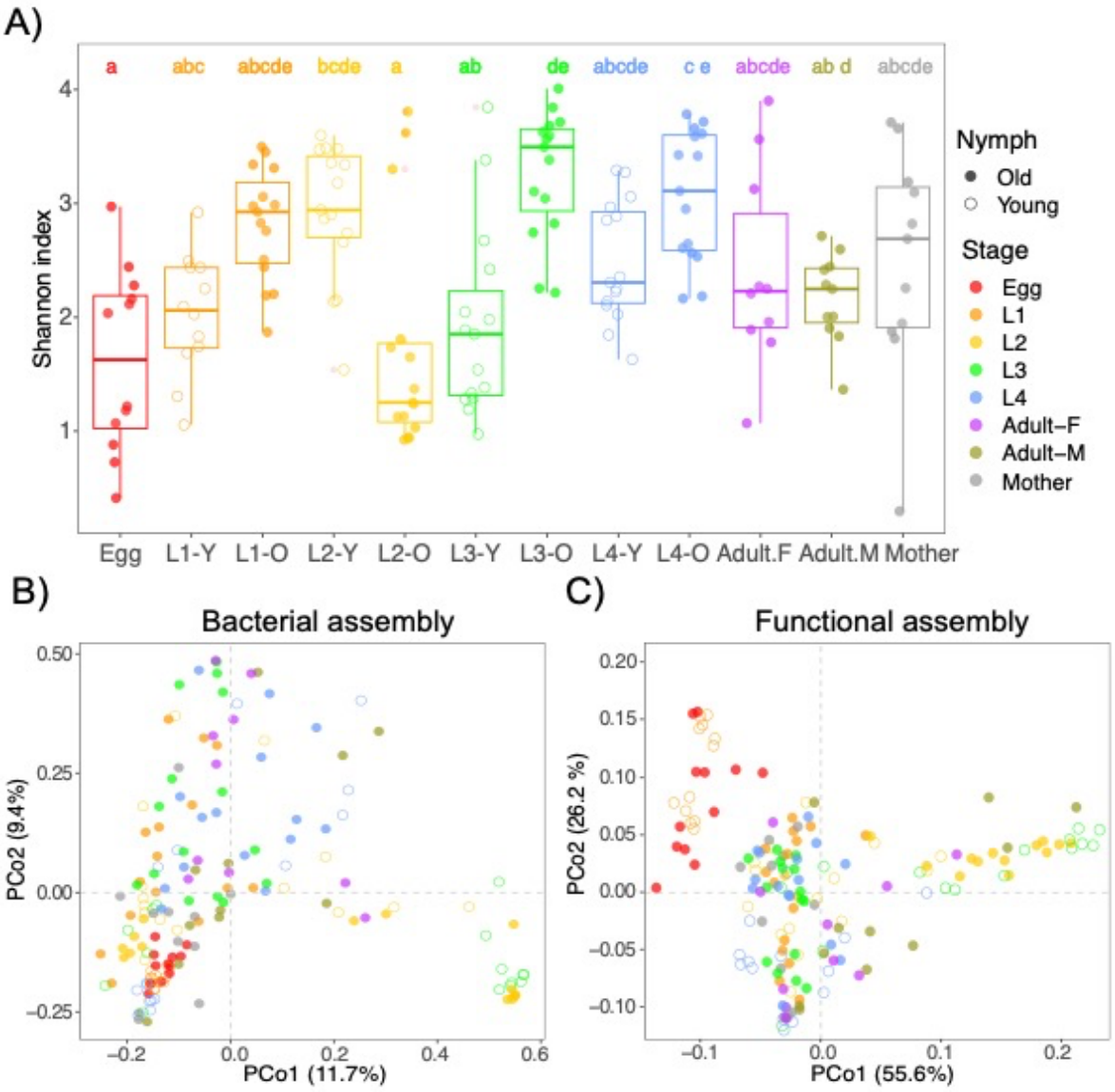


Figure 3: Microbiome variation during offspring development. The diversity is compared before (fill) and after moults (empty circles) and between each developmental stage and sex (coloured). **(A)** Shannon index comparisons for bacterial alpha diversity. Letters above the boxes indicate significant grouping contrasts ($P \leq 0.05$). Boxplots represent the median (middle bar) and the interquartile range (box) with whiskers representing the 1.5-fold the interquartile range. Dots represent the value for an individual (with red dot are outliers). **(B-C)** Principal coordinates analyses (PCoA) plots in the two first axes illustrating Bray-Curtis distances calculated between samples based on their ASVs and their KOs compositions.

Overall, moult was not associated with changes in offspring alpha diversity, except for the final moult from 4th instar to adult males. In old nymphs, alpha diversity was lower in 2nd compared to 3rd and 4th instars, whereas values were intermediate in 1st instars and adults (Figure 3A). In contrast, in newly moulted nymphs, alpha diversity was lower in 3rd instar compared to 2nd instar, while values were intermediate in 1st and 4th instar (Figure 3A). Finally, in adult offspring, alpha diversity was similar in males and females (Figure 3A, Figures S3, Supplemental File). As with alpha diversity, the beta diversity of the earwig gut microbiome changed as the offspring developed (PERMANOVA performed on all beta diversity metrics, Stage effect $0.074 < R^2 < 0.103$, all $P = 0.001$) (Table 1).

