1 **Gut microbial composition in different castes and developmental stages of the**
2 **invasive hornet *Vespa velutina nigrithorax***

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27

28 ## Abstract

29 Social insects are successful animal invaders. Their survival and success, and in some cases also
30 their impact on invaded ecosystem functioning, is often mediated by symbiosis with
31 microorganisms. Here, we report a comprehensive comparative characterization of the gut
32 microbial communities of different castes and developmental stages of the invasive hornet *Vespa*
33 *velutina nigrithorax*. The species recently colonized Europe, becoming a high ecological and
34 economic concern, as it threatens pollinator survival and competes with native hornet species. We
35 used targeted meta-genomics to describe the yeasts and bacteria gut communities of individuals
36 of different reproductive phenotypes (workers and future queens), life stages (larvae, newly
37 emerged individuals and adults) and colony non-living samples (nest paper and larval faeces).
38 *Bacilli*, *Gammaproteobacteria*, *Actinobacteria*, *Alphaproteobacteria* were the most abundant
39 classes of bacteria, and *Saccharomycetes*, *Dothideomycetes*, *Tremellomycetes* and *Eurotiomycetes*
40 were the most represented yeast classes. We found that the microbial compositions significantly
41 differ across developmental stages and castes, with yeast and bacterial communities switching in
42 frequency and abundance during ontogeny and according to reproductive phenotype. Moreover,
43 the gut microbial communities poorly mirror those found in the nest, suggesting that hornets
44 possess a specific microbial signature. Our results provide the first metagenomic resource of the
45 microbiome of *V. velutina* in Europe and suggests the importance of considering life stages,
46 reproductive phenotypes and nest influence in order to obtain a comprehensive picture of social
47 insect microbial communities.

48

49 **Keywords**: gut microbiome; Vespidae; social insects; alien species; yeasts; bacteria

50

51 ## Introduction

52

53 A major feature of the current 'Anthropocene' epoch is the fast and increasing loss of
54 biodiversity, which affects the functioning of natural ecosystems and threatens human well-being.
55 Insects are among the animal taxa that are suffering the most marked decrease in their abundance
56 and diversity: a recent study estimated a loss of 75% of the biomass of flying insects in protected
57 areas in Germany over the last 30 years (Leather, 2018). Such a huge loss is especially worrying
58 when regarding important ecosystem services' providers such as social insects. Many species of
59 bees, wasps, ants and termites are involved in pollination, predation, seed dispersal and nutrient
60 recycling to such an extent that they are often considered among "the little things that run the
61 world" (Wilson, 1987; Del Toro et al., 2012). In addition to providing essential ecosystem services,
62 some social insect species also cause severe negative impacts on the environment as well as on
63 human activities, as in the case of accidental species translocation from native to new habitats (i.e.
64 biological invasions) (Chapman and Bourke, 2001). The changes in social insect diversity,
65 abundance and community composition is likely mirrored by changes in ecosystem functioning,
66 potentially leading to what has been defined as an "Ecological Armageddon" (Leather, 2018; but
67 see also Wagner, 2019; Cardoso et al., 2019; Outhwaite et al., 2020).

68 The interaction between social insects and their environment, and consequently their
69 impact on it, can be mediated by symbiosis with microorganisms (Zientz et al., 2005; Hughes et al.,
70 2008). Social insects host a wide range of microorganisms, with a particularly rich community
71 inhabiting the intestinal tract. Gut microbial communities can strongly influence their survival and
72 fitness by mediating crucial life history traits such as nutritional ecology, immunity and
73 reproduction (Koch and Schmid-Hempel, 2011; Mutinelli, 2011; Rosengaus et al., 2011; Engel and
74 Moran 2013; Meriggi et al., 2019). Moreover, symbiosis with gut microorganisms underlie some of

75 the most astonishing and important functions, namely cellulose digestion from wood fibres in
76 termites as well as nitrogen fixation in herbivorous ants (Radek, 1999; Pinto-Tomás et al., 2009;
77 Suen et al., 2010; Brune and Dietrich, 2015). Furthermore, social insects may also positively affect
78 reproduction, survival and dispersal of gut microorganisms, as it has been suggested for the
79 economically significant *Saccharomyces* yeasts which inhabit social wasps guts (Stefanini et al.,
80 2012; Dapporto et al., 2016).

81 Understanding the diversity and composition of gut microbial communities, as well as how
82 they change and interact with their social insect host features, is therefore of overwhelming
83 importance to understand the interaction between social insects and their environment. There is
84 also potential to use this knowledge to manage the positive and negative impacts of social insects
85 on ecosystems and human activities. For example, the understanding of honeybee gut microbiota
86 is a promising tool to improve its health, thus securing and increasing its pollination services
87 (Anderson and Ricigliano, 2017). On the other hand, the manipulation of the microbiota in pest
88 and invasive species may result in significant practical applications for the development of
89 management strategies (Microbial Resource Management' (MRM) (Douglas, 2007; Crotti et al.,
90 2012).

91 To exploit gut microbial communities in a MRM approach, a first crucial step is to
92 undertake their description in terms of diversity and functionality (Crotti et al., 2012). Despite the
93 growing body of knowledge on social insect gut microbiota that has been gathered during the last
94 decades, the variability and functions of social insects' microbial communities still suffers from
95 some significant limitations. First, the taxonomic coverage is limited, with most knowledge
96 focused on model organisms or a few particularly economically relevant species (taxonomic
97 limitation). In fact, while more than 15000 species of social insects have been described, detailed
98 study on the gut microbiota have been carried out only for a handful of species (mainly the

99 honeybee *Apis mellifera*, and few species of ants and termites) (Engel and Moran, 2013). Current
100 studies suffer also from poor phenotypic coverage (phenotypic limitation); for instance only a few
101 studies examined in detail the intraspecific variation in microbial communities by sampling many
102 different phenotypes (such as different morphs, or different castes) and/or investigating the
103 dynamic changes through individual life (Berlanga et al., 2011; Diouf et al., 2015; Kapheim et al.,
104 2015; Otani et al., 2019). Finally, studies usually focus on either the bacterial or the yeast
105 communities, rarely describing both at the same time.

106 Here, we provide a comprehensive characterization of the gut microbial communities
107 (yeast and bacteria) of a social insect of recent economic and ecological interest, the invasive
108 yellow-legged hornet *Vespa velutina* Lepetier 1836 (hereafter Vv), with a detailed sampling which
109 considered intraspecific phenotypic variability and ontogenetic dynamics. Vv is an invasive hornet
110 species native of South East Asia (Monceau et al., 2014; Monceau et al., 2017). Its presence
111 (specifically the subspecies *V. velutina nigrithorax* DuBuysson 1905) was first recorded in the
112 South of France, in 2004 and has since then rapidly spread across most Europe (Villemant et al.,
113 2011a, b; Robinet et al., 2018; Granato et al., 2019). Vv has significant ecological, economic and
114 sanitary impacts, leading to it being listed among the invasive alien species of most concern for
115 Europe (Haxaire and Villemant, 2010; Requier et al., 2019) (COMMISSION IMPLEMENTING
116 REGULATION (EU) 2016/1141). The species' ecological impact is due to its heavy predation on a
117 vast array of insect species (Villemant et al., 2011b) and competition with native species sharing a
118 similar ecological niche (Cini et al., 2018). Economically, the main threat posed by Vv is on
119 beekeeping activities, as the yellow-legged hornet is a specialized predator of honeybees
120 (Monceau et al., 2014). Finally, Vv might also have an impact on human health since
121 envenomation of Vv can induce severe allergic or toxic reactions, resulting in organ failure and
122 death (Chugo et al., 2015; Liu et al., 2015).