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

	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**

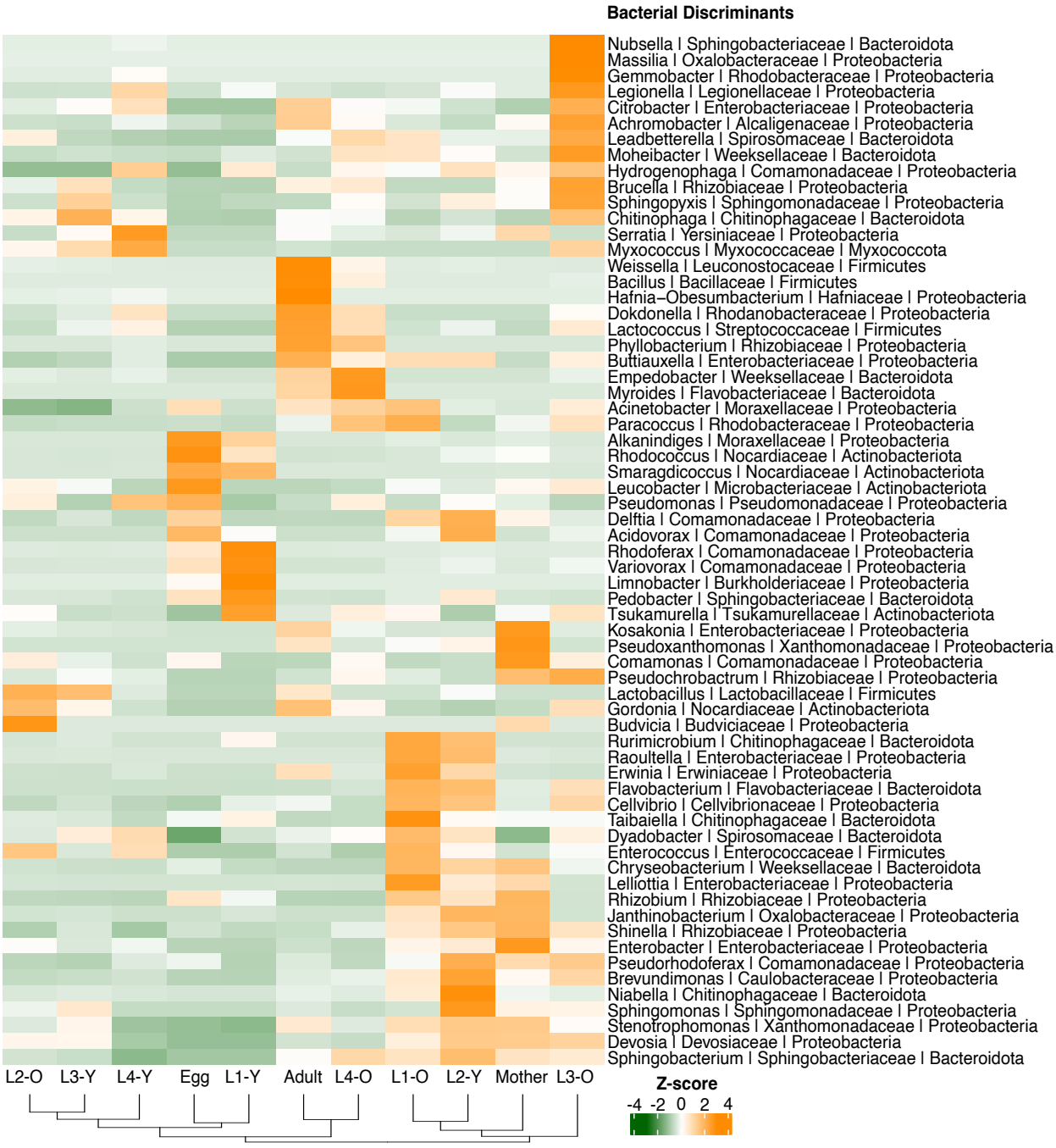
Here, however, the beta diversity of each collection time was different from all other collection times for at least one metric in 61 of the 65 pairwise comparisons ($P < 0.05$). Two major microbial turnovers occurred after hatching and during the passage

413 from the 2nd to the 3rd instar nymphs. The PCoA performed on taxonomical distance
414 matrices (*i.e.,* Bray-Curtis and Jaccard) showed a marked separation on the first axis
415 with both microbial assemblies of old 2nd instar nymphs (L2-O) and the young 3rd instar
416 nymphs (L3-Y) on one side, and all the microbial assemblies associated with the other
417 stages on the other side (Figure 3B, Figure S5A). The PCoA based on Unifrac
418 distances shows an additional separation with eggs and newly hatched nymphs (L1-
419 Y) on one side and the other stages on the other side (Figure S5B-C) showing a strong
420 phylogenetic turnover. The only four comparisons that did not present any beta
421 diversity difference were before and after the three first moults, i.e. between eggs and
422 newly hatched nymphs (L1-Y), between old 1st instar nymphs (L1-O) and young 2nd
423 instar nymphs (L2-Y), and between old 2nd instar nymphs (L2-O) and young 3rd instar
424 nymphs (L3-Y), as well as the comparison between adult males and females
425 (Supplemental File).

426

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

428 A total of 231 ASVs that belonged to 62 bacterial genera were stage-specific (Figure
429 4). The hierarchical clustering analysis based on the Z-score of these discriminants
430 showed a structure that does not strictly follow the chronology of the developmental
431 stages of the offspring, but rather clustered individuals at the end of one instar to
432 individuals at the beginning of the following instar. In particular, they grouped the eggs
433 with newly hatched nymphs (L1-Y), the old 1st (L1-O) with the young 2nd instar nymphs
434 (L2-Y), and the old 2nd instar (L2-O) with the young 3rd (L3-Y), and the old 4th instar
435 nymphs (L4-O) with adults (Figure 4).