123 The great concern due to Vv arrival, diffusion and impact in Europe, and other invaded
124 countries (Korea, Japan), has boosted research on life-history traits, ecology and behaviour of the
125 species. Currently, however, there is almost no information about gut microbial composition for
126 hornet species in general, and Vv in particular. The only study so far carried out on Vv has been
127 performed in another invaded region (Korea) and it investigated gut bacterial communities, but
128 not yeast (Kim et al., 2018).

129 By using a metataxonomic approach, we aim to address three main research questions
130 concerning the gut microbial composition of Vv. Firstly, we provide a comprehensive
131 characterization of bacterial and yeast gut communities of this species at the front of the invasion
132 in the newly introduced range (Europe), evaluating its richness and diversity. Secondly, we explore
133 to what extent the gut microbiota is conserved, or not, across life stages and across different
134 reproductive phenotypes. Finally, we are interested in understanding how much the species' core
135 microbiota is shared with the nest, which is one of the most likely environmental sources of
136 microbial communities for a hornet. These three research questions are instrumental in providing
137 information of generic importance (i.e. the first characterization of the gut microbiota of this
138 invasive pest species and the understanding of the dynamics of insect gut microbiota across life
139 stage and phenotypes and its resemblance with the nest) as well as opening potential future
140 avenues of research for applied perspectives on this species.

141

142 **Materials and Methods**

143

144 *The model species V. velutina: life-history and colonial cycle*

145 Vv is a social species with an annual colony cycle (Spradbery, 1973; Matsuura and Yamane, 1990;
146 Monceau et al., 2014). New colonies are founded in spring by single mated queens that survived
147 the wintering diapause (Monceau et al., 2014). By early summer workers start to emerge and take
148 over the duties of rearing the immature brood and providing for the growing colony. Adult
149 workers catch and manipulate preys and provide them to developing larvae, which, in exchange,
150 regurgitate drops of liquid, rich in semi-digested nutrients (aminoacids), for the colony adults.
151 Before reaching the pupal stage, larvae expel the entire content of their digestive tract which
152 remains at the bottom of the nest cell (meconium). At the end of summer, the colony reaches its
153 peak in terms of number of individuals and the new generations of sexuals (males and
154 reproductive females, i.e. gynes) are produced. After emergence reproductive individuals stay in
155 the nest, where interactions exchange frequently occurs also among adults; mating takes place in
156 autumn, after that, only mated future queens will survive and enter hibernation (Monceau et al.,
157 2014). As other Vespidae species, Vv build their nest combs with paper material made by
158 collecting plant fibers in the surrounding environment and then by mixing this plant material with
159 saliva (Matsuura and Yamane, 1990; Monceau et al., 2014).

160

161 *Hornets sampling*

162 Vv combs with sealed brood were collected during October and November 2016 in the
163 surroundings of Ventimiglia (Liguria, Italy), from 4 nests that were gathered by local beekeepers
164 (coordinates: N1: 43°49’00”N 7°46’00”E; N2: 43°51’44”N 7°51’14”E; N3: 43°53’01”N 7°50’73”E;
165 N4: 43°51'21" N 7°43'34"E). We collected the following samples from each nest: nest paper (a
166 piece of approximately 1 cm^2, from the wall of a cell randomly chosen); meconium, i.e. the waste

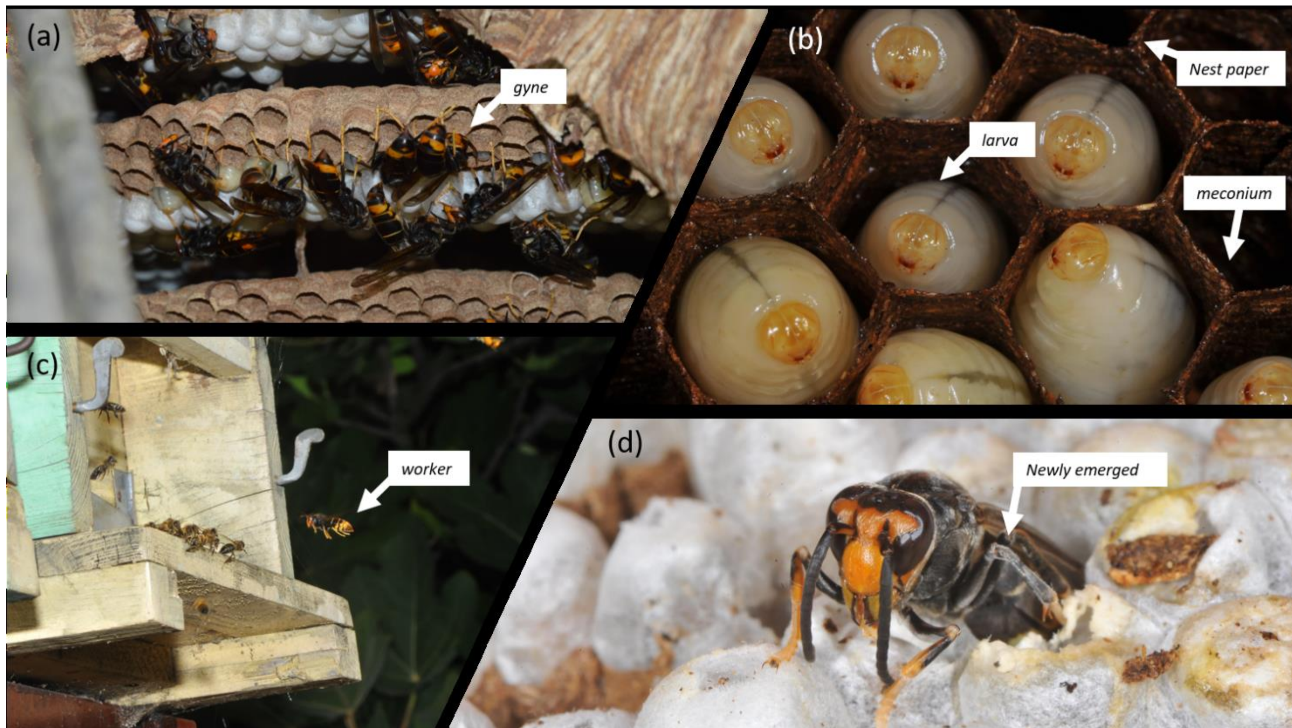
167 products of larvae, randomly chosen from a cell; larvae (L4-L5 instars); newly emerged females. To
168 be sure that newly emerged females did not have the chance to exchange liquids with other
169 individual or get “contaminated” in any other way, we we collected individuals which were ready
170 to emerge from caped cocoons, easily recognized by their exoscheleton consistence and colour,
171 and by the fact that they were moving. Moreover, we collected adult gynes and workers. Gynes
172 were sampled directly from the nest combs. We first considered as gynes the biggest females on
173 the comb which, during sampling, did not defend the comb but rather absonded behind it. Their
174 reproductive phenotype was then confirmed during dissection by looking at the abundance and
175 colour of fat bodies: as gynes have to overwinter while workers do not, fat storage allows to
176 distinguish between workers (no or almost absent fat storage) and gynes (abundant fat storage)
177 (Cini et al., 2018; Cappa et al., 2019). Workers were sampled at apiaries, in order to be sure about
178 their foraging phenotype (i.e. while they were foraging by preying on bees). Workers were
179 sampled in October 2017 in three apiaries (A1: 43°54'09"N, 7°50'34"E; A1: 43°59'17"N, 7°47'34"E;
180 A3: 43°49'24"N, 7°44'24"E) in the same area where colonies were collected. All collected samples
181 were immediately stored in RNAlater solution (Invitrogen – Thermo Fisher Scientific) at -20°C until
182 DNA extraction. In order to be protected by hornet stings we wore specific hornet protective suits
183 (EDILGRAPPA Commerciale S.r.l.) as normal beekeeper suits are not enough to protect from stings.
184 We gathered 32 samples in total, distributed as it follows: 8 from workers, 5 from adult
185 gynes, 3 from newly emerged females, 4 from larvae, and 12 non-insect samples (9 meconium
186 samples and 3 nest paper samples). All categories had samples from at least two different
187 nests/apiaries (details in Supplementary Material, file bacteria_samples.csv and fungi_sample.csv).