436

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

440

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

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

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

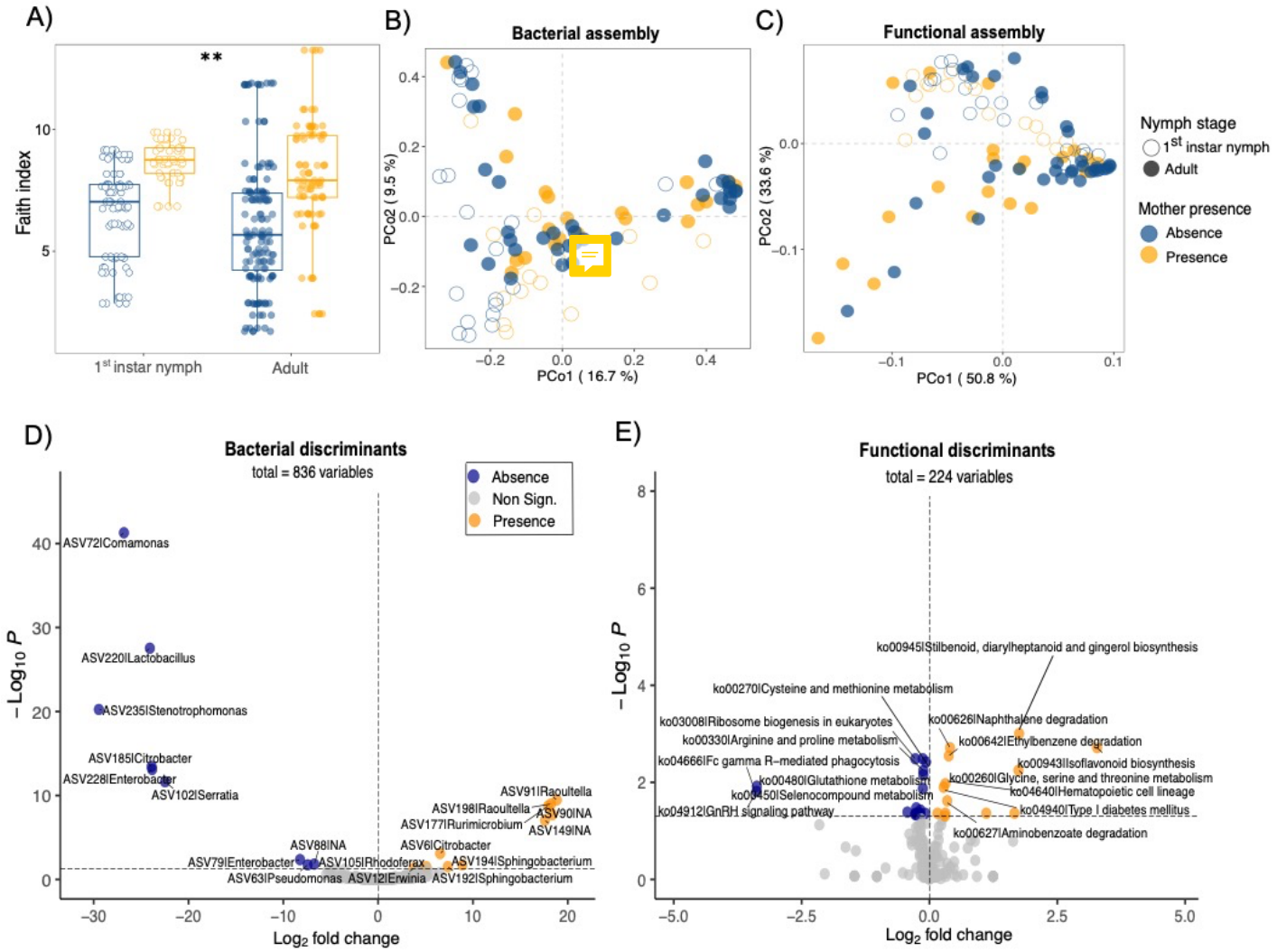


Figure 5: Effect of maternal presence (orange) or absence (blue) on the microbiome of 1st instar nymphs (empty circles) and adults (fill circles). (A) Alpha diversity is represented with the phylogenetic diversity index of Faith. **(B, C)** Beta diversity is showed through a two-dimensional PCoA according 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. P 0.001 < ** ≤ 0.01.

Predictions of potential functions of the different microbiomes

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

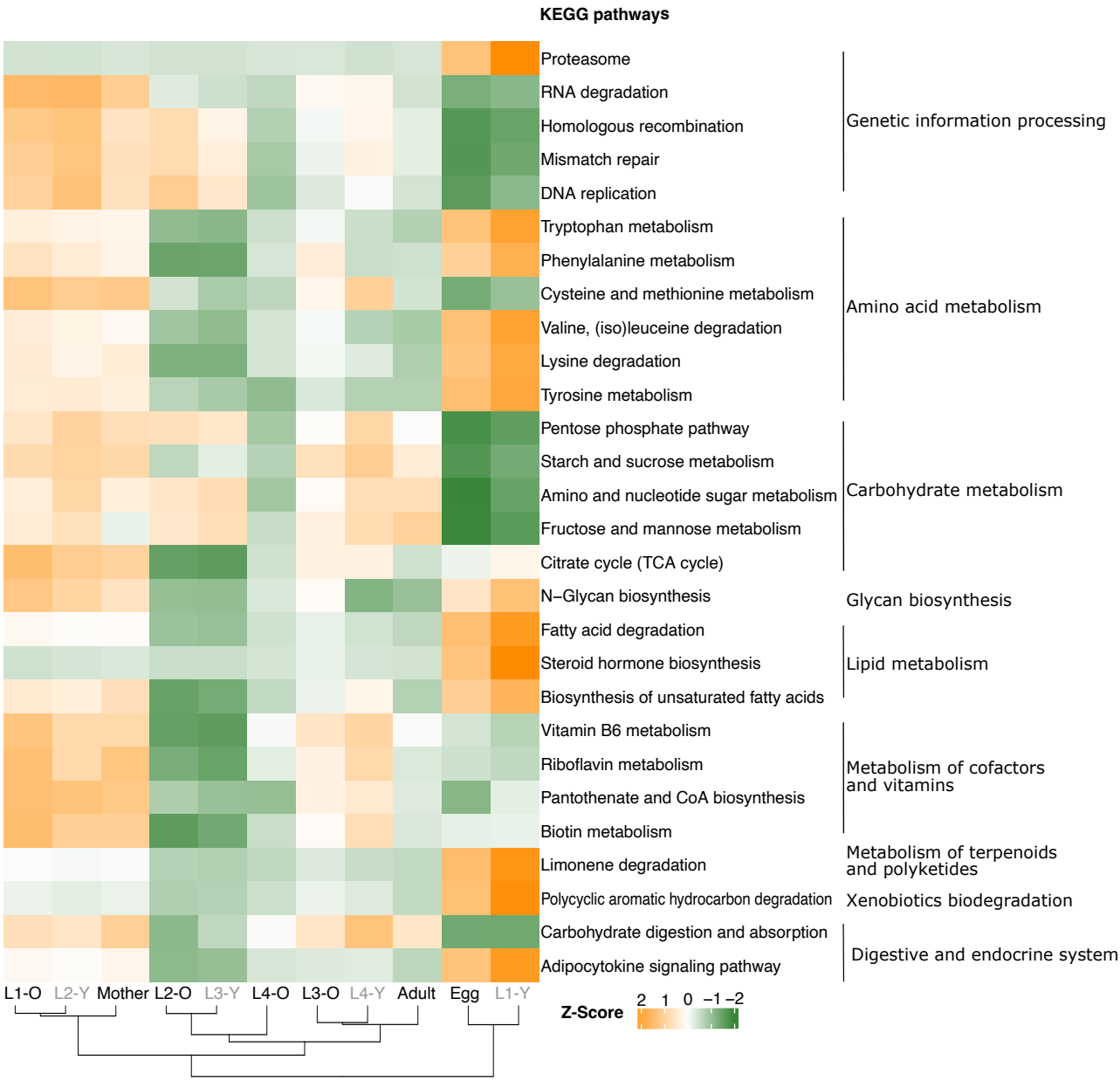


Figure 6: Heatmaps of hierarchical clustering based on the KEGG pathways indicators of developmental stages in the European earwig. Raw represent the Z-score associated to the pathway name in each sampling stage (young-nymphs are coloured in grey).

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

Table 2: Effect of access to (A) maternal care during family life and (B) offspring age on microbiome alpha and beta diversity at bacterial (white) and functional (grey) levels. Significant p-values (P <.005) are indicated in bold.

		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**

Discussion

The gradual and subtle changes that hemimetabolous juveniles undergo during moulting are often thought to limit profound changes in their microbiota during development (Carrasco and Pérez-Cobas, 2014; Manthey *et al.*, 2022). Our data show that this assumption is not always true, as these changes occur in the European earwig. Using 16S rRNA metabarcoding on 218 samples from egg to adult stages, we found that the microbiome of earwig offspring shows substantial variation throughout their development both in terms of beta diversity (thereafter called “structure”) and alpha diversity (thereafter called “diversity”). Interestingly, these changes did not occur during moulting, but rather between the beginning and end of certain developmental stages. In addition, we found that maternal care partly shapes the microbiome of offspring, even if this behaviour is facultative in the European earwig. Access to maternal care during the first few weeks after hatching affected not only the microbiome of first instar nymphs during family life, but also that of the resulting adults two months after family life has ended.

Offspring microbiome varies during development

Our data first reveal that the microbiome diversity (alpha) and structure (beta) of earwig changed during offspring development. This is striking for two main reasons. First, these changes were not linear and only occurred at certain stages of offspring development, which contrasts with the general pattern where microbiome diversity gradually increases with natural growth in offspring body size (Sherrill-Mix *et al.*, 2018). Second, some of these changes occurred in the second and third nymphal instars, even though all instars were fed the same diet and developed in the same laboratory conditions throughout their development.

The first marked change in microbiome structure occurred shortly after the nymphs hatched. We showed that both eggs and newly hatched nymphs had a microbiome structure different from that of all other stages. From the egg stage to the freshly moulted 2nd instar nymphs, we observed an increase in diversity due to the gradual colonization of the 1st instar nymphs by new genera, including *Erwinia, Raoultella, Flavobacterium* and *Lactobacillus*. These new genera are present in a wide range of insects, where they are often known to perform beneficial functions in juveniles (Malacrinò, 2022). For instance, *Erwinia* is known to reduce the maturation time of the bark beetle *Ips typographus* (Peral-Aranega *et al.*, 2023).

The second marked change in microbiome diversity occurred during the development of the 2nd instar nymphs. We found that the diversity drops to its lowest level during the transition from newly moulted to old 2nd instar nymphs and remains low in the newly moulted 3rd instar nymphs (Figure 3A). This fall is reflected by the loss of several genera, including *Smaragdicoccus, Rhodococcus* or *Alkanindiges* (Figure 4). The bacterial communities of the newly moulted 3rd instar nymphs are very different from those of any other stage, because of its structural monotony where *Lactobacillus* largely dominates the bacterial composition (Figures 1, 4). Such a decrease in microbiome diversity has been reported in the 3rd instar larvae of the German cockroach *Blattella germanica* (Carrasco and Pérez-Cobas, 2014), where it has been suggested to result from the physiological state of the host at that particular stage (Kirkland *et al.*, 2020). In lower termite workers, the gut flagellates are totally lost prior the ecdysis, probably due to a combination of host starvation and hormonal variation linked to changes in the host social status (Cleveland, 1949; Raina *et al.*, 2008; Nalepa, 2017). This may also be the case with earwigs, although our knowledge of the physiological and hormonal peculiarities of each instar is still very limited (Meunier,

549 2024). This decrease may also result from the end of mother-offspring interactions, as
550 we removed mothers from their nymphs 14 days after hatching, which is often shortly
551 after they have reached the 2nd instar (Thesing *et al.*, 2015). This end of family life
552 inherently means that any potential form of coprophagy and trophallaxis between
553 mother and offspring (and thus potential bacterial vertical transmission) ceases, and
554 the juveniles begin to process the food source exclusively on their own. Whether
555 physiological peculiarities of 2nd instar nymphs, suppression of maternal bacterial
556 transfer and/or changes in dietary habits are the drivers of the reported changes in the
557 microbiome diversity remains to be further investigated.

558 The last marked change in microbiome diversity was an increase during the
559 development of the 3rd instar nymphs, mostly explained by the colonization of new
560 genera, such as *Nubsella, Massilia, Gemmobacter* or *Chitinophaga.* These genera are
561 common colonizers of insect guts (Da Silva Correia *et al.*, 2018; Guégan *et al.*, 2018;
562 Paddock *et al.*, 2022) but can also be found in the environment (Mayoral-Peña *et al.*,
563 2022). For example, bacteria of the genus *Nubsella* are mutualistic with certain
564 phytophagous insects and are likely to be important for the fitness of walking sticks (Lü
565 *et al.*, 2019; Li *et al.*, 2020). Interestingly, the genus *Chitinophaga* is known to be
566 involved in the degradation of chitin, a major component of the insect skeleton (Glavina
567 Del Rio *et al.*, 2010). Whether their presence is a mutualistic association with earwigs
568 aiding in cuticle digestion during cannibalism or during the reingestion of their own
569 exuviae is an open question and will be developed below.

570

571 **Moulting does not alter microbiome diversity**

572 Moulting did not induce any changes in the microbiome diversity and structure of
573 earwig offspring, except for the moults between the 3rd and 4th instar nymphs. This

overall lack of moulting effect is in line with studies carried out in other hemimetabolous insects showing that the gut microbiome remains stable during development (Manthey *et al.*, 2022). For earwigs, we propose several possible explanations. First, nymphs could possess a microbial reservoir that is preserved during moulting. Such a reservoir is present, for example, in the bean bug *Riptortus clavatus* to maintain their *Burkholderia* symbionts during moult (Kikuchi and Yumoto, 2013). However, the presence of such a reservoir has never been documented in earwigs. Another possibility is that earwig nymphs re-inoculate themselves with their own bacteria by eating their shed cuticle after moulting, as reported in cockroaches (Mira, 2000). Even if earwig nymphs are regularly observed eating their shed cuticle during laboratory rearing (J Meunier, pers. obs), the role of this behaviour to inoculate a lost microbiome has never been investigated in this species. Finally, earwig nymphs may be able to re-inoculate themselves through their frequent (in)direct contact with their siblings by means of coprophagy and trophallaxis (Falk *et al.*, 2014; Kramer and Meunier, 2016). These social acquisitions allow the persistence and the (re)acquisition of lost bacteria in numerous arthropods, such as termites (Raina *et al.*, 2008; Michaud *et al.*, 2020), cockroaches (Nalepa, 2020), and spiders (Rose *et al.*, 2023). Overall, these data call for future experiments to disentangle which parameter explains the resistance of the earwig microbiome to moulting, and to understand why they are no longer efficient for the moult between the 3rd and 4th instar nymphs.