188

189 **Figure 1:** The model system, *Vespa velutina*, and the several living and non-living samples
190 analysed: a) view of Vv combs from inside the nest, with adults and pupal cocoons, a gyne (see

191 material and methods for identification) is indicated; b) close-up photo of a comb, with several Vv
192 larvae; larva, nest paper and meconium (i.e. larval faeces) are indicated; c) a Vv worker is foraging
193 in front of a honey bee hive; d) a newly emerged Vv (photo credits: a) Laura Bortolotti; b), c) and
194 d) Antonio Felicioli).

195



196

197 *DNA extraction and rRNA genes sequencing*

198 The insect surface was sterilized with 70% ethanol. All insect samples were dissected and the guts
199 (midgut and hindut for adults, whole intestinal sac in larvae) were collected in RNAlater storage
200 and stabilization solution (Invitrogen – Thermo Fisher Scientific) in sterile microcentrifuges tubes
201 and stored at -20°C until DNA extraction. Meconium and nest paper samples were collected and
202 stored using the same protocol. Microbial DNA extraction from all samples were performed using
203 DNeasy PowerLyzer PowerSoil Kit (QIAGEN) following the manufacturer's protocol. Total genomic
204 DNA was then quantified by Tecan Quantification Device (Life Sciences). DNA sequencing was
205 carried out by MiSeq - Illumina platform at BMR Genomics (BMR Genomics sequencing service of

206 University of Padova, Italy, https://www.bmr-genomics.it/). For the study of Bacteria
207 communities, DNA sequencing was carried out on V3-V4 region of the 16S rRNA. V3-V4 region of
208 the 16S rRNA genes was amplified using the primers Pro341F: 5′-CCTACGGGNBGCASCAG-3′ and
209 Pro805R: 5′-GACTACNVGGGTATCTAATCC-3′ (Takahashi et al., 2014). For the study of Fungi
210 communities, sequencing was carried out targeting the second internal transcribed spacer (ITS2
211 region). In this case amplification was performed usign the primers ITS3: 5′-
212 GCATCGATGAAGAACGCAGC-3′ and ITS4: 5′- TCCTCCGCTTATTGATATGC-3′ (White et al. 1990).

213

214 *Sequences pre-treatment and OTUs picking*

215 Overall sample quality was evaluated with FastQC program (Andrews, 2010). For raw reads quality
216 filtering, primers and other Illumina adapters were removed with Cutadapt (Marcel, 2011), and
217 low-quality end of forward and reverse reads were trimmed using Sickle (Joshi and Fass, 2011;
218 quality cut-off of 20 and a length threshold after trimming of 200). MICCA pipeline (ver. 1.7.2,
219 Albanese et al., 2015) was used for OTUs picking as follows: forward and reverse reads were
220 joined with "*micamergepairs*" command and default parameters, reads with N bases were
221 discarded with command *micca filter*. OTUs picking, denoising, and chimera checking was
222 performed with "*micca otu*" command and using the UNOISE3 protocol (Edgar, 2016) as
223 picking/denoising algorithm. UNOISE3 performs exact amplicon sequence variants (ASV)
224 reconstruction based on single sequences abundance (i.e. for each sequence, if the abundance is
225 above a threshold the sequence represents a new ASV, if not it is joined with the nearest ASV). In
226 this text, we will use the "OTU" word as synonym of ASV, not with the meaning of similarity-based
227 OTUs but with the meaning of the smaller taxonomic unit considered. Taxonomy was assigned to
228 the representative sequences of the identified OTUs using the RDP classifier and database (ver

229 2.11, Wang et al., 2007) for 16S rRNA sequences, and using the RDP classifier and the UNITE
230 database (Nilsson et al., 2018) for ITS sequences.

231

232 *Statistical analysis*

233 All statistical analyses were performed into R environment (version 3.4.4, R Core Team 2017).
234 Ordinational analyses were computed using Principal Coordinate Analysis based on Bray-Curtis
235 distance with “ordinate” function (phyloseq package version 1.23). Singletons, namely OTUs
236 counting to one in all the dataset, were removed before distance calculation to dampen the
237 hypothesis that extremely rare species may impact the overall distribution of samples. After
238 singleton removal, counts were scaled using DESeq2 package (Love et al., 2014) to account for
239 different sample depth across all samples (‘counts’ function with ‘norm = TRUE’). Scaled counts
240 were used for Bray-Curtis calculation whereas binary data (presence/absence matrix) were used
241 for Sorensen index calculation. Ordinations were analysed using Permutational Multivariate
242 Analysis of Variance (PERMANOVA) using distance matrices as implemented in the “adonis2”
243 function (vegan package version 2.5). Nest origin was used as blocking factor far PERMANOVA
244 analysis to inspect whether different environments had a different microbiome distribution while
245 controlling for the effect of nest. Variation in diversity and composition between sample groups
246 were also assessed using by PERMANOVA pairwise. Beta diversity between developmental stages
247 was computed using the Sorensen index as S = (B + C)/(2A + B + C), with A = shared species among
248 groups, B = species from group B and C = species from groups C (“betadiver” function of vegan
249 package 2.5). Beta diversity indices were calculated using the veg dist function (vegan package
250 2.5). The influence of phenotypes on alpha diversity was inspected thorough one-way analysis of
251 variance (ANOVA) as implemented in the “aov” function of stats native R package. Different alpha
252 diversity indexes were tested: richness (expressed as the number of OTUs detected in a sample),

253 Inverse Simpson index (expressed as 1/D, where D is the Simpson index (Simpson 1949)), and
254 Sorensen index (Shannon 1948). Species evenness was also calculated and tested using the
255 Pielou's formulation: H/log(R); where H is the Shannon diversity index and R is the richness
256 (Pielou EC (1966). All alpha diversity indexes were computed using the vegan package, version 2.5
257 (Oksanen et al., 2019). Differentially abundance OTUs detected assessed using DESeq2 (Love et al.,
258 2014). The influence of sample type was assessed using likelihood ratio test (LRT, "deseq"
259 function) and p-values were adjusted using the Benjamini and Hochberg approach (also known as
260 "false discovery rate") to reduce type I errors. Colony or apiary of origin was included in the
261 analyses as "origin".

262

263 Results

264 *Microbial community composition*

265 After quality filtering, a total of 2,609,129 16S rRNA and 953,185 ITS reads were obtained, ranging
266 from 3,055 to 143,037 and from 799 to 154,965 in all samples, respectively. The number of reads
267 reported a standard deviation of 30,901.17 (for 16S rRNA) and of 37,901.26 (for ITS). A summary
268 of the number of pre-filtering, post-filtering reads for each *sample type* is reported in
269 supplementary file 1. After singleton removal a total of 2,655 OTUs from 16S dataset and 999
270 OTUs from ITS dataset were obtained (details were reported in supplementary file 1).

271 The bacterial taxa detected in all samples corresponded to the following main phyla by the order
272 of rank abundance: *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. At the class-level
273 the main taxa were represented by *Bacilli*, *Gammaproteobacteria*, *Actinobacteria*,
274 *Alphaproteobacteria* (Figure 2f). The fungal phyla detected in all samples corresponded to

275 *Ascomycota*, *Basidiomycota and Zygomycota.* The main related class were *Saccharomycetes*,

276 *Dothideomycetes*, *Tremellomycetes* and *Eurotiomycetes* (Figure 3f).

277

278 *Gut microbiota shapes across life stages and phenotypes*

279 *Sample type* (life stage samples, as well as non-insect samples) significantly shaped the community

280 structure for both bacteria and fungi. PERMANOVA using distance matrices was statistically

281 significant for the *sample type* ($p < 0.001$ and R^2 statistic indices 0.45 and 0.4 respectively for

282 bacteria and fungi; Table 1). Thus, the analysis showed that the *sample type* influenced the

283 community structure, and this occurred for each developmental stage and for the non-insect

284 samples (meconium and nest paper).

285

286 **Table 1**: PERMANOVA performed on the *sample type* for both bacterial and fungal communities.

		Df	**SumOfSqs**	**R^2**	**F**	**Pr(>F)**
16S	Sample type	5	5.240	0.459	4.417	<0.001
	Residual	26	6.169	0.541	/	/
	Total	31	11.409	1	/	/
ITS	Sample type	5	4.632	0.401	3.486	<0.001
	Residual	26	6.910	0.599	/	/
	Total	31	11.542	1	/	/

287

288

289 In accordance with PERMANOVA, Principal Coordinate Analysis (PCoA; Bray-Curtis distance)

290 suggested that both Bacteria and Fungi communities differed on the basis of *sample type*, as

291 demonstrated by the separation of points on the ordination plane, roughly based on *sample type*

292 groups (i.e. see color of points) as showed in Figure 2a,b,c and Figure 3a,b,c. When all the samples

293 were included in the analysis, bacterial community on nest paper clearly differed compared to all
294 other sample types (Figure 2a), while the same condition was less evident for Fungi (Figure 3a),
295 where a partial overlap between nest paper and meconium (i.e. the not-living sample) was
296 evident. Despite all cross-comparisons between *samples type* were significant, nest paper sample
297 showed the most pronounced differences compared to all other samples (Figure 2d and Figure 3d,
298 see color intensity of the first column in the heatmap of R^2 , reported for all pairwise PERMANOVA
299 comparisons). In addition, nest paper samples showed high within-sample similarity in community
300 structure, as suggested by the complete overlap of samples coming from different nests in the
301 ordination plane (Figure 2a,b and Figure 3a,b, see the position of the blue points in overlap to each
302 other in the PCoA; Figure2e, Figure 3e, see the high level of similarity showed by Sorensen index,
303 as indicated by the yellow color, and the value distributions represented by ridgeline plot in the
304 nest-nest cross-comparison). On the other hand, a clear separation on the basis of developmental
305 stage can be observed when only living samples were considered (Figure 2c and Figure 3c),
306 showing a sharp stage -related clusters. Overall, adult stages (i.e. workers and gynes) appeared
307 separated from immature stages (i.e. larvae and newly emerged) as displayed by the first PCoA
308 (Figure 2c, Figure 3c, explaining 21.4 and 20.2% of variance, respectively) and hierarchical
309 clustering analysis (UPGMA method based on Bray-Curtis distance; Figure 2f, Figure 3f), and this
310 was especially evident for Bacteria, while Fungi seemed to hold a more stage-specific community
311 signature.

312 Results from PCoA were in accordance with beta diversity analysis performed using Sorensen
313 similarity index that showed profiles of similarity based on the distribution of values from
314 Sorensen index plotted by ridgeline plot that showed a cross-comparison between all sample
315 groups (Figure 2e and Figure 3e). Panel e of figure 2 and 3 shows the distribution of the Sorensen
316 index for all sample types. The analysis showed a distribution similar to the one reported in the

317 PCoA analysis (panels a, b, and c of figure 2 and 3). Nest paper samples were highly conserved (i.e.
318 contrasts within samples of the same type reported a high similarity level whereas they displayed
319 a high level of dissimilarity compared to other sample types). Gynes paired in similarity with newly
320 emerged and the same condition was visible for larvae and meconium. Gynes and newly emerged
321 females showed more shared phylotypes compared to the other samples and the same condition
322 was reported for larvae and meconium (Sorensen similarity index, Figure 2 and 3 panel e).

323 *Sample type* affected alpha-diversity indices though the effect was more relevant regarding the
324 mycobiota (Table 2) and no significant effect has emerged considering the association with the
325 nest (origin). Alpha diversity indices in fungi were much more influenced by the *sample type*
326 variable than Bacteria, indicating that yeasts constitute a better signature of gut microbiota
327 comparing to the Bacteria. The worker and gyne stages resulted to be dominated by
328 *Saccharomycetes* that hierarchically decreased in newly emerged, larvae, meconium, and finally
329 appear almost absent in nest paper samples. On the other side, the most represented bacterial
330 classes across all samples results to be the following class, *Bacilli* and *Gammaproteobacteria*
331 mainly represented in workers, gynes and newly emerged.

332

333 **Table 2**: Alpha diversity indices table reporting Evenness, Inverse Simpson, Richness (referred as
334 Number of OTUs) and Shannon diversity for 'sample type' and 'origin' variables. All diversity
335 indices were reported for both 16S and ITS datasets. (∗ $p < 0.05$, ∗∗ $p < 0.01$).

	variable	**term**	**df**	**sumsq**	**meansq**	**statistic**	**p.value**	**adj.p**	**sign**
16S	Evenness	Sample type	5	0.11	0.02	0.77	0.58	0.58	
	Evenness	Origin	5	0.21	0.04	1.43	0.26	0.51	
	InvSimpson	Sample type	5	4900.48	980.10	4.20	0.01	0.02	*
	InvSimpson	Origin	5	1140.05	228.01	0.98	0.46	0.46	
	Number of OTUs	Sample type	5	428144.98	85629.00	5.71	0.00	0.00	*

									*
	Number of OTUs	Origin	5	61735.93	12347.19	0.82	0.55	0.55	
	Shannon diversity	Sample type	5	4.41	0.88	0.84	0.53	0.53	
	Shannon diversity	Origin	5	6.15	1.23	1.18	0.35	0.53	
ITS	Evenness	Sample type	5	0.90	0.18	4.57	0.01	0.01	*
	Evenness	Origin	5	0.35	0.07	1.77	0.16	0.16	
	InvSimpson	Sample type	5	3429.66	685.93	11.21	$2.33E^{-05}$	$4.65E^{-05}$	**
	InvSimpson	Origin	5	270.51	54.10	0.88	0.51	0.51	
	Number of OTUs	Sample type	5	180574.35	36114.87	37.79	$8.31E^{-10}$	$1.66E^{-09}$	**
	Number of OTUs	Origin	5	9512.39	1902.48	1.99	0.12	0.12	
	Shannon diversity	Sample type	5	36.27	7.25	12.20	$1.25E^{-05}$	$2.50E^{-05}$	**
	Shannon diversity	Origin	5	5.37	1.07	1.81	0.16	0.16	