Bacterial functions could play a role throughout juvenile development

The reported changes in the structure of the offspring microbiome during development are associated with changes in the predicted potential functions of their bacteria that could be beneficial to the host. For instance, many of the bacteria overrepresented in

eggs and newly hatched nymphs are known to produce and accumulate lipids such as *Rhodococcus, Delftia* and *Pedobacter* (Alvarez *et al.*, 1997; Liu *et al.*, 2016; Franks *et al.*, 2021). As completing the transition from eggs to nymphs is a highly energetic process, our data suggest that earwig embryos may not only obtain this energy from egg lipid reserves, but also from these bacteria (Ziegler and Vanantwerpen, 2006; Diether and Willing, 2019). Moreover, this would be consistent with the predictions of PICRUST2, which show a strong positive correlation in lipogenesis processes such as adipocytokine signalling pathway and unsaturated fatty acid biosynthesis (Figure 6). The bacterial loss observed in the third nymph instar was also found in terms of predicted functions, as all pathways except cell developmental pathways, amino acids and sugar metabolism were underrepresented in this developmental stage compared to the subsequent ones. Finally, the acquisition of new genera such as *Nubsella, Massilia, Gemmobacter* or *Chitinophaga* during the development of the third nymphal instar came with new predicted functions linked to energy uptake, amino acids and vitamins B biosynthesis, which act as a coenzyme in numerous pathways involved in the fatty acid synthesis, glucogenesis or amino acids synthesis. These vitamins are essential during insect development, but they cannot be synthetized by animals themselves and are often acquired through alimentation or provided by the microbiota (Douglas, 2017; Kinjo *et al.*, 2022). Although these functions may provide insights into our general understanding of the driver of microbiome changes during offspring development, they must be considered with caution as the validity of the approach is debatable due to the short length of the amplicons and the lack of genome reference concerning insect-associated microbial communities (Djemiel *et al.*, 2022). Future studies are thus needed to confirm these potential functions in earwigs, for example using transcriptomic analyses.

Maternal care shapes the microbiomes structure of offspring

In animals, maternal care is often considered to be an important mediator of microbial transmission from parents to offspring (Bright and Bulgheresi, 2010; Sachs *et al.*, 2011; Hosokawa and Fukatsu, 2020). Our data suggests that the European earwig is no exception. Access to maternal care not only shapes the microbiome structure, phylogenetic diversity and functions of 1st instar nymphs, but also those of the resulting adults, even though none of these adults had any contact with their mothers in the previous two months. We found that the microbiome of nymphs with maternal care contained a higher diversity of phylogenetically distant bacteria compared to their orphaned counterparts. Not surprisingly, the bacteria found in these nymphs were also found in the microbiome of their mothers (including *Raoultella*, *Rurimicrobium* and *Sphingobacterium*). This is likely due to direct or indirect maternal transmission during post-hatching family life. However, even if maternal transmission contributes to the shape of the microbiome, the non-significant effect of maternal care on alpha diversity in terms of taxonomic richness, Shannon entropy or Allen index, combined with the small, albeit significant effect on microbial structure, suggests that maternal presence is not the main route of bacterial acquisition in this species. Indeed, we also found an overabundance of some ASVs in the orphan offspring, such as sequences related to *Comamonas*, *Lactobacillus* or *Serratia*. Since these bacteria are often generalists, associated with laboratory rearing conditions (Malacrinò, 2022), and common in the mothers tested, they are likely to come from the rearing environment. However, their overabundance in orphaned nymphs suggests that they were outcompeted by maternally transmitted bacteria in non-orphaned nymphs. In addition to these differences, we found one major discriminant (potential) function in the tended nymphs,

649 related to isoflavonoid biosynthesis, and two in the orphan nymphs, related to the
650 endocrine and immune systems. These predicted functions are consistent with
651 previous phenotypic studies showing that orphaned nymphs develop faster to adults,
652 produce larger adults with longer male appendages, but contradict with other studies
653 showing that orphaning has limited long-term effects on the basal immunity of the
654 nymphs and resulting adults (Meunier and Kölliker, 2012; Thesing *et al.*, 2015;
655 Vogelweith *et al.*, 2017; Körner *et al.*, 2020). Here again, these predicted functions
656 need to be taken with caution. Whether all or some of these offspring phenotypes are
657 indeed due to maternally derived bacteria remains to be further explored, *e.g.* with the
658 use of gnotobiotic lineages.
659

660 ## Conclusion

661 Overall, we showed that the European earwig microbiome changes multiple times
662 during offspring development. Interestingly, these changes were independent of
663 moulting. The fact that moulting did not induce any purge or shift in the bacterial
664 communities of the nymphs calls for future studies to test whether this is due to the
665 presence of a bacterial reservoir, moult consumption and/or social interactions with
666 siblings. Our data suggest that the predicted functions of some components of these
667 microbiomes are relevant to the developmental stage at which they occur, such as
668 lipogenesis or steroid synthesis in early stages, and nutrient and vitamin synthesis in
669 late stages. However, future studies are required to confirm the functional role of the
670 microbiome changes in this species. Finally, we showed that maternal care is an
671 important short- and long-term determinant of the offspring microbiome. Given that
672 earwig nymphs do not require maternal care to develop and survive (Kölliker, 2007;
673 Thesing *et al.*, 2015; Kramer and Meunier, 2016) and that nymph can also develop in

absence of any social interactions (Van Meyel and Meunier, 2022), our results call for future studies to investigate the role of these socially-acquired bacteria (and other potential members of their microbiota such as fungi, viruses and other microorganisms) in the biology of the European earwig and, more generally, in the early evolution and maintenance of facultative family life in insects (Archie and Tung, 2015; Körner *et al.*, 2023).

Acknowledgements

We thank Franck Dedeine, Maximilian Körner and Jean-Claude Tourneur for their comments on this manuscript. We also thank all members of the EARWIG group at IRBI for their help with animal rearing and setup installation, Marina Querejeta for her help on bioinformatical analyses, and the Bio-environment platform of the University of Perpignan (France) for providing the MiSeq Illumina sequencing. Finally, we thank Armand Guillermin and the INRAE unité expérimentale Recherche Intégrée in Gotheron for giving us access to their orchards for earwig field sampling. Graphical abstract was made with Biorender.com.

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.

Funding stattemen

This research has been supported by a research grant from the Agence Nationale de la Recherche (ANR-20-CE02-0002 to J.M.).

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.

References

Alvarez, H.M., Kalscheuer, R., and Steinbüchel, A. (1997) Accumulation of storage lipids in species of *Rhodococcus* and *Nocardia* and effect of inhibitors and polyethylene glycol. *Fett/Lipid* **99**: 239–246.

Arce, A.N., Johnston, P.R., Smiseth, P.T., and Rozen, D.E. (2012) Mechanisms and fitness effects of antibacterial defences in a carrion beetle: Antibacterial defence in a carrion beetle. *Journal of Evolutionary Biology* **25**: 930–937.

Archie, E.A. and Tung, J. (2015) Social behavior and the microbiome. *Current Opinion in Behavioral Sciences* **6**: 28–34.

Barbera, P., Kozlov, A.M., Czech, L., Morel, B., Darriba, D., Flouri, T., and Stamatakis, A. (2019) EPA-ng: Massively parallel evolutionary placement of genetic sequences. *Systematic Biology* **68**: 365–369.

Bartoń, K. (2022) MuMIn: Multi-Model Inference.