336

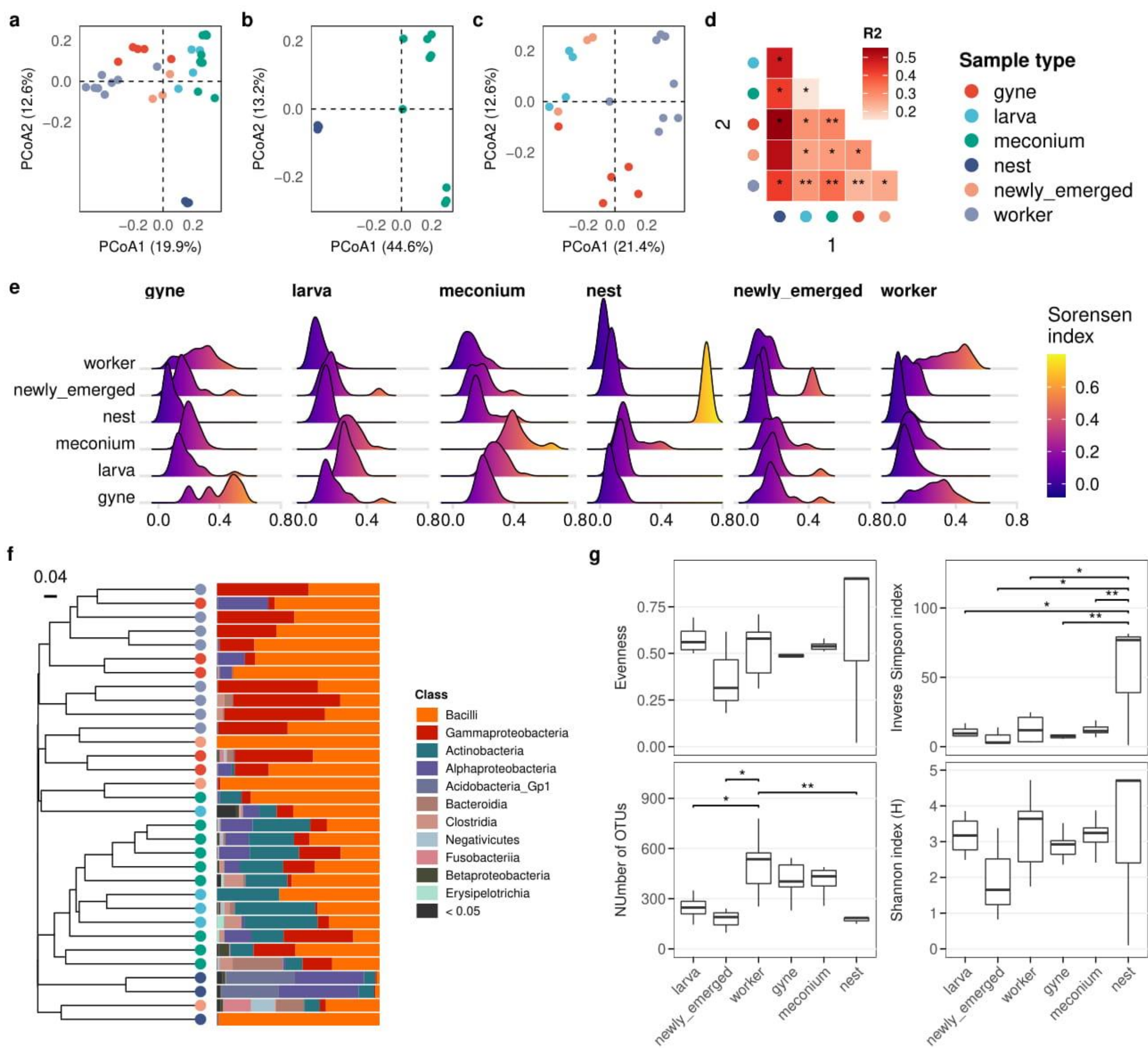
337

338 Workers showed a significant higher richness rank compared to the meconium, newly emerged
339 and nest paper samples but this does not result in a highest level of diversity compared to the
340 other *sample types* (Figure 2g). Richness and diversity of bacterial communities significant differed
341 among *sample types*, with nest communities showing higher differences compared to all other
342 *sample types*, as displayed by Inverse Simpson index (Figure 2g).

343

344 **Figure 2**: Bacterial community diversity and composition: Principal coordinate analysis (PCoA)
345 based on Bray Curtis distance reporting all sample types (a), only non-living samples (meconium
346 and nest paper samples) (b) and only living samples (gynes, workers, newly emerged and larvae
347 samples) (c). R^2 values from pairwise adonis Permutational analysis of variance test on community
348 composition displayed as a red gradient cells, from light red (lower R values) to dark red (higher R
349 values) in a combination matrix with cross-comparisons between all samples (d) together with the
350 p-value of each contrast (*, p < 0.05; **, p < 0.01). Pattern of similarity between all different

351 sample types performed using Sorensen index. Similarity was mapped as a color gradient, from
352 blue (lower similarity index) from yellow (higher similarity index); the distribution of values was
353 displayed by Ridgeline plot (e). Relative abundance (%) taxa bar plot of bacterial community
354 composition at class level reported for all sample types (f); the dendrogram above the bar plot
355 hierarchically represents the clustering of the microbiota profiles based on Bray-Curtis distance.
356 Box-plots showed alpha diversity indices, including Evenness, Inverse Simpson, Number of OTUs
357 and Shannon diversity (g) (∗ $p < 0.05$, ∗∗ $p < 0.01$).



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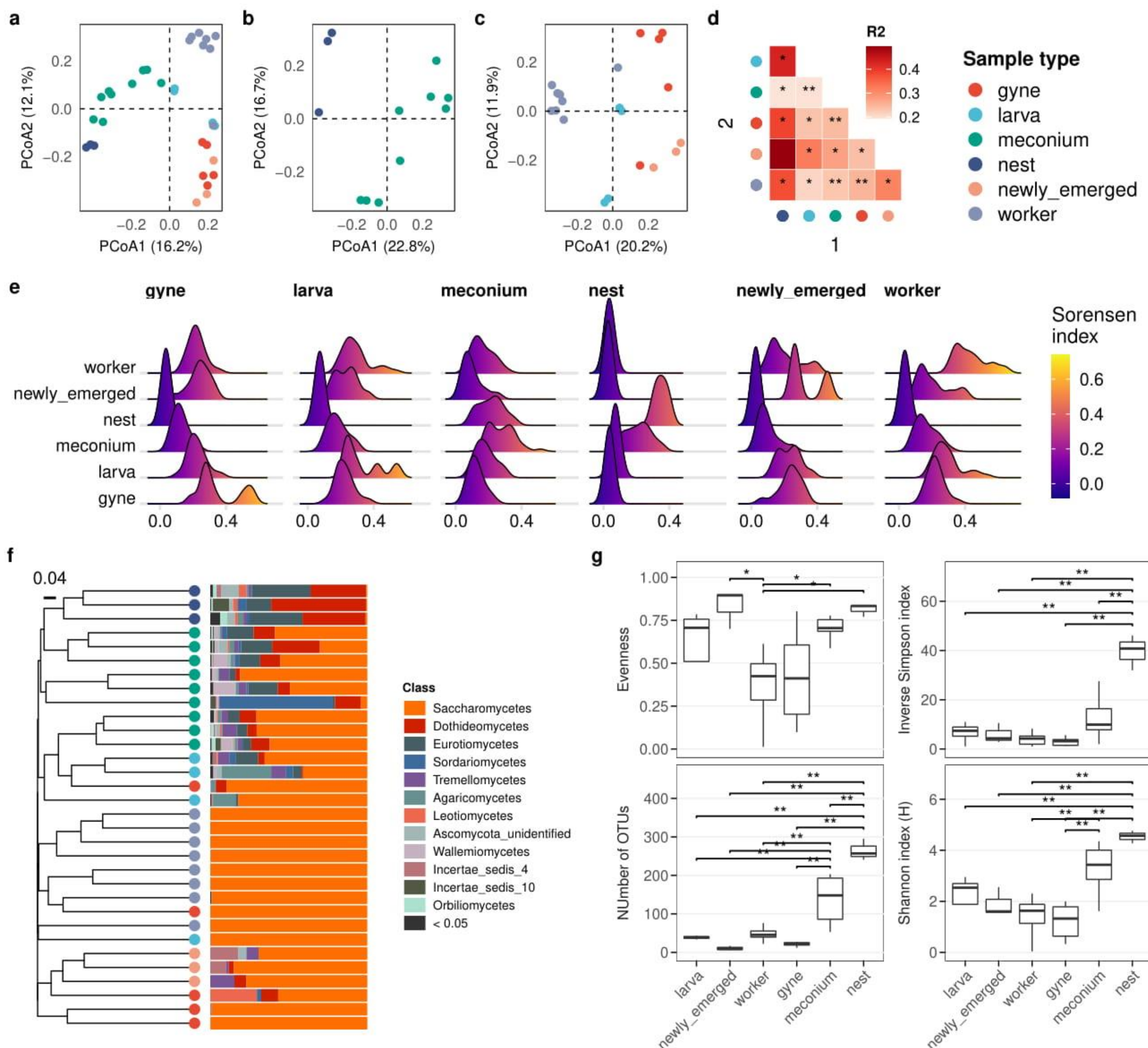
361 **Figure 3**: Fungal community diversity and composition: Principal coordinate analysis (PCoA) based
362 on Bray Curtis distance reporting all sample types (a), only meconium and nest paper samples (b)
363 and only gynes, workers, newly emerged and larvae samples (c). R^2 values from pairwise adonis
364 Permutational analysis of variance test on community composition displayed as a red gradient
365 cells, from light red (lower R values) to dark red (higher R values) in a combination matrix with
366 cross-comparisons between all samples (d) together with the p-value of each contrast (*, $p < 0.05$;
367 **, $p < 0.01$). Pattern of similarity between all different sample types performed using Sorensen
368 index. Similarity was mapped as a color gradient, from blue (lower similarity index) from yellow
369 (higher similarity index); the distribution of values was displayed by Ridgeline plot showing the
370 distribution of Sorensen index values according to the sample types (e). Relative abundance (%)
371 taxa bar plot of fungal community composition at class level reported for all sample types (f); the
372 dendrogram above the bar plot hierarchically represents the clustering of the fungal profiles based
373 on Bray-Curtis distance. Box-plots showed alpha diversity indices, including Evenness, Inverse
374 Simpson, Number of OTUs and Shannon diversity (g) (∗ $p < 0.05$, ∗∗ $p < 0.01$)



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378 *Relative abundance and taxonomic distribution*

379 We detected differential relative abundant OTUs among all sample types using DESeq2 likelihood-

380 ratio-test (LRT, alpha < 0.05). *Lactobacillus* and *Bifidobacterium* showed the most differently

381 abundant pattern mainly represented in gynes, larvae, and meconia (Figure 4 G, L, M). Similarly,

382 *Lactococcus* was one of the most represented genera, together with *Lactobacillus* and

383 *Bifidobacterium. Actinobacteria* (*Bifidobacterium*, *Alphaproteobacteria*) and genera inside *Bacilli*

384 phylum (*Lactobacillus, Fructobacillus, Leuconostoc, Enterococcus, Staphylococcus, Streptococcus*
385 *and Bacillus*), with the exception of *Lactococcus,* represented the most abundant pattern
386 associated with meconia. Furthermore, it showed a similar abundance distribution of
387 *Betaproteobacteria, Clostridia*, and genera from *Gammaproteobacteria* (*Frischella*, *Gilliamella* and
388 *Diplorickettsia*)*,* thus representing the microbial communitites displayed among all sample types
389 (Figure 4). Larvae and meconia showed the most similar pattern of abundance, even if the larval
390 samples were less rich in *Alphaproteobacteria*, *Betaproteobacteria*, *Gilliamella* and *Diplorickettsia*
391 (Figure 4 L, M). Interestingly, newly emerged females and nest paper samples showed the poorest
392 relative abundance distribution of the entire dataset (Figure 4 N, NE). Gyne and worker samples
393 harbored as main abundant phyla *Bacilli* and *Gammaproteobacteria* (Figure 2f and Figure 3f),
394 however, both showed some quite distinct imbalance in characteristic differentially abundant
395 taxonomic groups. Indeed the workers were less abundant in *Lactobacillus*, *Alpharoteobacteria*
396 and *Actinobacteria* but richer in the genera from phylum *Gammaproteobacteria*, such as
397 *Buttiauxella* (Figure 4 G, W). Looking inside *Gammaproteobacteria*, overall mainly represented in
398 workers, we found as the most abundant taxa, *Klebsiella*, *Enterobacter* and *Escherichia/Shigella*,
399 also widely represented in gynes with the exception of *Buttiauxella,* one of the most abundant
400 genus reported in worker samples (Figure 4 G, W). Concerning the genera inside *Bacilli* class, it is
401 noteworthy the difference between adult stages (gynes and workers), with respect to the larvae
402 and meconium samples. In fact, a different abundance distribution of taxa classified as genus
403 *Lactobacillus* was evident among those groups (with most Lactobacillus taxa in larvae and
404 meconial), as well as a substantial depletion of OTUs from the *Lactocossus* genus in meconium and
405 larvae.

406 **Figure 4**:

407 Results of likelihood-ratio test (LRT) on 16S rRNA gene distribution. Figure reports all OTUs with a
408 significant effect of the sample type on their distribution according to the LRT test of DESeq2.
409 Point size represents the abundance (normalized number of reads in logarithmic scale, see
410 Statistical analysis section for additional details about normalization procedures) of selected OTUs
411 whereas colors reflect the Family attribution. OTUs with no classification at a given taxonomic
412 level were reported using the label “unknown”. Panels show the abundance in all sample types: G:
413 gynes, L: larvae, M: meconia, N: nests, NE: Newly emerged individuals, W: workers.

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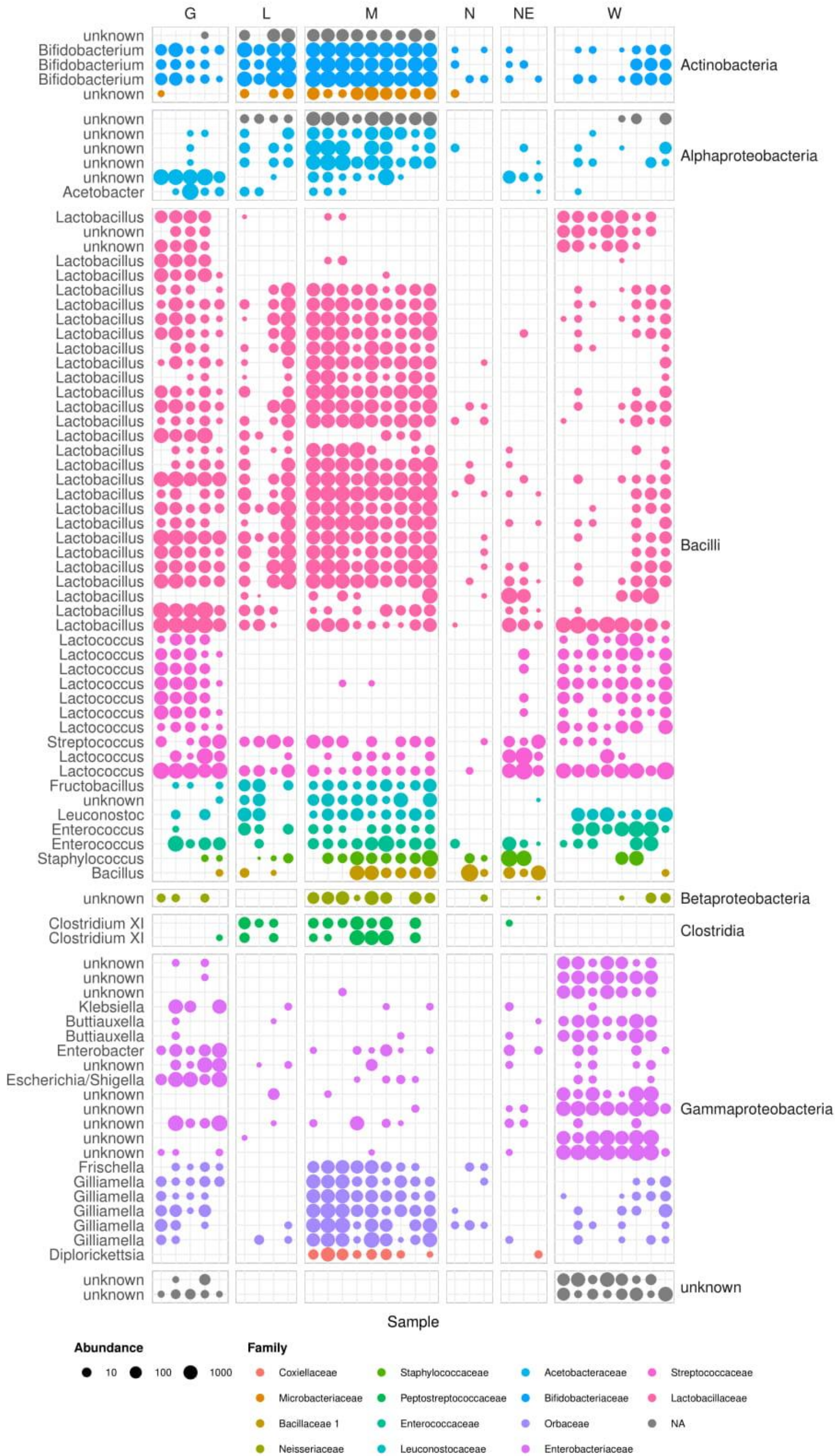