Berenbaum, M. (2017) Insect biodiversity - Millions and millions. In *Insect Biodiversity*. Foottit, R.G. and Adler, P.H. (eds). Chichester, UK: John Wiley & Sons, Ltd, pp. 783–792.

Berlanga, M. and Guerrero, R. (2016) The holobiont concept: the case of xylophagous termites and cockroaches. *Symbiosis* **68**: 49–60.

Boos, S., Meunier, J., Pichon, S., and Kölliker, M. (2014) Maternal care provides antifungal protection to eggs in the European earwig. *Behavioral Ecology* **25**: 754–761.

Bright, M. and Bulgheresi, S. (2010) A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol* **8**: 218–230.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., and Holmes, S.P. (2016) DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* **13**: 581–583.

Cameron, E.S., Schmidt, P.J., Tremblay, B.J.-M., Emelko, M.B., and Müller, K.M. (2021) Enhancing diversity analysis by repeatedly rarefying next generation sequencing data describing microbial communities. *Sci Rep* **11**: 22302.

Carrasco, P. and Pérez-Cobas, A.E. (2014) Succession of the gut microbiota in the cockroach *Blattella germanica*. *International Microbiology* 99–109.

Caspi, R., Billington, R., Keseler, I.M., Kothari, A., Krummenacker, M., Midford, P.E., et al. (2020) The MetaCyc database of metabolic pathways and enzymes - a 2019 update. *Nucleic Acids Research* **48**: D445–D453.

Chao, A., Chiu, C.-H., and Jost, L. (2010) Phylogenetic diversity measures based on Hill numbers. *Phil Trans R Soc B* **365**: 3599–3609.

Cheutin, M.-C., Villéger, S., Hicks, C.C., Robinson, J.P.W., Graham, N.A.J., Marconnet, C., et al. (2021) Microbial shift in the enteric bacteriome of coral reef fish following climate-driven regime shifts. *Microorganisms* **9**: 1711.

Cleveland, L.R. (1949) Hormone-induced sexual cycles of flagellates. I. Gametogenesis, fertilization, and meiosis in *Trichonympha*. *Journal of Morphology* **85**: 197–295.

Da Silva Correia, D., Passos, S.R., Proença, D.N., Morais, P.V., Xavier, G.R., and Correia, M.E.F. (2018) Microbial diversity associated to the intestinal tract of soil invertebrates. *Applied Soil Ecology* **131**: 38–46.

Diehl, J.M.C. and Meunier, J. (2018) Surrounding pathogens shape maternal egg care but not egg production in the European earwig. *Behavioral Ecology* **29**: 128–136.

Diether, N. and Willing, B. (2019) Microbial fermentation of dietary protein: An important factor in diet–microbe–host interaction. *Microorganisms* **7**: 19.

Djemiel, C., Maron, P.-A., Terrat, S., Dequiedt, S., Cottin, A., and Ranjard, L. (2022) Inferring microbiota functions from taxonomic genes: a review. *GigaScience* **11**: giab090.

Douglas (2017) The B vitamin nutrition of insects: the contributions of diet, microbiome and horizontally acquired genes. *Current Opinion in Insect Science* **23**: 65–69.

Douglas, G.M., Maffei, V.J., Zaneveld, J.R., Yurgel, S.N., Brown, J.R., Taylor, C.M., et al. (2020) PICRUSt2 for prediction of metagenome functions. *Nat Biotechnol* **38**: 685–688.

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

Falk, J., Wong, J.W.Y., Kölliker, M., and Meunier, J. (2014) Sibling cooperation in earwig families provides insights into the early evolution of social life. *The American Naturalist* **183**: 547–557.

Fillol, M., Auguet, J.-C., Casamayor, E.O., and Borrego, C.M. (2016) Insights in the ecology and evolutionary history of the *Miscellaneous Crenarchaeotic Group* lineage. *The ISME Journal* **10**: 665–677.

Franks, K., Kooienga, E., Sanders, M., Pendarvis, K., Yang, F., Tomberlin, J.K., and Jordan, H.R. (2021) The effect of *Rhodococcus rhodochrous* supplementation on black soldier fly (Diptera: Stratiomyidae) development, nutrition, and waste conversion. *JIFF* **7**: 397–408.

Girard, M., Luis, P., Valiente Moro, C., and Minard, G. (2023) Crosstalk between the microbiota and insect postembryonic development. *Trends in Microbiology* **31**: 181–196.

Glassman, S.I. and Martiny, J.B.H. (2018) Broadscale ecological patterns are robust to use of Exact Sequence Variants versus Operational Taxonomic Units. *mSphere* **3**: e00148-18.

Glavina Del Rio, T., Abt, B., Spring, S., Lapidus, A., Nolan, M., Tice, H., et al. (2010) Complete genome sequence of *Chitinophaga pinensis* type strain (UQM 2034T). *Stand Genomic Sci* **2**: 87–95.

González-Miguéns, R., Muñoz-Nozal, E., Jiménez-Ruiz, Y., Mas-Peinado, P., Ghanavi, H.R., and García-París, M. (2020) Speciation patterns in the *Forficula auricularia* species complex: cryptic and not so cryptic taxa across the western Palaearctic region. *Zoological Journal of the Linnean Society* **190**: 788–823.

Greer, J.A., Swei, A., Vredenburg, V.T., and Zink, A.G. (2020) Parental care alters the egg microbiome of maritime earwigs. *Microb Ecol* **80**: 920–934.

Guégan, M., Zouache, K., Démichel, C., Minard, G., Tran Van, V., Potier, P., et al. (2018) The mosquito holobiont: fresh insight into mosquito-microbiota interactions. *Microbiome* **6**: 49.

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

Hartig, F. (2022) DHARMa: Residual diagnostics for hierarchical (multi-Level / mixed) regression models. *R package version 046*.

Hosokawa, T. and Fukatsu, T. (2020) Relevance of microbial symbiosis to insect behavior. *Current Opinion in Insect Science* **39**: 91–100.

Jeanbille, M., Gury, J., Duran, R., Tronczynski, J., Agogué, H., Ben Saïd, O., et al. (2016) Response of core microbial consortia to chronic hydrocarbon contaminations in coastal sediment habitats. *Front Microbiol* **7**:.

Johnston, P.R., Paris, V., and Rolff, J. (2019) Immune gene regulation in the gut during metamorphosis in a holo- versus a hemimetabolous insect. *Phil Trans R Soc B* **374**: 20190073.

Katoh, K. (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research* **30**: 3059–3066.

Kikuchi, Y. and Yumoto, I. (2013) Efficient colonization of the bean bug *Riptortus pedestris* by an environmentally transmitted *Burkholderia* symbiont. *Appl Environ Microbiol* **79**: 2088–2091.