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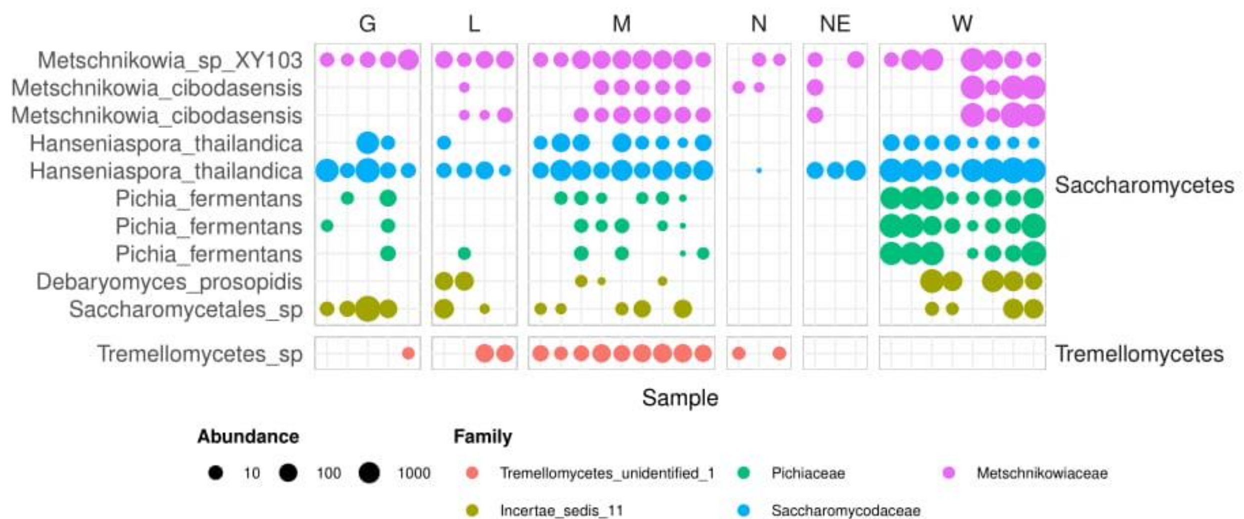
416 DESeq2 likelihood-ratio-test (LRT, alpha < 0.05) performed on fungal communities showed
417 *Saccharomycetes* as the main representative class of the entire ITS dataset. *Metschnikowia* and
418 *Hanseniaspora thailandica* proved to be widespread across all sample types, even if they resulted
419 to be less abundant in the nest paper samples (Figure 5 N, E). The analyses also showed
420 differentially abundant species within sample types, *Metschnikowia cibodasensis* absent in gynes
421 resulted mostly related to meconium and workers, while less present in the other sample types.
422 *Pichia fermentans* was one of the main abundant genera in the workers, it decreased in meconium
423 and gynes, while it was absent in newly emerged individuals and nest paper samples (Figure 5
424 M,G,NE). *Debaryomyces prosopidis* was strongly represented in larvae, meconium and worker
425 samples; similarly *Saccharomycetales* order mirrors this abundance pattern with the exception for
426 gynes; where this taxa was strongly represented. *Tremellomycetes* were the only *Basidiomycetes*
427 in the entire ITS dataset showing a high abundance level in meconium, larvae, nest and in only one
428 gyne sample. As it happened for the microbiota, also in the mycobiota the meconium is still the
429 most representative sample, in term of diversity, of the entire stage dataset.

430 **Figure 5**: Results of likelihood-ratio test (LRT) on ITS sequence distribution. Figure reports all OTUs
431 with a significant effect of the sample type on their distribution according to the LRT test of
432 DESeq2. Point size represents the abundance (normalized number of reads in logarithmic
433 scale, see Statistical analysis section for additional details about normalization procedures) of
434 selected OTUs whereas colors reflect the Family attribution. OTUs with no classification at a
435 given taxonomic level were reported using the label "unknown". Panels show the abundance
436 in all sample types: G: gynes, L: larvae, M: meconia, N: nests, NE: Newly emerged individuals,
437 W: workers.



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440 ## Discussion

441 Here we provided the first comprehensive characterization of the bacterial and fungal
442 communities of an introduced European population of the Asian hornet *V. velutina nigrithorax.*
443 We carried out an extensive metataxonomic investigation, describing both the microbiota and
444 mycobiota communities of different hornet life stages (larvae, newly emerged and adult females),
445 including two different reproductive phenotypes (workers and gynes). Our analysis also included

446 two types of not-living colonial samples, namely the meconial and the nest material (referred as
447 nest paper).

448 Overall, we identified *Bacilli*, *Gammaproteobacteria*, *Actinobacteria*, *Alphaproteobacteria*
449 as the most abundant classes of Bacteria, and *Saccharomycetes*, *Dothideomycetes*,
450 *Eurotiomycetes, Sordariomycetes* and *Tremellomycetes* as the most abundant Fungi classes. Alpha
451 diversity indices showed that the worker stage harbours a significantly higher number of Bacterial
452 OTUs compared to all other sample types, however there are no differences displayed by Shannon
453 and Evenness indices among all sample types, except for Inverse Simpson index that suggests a
454 significantly higher diversity composition of the nest compared to the other sample types.
455 Nevertheless, the alpha diversity indices suggest that the number and distribution of bacterial
456 species among all sample types are rather uniform, with the exception of some significant
457 differences as shown in Figure 2f and Figure 3f. Similarly, the same indication is displayed in the
458 alpha diversity indices analysis of Fungi community composition that follows the trend described
459 above but includes meconium as well as nest sample. Overall, alpha diversity was affected by the
460 sample type and not by the colony of origin.

461 A striking result of this study is that microbial communities showed significant sample-
462 specific signature for both Bacteria and Fungi, showing differences in composition across life
463 stages and among reproductive phenotypes. Indeed, beta diversity analyses showed a pattern of
464 sample-related clustering composition among all sample types in agreement with the specific life-
465 style of each developmental stage, indicating the presence of sample-type related microbiota and
466 mycobiota. Moreover, all sample's community composition grouped according to the hornet's
467 phenotypic change during ontogeny, showing a displacement of the communities along a
468 succession from the larval stage and the meconium up to the adult phase (workers and gynes),
469 passing through the newly emerged females. We can therefore speculate that the ontogenetic

470 development of hornets from the larva to the adult stages leads to a clear change in the intestinal
471 microbial diversity and composition. The adult stages (gynes and workers) show an increase in gut
472 bacterial richness (e.g. number of OTUs), suggesting that the hornet gut microbiota reaches the
473 higher level of bacterial load in the adult stage rather than during the larval stage. The striking
474 difference in diet of adults (sugar-based) and larvae (protein-based) is likely to play a role and
475 needs to be investigated in the future as a possible driver of such variation.