Kinjo, Y., Bourguignon, T., Hongoh, Y., Lo, N., Tokuda, G., and Ohkuma, M. (2022) Coevolution of metabolic pathways in Blattodea and their *Blattabacterium* endosymbionts, and comparisons with other insect-bacteria symbioses. *Microbiol Spectr* **10**: e02779-22.

Kirkland, L.S., Maino, J., Stuart, O., and Umina, P.A. (2020) Ontogeny in the European earwig (*Forficula auricularia*) and grain crops interact to exacerbate feeding damage risk. *J Applied Entomology* **144**: 605–615.

827 Klass, K.-D., Nalepa, C., and Lo, N. (2008) Wood-feeding cockroaches as models for
828 termite evolution (Insecta: Dictyoptera): *Cryptocercus* vs. *Parasphaeria*
829 *boleiriana*. *Molecular Phylogenetics and Evolution* **46**: 809–817.
830 Koch, L.K. and Meunier, J. (2014) Mother and offspring fitness in an insect with
831 maternal care: phenotypic trade-offs between egg number, egg mass and egg
832 care. *BMC Evol Biol* **14**: 125.
833 Kölliker, M. (2007) Benefits and costs of earwig (Forficula auricularia) family life. *Behav*
834 *Ecol Sociobiol* **61**: 1489–1497.
835 Körner, M., Foitzik, S., and Meunier, J. (2018) Extended winters entail long-term costs
836 for insect offspring reared in an overwinter burrow. *Journal of Thermal Biology*
837 **74**: 116–122.
838 Körner, M., Steiger, S., and Shukla, S.P. (2023) Microbial management as a driver of
839 parental care and family aggregations in carrion feeding insects. *Front Ecol Evol*
840 **11**: 1252876.
841 Körner, M., Vogelweith, F., Libbrecht, R., Foitzik, S., Feldmeyer, B., and Meunier, J.
842 (2020) Offspring reverse transcriptome responses to maternal deprivation when
843 reared with pathogens in an insect with facultative family life. *Proc R Soc B* **287**:
844 20200440.
845 Kramer, J. and Meunier, J. (2016) Kin and multilevel selection in social evolution: a
846 never-ending controversy? *F1000Res* **5**: 776.
847 Kramer, J., Thesing, J., and Meunier, J. (2015) Negative association between parental
848 care and sibling cooperation in earwigs: a new perspective on the early
849 evolution of family life? *J of Evolutionary Biology* **28**: 1299–1308.
850 Krebs, C. (1999) Ecological methodology, 2nd ed. New York: Harper and Row.
851 Lamb, R.J. (1976) Parental behavior in the Dermaptera with special reference to
852 *Forficula auricularia* (Dermaptera: Forficulidae). *Can Entomol* **108**: 609–619.
853 Lenth, R. (2022) emmeans: Estimated Marginal Means, aka Least-Squares Means.
854 Li, Y., Huang, Y., Chen, T., Wu, S., Tang, H., Hsiao, C., et al. (2020) Comparison of gut
855 microbiota of healthy and diseased walking sticks, *Phasmotaenia lanyuhensis*.
856 *Arch Insect Biochem Physiol* **105**: e21749.
857 Liu, W., Li, Y., Guo, S., Yin, H., Lei, C.-L., and Wang, X.-P. (2016) Association between
858 gut microbiota and diapause preparation in the cabbage beetle: a new
859 perspective for studying insect diapause. *Sci Rep* **6**: 38900.
860 Lombardo, M.P. (2008) Access to mutualistic endosymbiotic microbes: an
861 underappreciated benefit of group living. *Behav Ecol Sociobiol* **62**: 479–497.
862 Louca, S. and Doebeli, M. (2018) Efficient comparative phylogenetics on large trees.
863 *Bioinformatics* **34**: 1053–1055.
864 Love, M.I., Huber, W., and Anders, S. (2014) Moderated estimation of fold change and
865 dispersion for RNA-seq data with DESeq2. *Genome Biol* **15**: 550.
866 Lü, J., Guo, W., Chen, S., Guo, M., Qiu, B., Yang, C., et al. (2019) Host plants influence
867 the composition of the gut bacteria in *Henosepilachna vigintioctopunctata*. *PLoS*
868 *ONE* **14**: e0224213.
869 Magurran, A.E. and Henderson, P.A. (2003) Explaining the excess of rare species in
870 natural species abundance distributions. *Nature* **422**: 714–716.
871 Malacrinò, A. (2022) Host species identity shapes the diversity and structure of insect
872 microbiota. *Molecular Ecology* **31**: 723–735.
873 Manthey, C., Johnston, P.R., Nakagawa, S., and Rolff, J. (2022) Complete
874 metamorphosis and microbiota turnover in insects. *Molecular Ecology*
875 mec.16673.

Martinez Arbizu, P. (2017) pairwiseAdonis: Pairwise Multilevel Comparison using Adonis.

Mayoral-Peña, Z., Lázaro-Vidal, V., Fornoni, J., Álvarez-Martínez, R., and Garrido, E. (2022) Studying plant–insect interactions through the analyses of the diversity, composition, and functional inference of their bacteriomes. *Microorganisms* **11**: 40.

McFrederick, Q.S., Wcislo, W.T., Hout, M.C., and Mueller, U.G. (2014) Host species and developmental stage, but not host social structure, affects bacterial community structure in socially polymorphic bees. *FEMS Microbiol Ecol* **88**: 398–406.

McMurdie, P.J. and Holmes, S. (2013) phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **8**: e61217.

Meunier, J. (2024) The biology and social life of Earwigs (Dermaptera). *Annu Rev Entomol* **69**: annurev-ento-013023-015632.

Meunier, J. and Kölliker, M. (2012) When it is costly to have a caring mother: food limitation erases the benefits of parental care in earwigs. *Biol Lett* **8**: 547–550.

Meunier, J., Körner, M., and Kramer, J. (2022) Parental care. In *Reproductive Strategies in Insects*. Boca Raton: CRC Press.

Meunier, J., Wong, J.W.Y., Gómez, Y., Kuttler, S., Röllin, L., Stucki, D., and Kölliker, M. (2012) One clutch or two clutches? Fitness correlates of coexisting alternative female life-histories in the European earwig. *Evol Ecol* **26**: 669–682.

Michaud, C., Hervé, V., Dupont, S., Dubreuil, G., Bézier, A.M., Meunier, J., et al. (2020) Efficient but occasionally imperfect vertical transmission of gut mutualistic protists in a wood-feeding termite. *Molecular Ecology* **29**: 308–324.

Mira, A. (2000) Exuviae eating: a nitrogen meal? *Journal of Insect Physiology* **46**: 605–610.

Mora, C., Tittensor, D.P., Adl, S., Simpson, A.G.B., and Worm, B. (2011) How many species are there on Earth and in the Ocean? *PLoS Biol* **9**: e1001127.

Muyzer, G., de Waal, E.C., and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**: 695–700.

Nalepa (2020) Origin of mutualism between termites and flagellated gut protists: transition from horizontal to vertical transmission. *Front Ecol Evol* **8**: 14.

Nalepa, C. (2017) What kills the hindgut flagellates of lower termites during the host molting cycle? *Microorganisms* **5**: 82.

Neu, A.T., Allen, E.E., and Roy, K. (2021) Defining and quantifying the core microbiome: Challenges and prospects. *Proc Natl Acad Sci USA* **118**: e2104429118.

Onchuru, T.O., Javier Martinez, A., Ingham, C.S., and Kaltenpoth, M. (2018) Transmission of mutualistic bacteria in social and gregarious insects. *Current Opinion in Insect Science* **28**: 50–58.

Paddock, K.J., Finke, D.L., Kim, K.S., Sappington, T.W., and Hibbard, B.E. (2022) Patterns of microbiome composition vary across spatial scales in a specialist insect. *Front Microbiol* **13**: 898744.

Peral-Aranega, E., Saati-Santamaría, Z., Ayuso-Calles, M., Kostovčík, M., Veselská, T., Švec, K., et al. (2023) New insight into the bark beetle *Ips typographus* bacteriome reveals unexplored diversity potentially beneficial to the host. *Environmental Microbiome* **18**: 53.

Powell, J.E., Martinson, V.G., Urban-Mead, K., and Moran, N.A. (2014) Routes of acquisition of the gut microbiota of the honey bee *Apis mellifera*. *Appl Environ Microbiol* **80**: 7378–7387.
Price, M.N., Dehal, P.S., and Arkin, A.P. (2010) FastTree 2 – Approximately maximum-likelihood trees for large alignments. *PLoS ONE* **5**: e9490.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2012) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* **41**: D590–D596.
R Core Team (2022) R: A language and environment for statistical computing.
Raina, A., Park, Y.I., and Gelman, D. (2008) Molting in workers of the formosan subterranean termite *Coptotermes formosanus*. *Journal of Insect Physiology* **54**: 155–161.
Rose, C., Lund, M.B., Søgård, A.M., Busck, M.M., Bechsgaard, J.S., Schramm, A., and Bilde, T. (2023) Social transmission of bacterial symbionts homogenizes the microbiome within and across generations of group-living spiders. *ISME Communications* **3**: 60.
Sachs, J.L., Skophammer, R.G., and Regus, J.U. (2011) Evolutionary transitions in bacterial symbiosis. *Proc Natl Acad Sci USA* **108**: 10800–10807.
Salem, H., Florez, L., Gerardo, N., and Kaltenpoth, M. (2015) An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proc R Soc B* **282**: 20142957.
Samways, M.J. (2018) Insect conservation for the twenty-first century. In *Insect science-diversity, conservation and nutrition*. IntechOpen.
Sandrin, L., Meunier, J., Raveh, S., Walser, J.-C., and Kölliker, M. (2015) Multiple paternity and mating group size in the European earwig, *Forficula auricularia*. *Ecol Entomol* **40**: 159–166.
Schliep, K.P. (2011) phangorn: phylogenetic analysis in R. *Bioinformatics* **27**: 592–593.
Shapira, M. (2016) Gut microbiotas and host evolution: Scaling up symbiosis. *Trends in Ecology & Evolution* **31**: 539–549.
Sherrill-Mix, S., McCormick, K., Lauder, A., Bailey, A., Zimmerman, L., Li, Y., et al. (2018) Allometry and ecology of the bilaterian gut microbiome. *mBio* **9**: e00319-18.
Sudakaran, S., Kost, C., and Kaltenpoth, M. (2017) Symbiont acquisition and replacement as a source of ecological innovation. *Trends in Microbiology* **25**: 375–390.
Sudakaran, S., Salem, H., Kost, C., and Kaltenpoth, M. (2012) Geographical and ecological stability of the symbiotic mid-gut microbiota in European firebugs, *Pyrrhocoris apterus* (Hemiptera, Pyrrhocoridae). *Molecular Ecology* **21**: 6134–6151.
Thesing, J., Kramer, J., Koch, L.K., and Meunier, J. (2015) Short-term benefits, but transgenerational costs of maternal loss in an insect with facultative maternal care. *Proc R Soc B* **282**: 20151617.
Tourneur, Cole, C., and Meunier, J. (2020) The first of five moults of *Forficula auricularia* L. (Dermaptera: Forficulidae). *Can Entomol* **152**: 783–789.
Tourneur and Gingras, J. (1992) Egg laying in a northeastern north american (Montreal, Quebec) population of *Forficula auricularia* L. (Dermaptera: Forficulidae). *Can Entomol* **124**: 1055–1061.
Tourneur, J. and Meunier, J. (2020) Variations in seasonal (not mean) temperatures drive rapid adaptations to novel environments at a continent scale. *Ecology* **101**: e02973.

Van Meyel, S. and Meunier, J. (2022) Costs and benefits of isolation from siblings during family life in adult earwigs. *Animal Behaviour* **193**: 91–99.
Van Meyel, S. and Meunier, J. (2020) Filial egg cannibalism in the European earwig: its determinants and implications in the evolution of maternal egg care. *Animal Behaviour* **164**: 155–162.
Vogelweith, F., Körner, M., Foitzik, S., and Meunier, J. (2017) Age, pathogen exposure, but not maternal care shape offspring immunity in an insect with facultative family life. *BMC Evol Biol* **17**: 69.
Wang, Y. and Rozen, D.E. (2017) Gut microbiota colonization and transmission in the burying beetle *Nicrophorus vespilloides* throughout development. *Appl Environ Microbiol* **83**: e03250-16.
Wirth, T., Le Guellec, R., Vancassel, M., and Veuille, M. (1998) Molecular and reproductive characterization of sibling species in the European earwig (*Forficula auricularia*). *Evolution* **52**: 260–265.
Yang, Z., Xu, F., Li, H., and He, Y. (2021) Beyond samples: a metric revealing more connections of gut microbiota between individuals. *Computational and Structural Biotechnology Journal* **19**: 3930–3937.
Ye, Y. and Doak, T.G. (2009) A parsimony approach to biological pathway reconstruction/inference for genomes and metagenomes. *PLoS Comput Biol* **5**: e1000465.
Zhukova, M., Sapountzis, P., Schiøtt, M., and Boomsma, J.J. (2017) Diversity and transmission of gut bacteria in *Atta* and *Acromyrmex* leaf-cutting ants during development. *Front Microbiol* **8**: 1942.
Ziegler, R. and Vanantwerpen, R. (2006) Lipid uptake by insect oocytes. *Insect Biochemistry and Molecular Biology* **36**: 264–272.