476 The main bacterial classes found in living samples were *Bacilli* and *Gammaproteobacteria*.
477 *Actinobacteria* are mostly present in the meconial samples, they decrease in larvae and adult
478 stages. The presence of *Actinobacteria* in larval stages may play a role in the developmental
479 process or nutrients digestion and defence against parasites. Cases of *Actinobacteria* symbionts
480 have been found in pyrrhocorid bugs demonstrating their role in the sterilization of the egg
481 surface (Salem et al., 2013). Different phylotypes of *Actinobacteria* have been shown to defend
482 ants, beetles and wasps against detrimental microorganisms through the production of antibiotics
483 (Kaltenpoth, 2009; Seipke et al., 2012; Visser et al., 2012) and they might also be involved in the
484 nutrient processing and acquisition (Hanshew et al., 2014; Salem et al., 2014). *Orbacea*e,
485 *Lactobacillus* and *Acetobacteraceae* were reported as the main enriched families in adult stages
486 (gynes and workers). As previously found in *Drosophila melanogaster*, bacterial communities
487 affected by fruit-sugar rich diet were also involved in the maintenance of the immune system and
488 mating preferences (Broderick and Lemaitre, 2012). These communities are often associated with
489 a fruit-based diet, which agrees with the hornet lifestyle. Indeed, adult hornets are essentially
490 generalist sugar-feeding insects and the main carbohydrate sources are: tree sap, honeydew,
491 flower nectar, ripe fruit, mushrooms and other sugary secretions (Matsuura and Yamane, 1990),
492 while larvae are carnivorous and feed on arthropods such as bees, preyed by adult stages.

493 Interestingly, bacterial genera associated with bees, such as *Frischella* and *Gilliamella,*
494 appeared heavily enriched in the meconium samples (probably due to the accumulation effect, as
495 the meconium is made up of several layers of larvae faeces). This is probably because these
496 microorganisms acquired with the diet (predation on bees) are “transient” in the gut of the larvae
497 accumulating in the meconium stratifications. Their presence in adult stages (gynes and workers),
498 quantitatively lower than in the meconium, might be explained by predation on bees.

499 *Bifidobacterium* and *Lactobacillus* were the main abundant genera displayed in our
500 dataset. Already extensively described in *Apis mellifera* as the main bacterial communities
501 responsible for a subset of functions, they are essential for the insect development and overall for
502 the maintenance of the health of the entire colony (Forsgren et al., 2012). Here, *Bifidobacterium*
503 and *Lactobacillus* were transversally distributed through the insect samples but mostly abundant
504 in the adult stages, consolidating their presence as one of the leading bacterial communities in the
505 gut of social Hymenoptera. However, the same result was not described in the gut microbiota of *V.*
506 *velutina* sampled in Korea as reported in Kim et al. 2018. The Asian hornets sampled in Korea did
507 not showed the communities described above, instead they were mainly characterized by the
508 genera Flavobacterium (24%), Aeromonas (17%), Pseudomonas (13%) and Pedobacter (12%) (Kim
509 et al. 2018). The only genera shared between our dataset and that of Kim et al. were Enterobacter
510 and Clostridium, however, in the work by Kim et al., these phylotypes were minimally represented
511 (0.08% and 0.01%, respectively), reinforcing the substantial dissimilarity of the microbiota shown
512 by the Korean and European VV vpopulations.

513 The most striking discovery is that the fungal component of the microbiome is the major
514 driver of change, clearly differentiating during the different life stages. Variation in the microbial
515 diversity indices linked to the sample types were much more related to the Fungi than Bacteria,
516 qualifying the Fungi as a better signature of the different life stages. Results of *V. velutina*

517 mycobiota in workers and gynes stages were mainly characterized by *Saccharomycetes*, which are
518 conversely poorly represented in paper nest and meconium. Instead, the paper nest and
519 meconium are dominated by *Dothidomycetes*, *Tremellomycetes* and *Eurotiomycetes*. The nest
520 material showed a completely different fungal community composition compared to the other
521 sample types as displayed by diversity and composition analyses.

522 We can assert that the hornet gut mycobiota, as well as the microbiota, was not affected
523 by the nest-associated microbial communities. This aspect is extremely interesting given that the
524 nest represents the environment where the larvae grow and develop into adult stages and take
525 part in the expansion, nursing and maintenance of the colony. The diet is potentially the best
526 explanation for the finding that *Saccharomycetes* are most abundant in adult stages, as adult
527 hornets feed on sugary sources, such as fruits. This reinforces the importance of the Hymenoptera
528 in the dispersion and overall in the ecology of these sugar-related yeast (Stefanini, 2018).

529 The main relatively abundant taxa representative of the ITS dataset were *Metschnikowia*
530 *cibodasensis*, *Hanseniaspora thailandica*, *Pichia fermentans*, *Debaryomyces prosopidis*,
531 *Metschnikowia*, *Saccharomycetales* and *Tremellomycetes.*

532 *Pichia fermentans* is an Ascomycete known for its fermentative traits and its presence in
533 many fermentative substrates but recently was also found in association with insects, such as *D.*
534 *melanogaster* and social wasps (Fogleman et al., 2014; Stefanini et al., 2012). This makes *P.*
535 *fermentans* a yeast capable of living and adapting to the gut of different insect species. *P.*
536 *fermentans,* as well as yeast from genera *Metschnikowia*, and *Hanseniaspora*, are often isolated
537 from fruit trees (Vadkertiová et al., 2012), in accordance with the diet of the adult stages, which
538 feed on sugary sources such as fruits, thus sharing the adherent microorganisms and providing
539 fermentable substrate to those already present. These results support the hypothesis that the
540 hornet gut mycobiota is affected by the life stage–related diet. Indeed, after the elimination of the

541 peritrophic sac at the end of immature life, the intestine shows a low number of communities (as
542 we found in the newly emerged stage), and then it can be easily colonized. This might explain the
543 process of colonization of the intestinal tract by *Saccharomycetes* and their strong predominance
544 in the adult stages

545 *Tremellomycetes,* found in the nest paper samples and in the meconium, is known as a
546 class that includes plant pathogen strains (Weiss et al., 2014). The presence of *Tremellomycetes* in
547 the nest paper is in accordance with the nest building matrix usually made by environmental wood
548 debris and this offers interesting insights to consider the analysis of the nest microbial
549 composition as a biomarker assay in the study of plant pathogens.

550 Finally, the discovery that the species' core microbiota in adults and larvae differ almost
551 completely with the nest's microbiota, suggests that the social wasps have a proprietary
552 microbiome. In addition to providing shelter and protection for the brood, the nest also represents
553 the locus of *Vespidae* social life (Starr, 2019). At the same time, the nest is a product of wasp
554 activity (the paper of the nest is made by vegetal fibres chewed by wasps) and is filled with hornet
555 products, the principal of which is the meconium, for instance, the residues of larval faeces. Thus,
556 our findings that nest's microbiome does not reflect the microbiota harboured by hornet guts
557 indicates that the environment in which hornets live most of their life is not a source of the
558 microbes that make the hornet's microbiome. The evolution of microbial communities during
559 ontogenetic development was previously described in other social insects, such as *Apis mellifera*
560 (Martinson et al., 2012). Our results confirm the findings that variation in the physiochemical
561 conditions of the gut in invertebrates at different life stages are a driver of development of
562 microbial communities (Lemke et al., 2003). Our results provide the "first metagenomic resource"
563 of the microbiome of *V. velutina* in Europe, and also suggests the importance of considering life
564 stages and reproductive phenotypes as well as nest influence in order to obtain a comprehensive

565 picture of insect microbial communities. The detailed information we provide is instrumental to
566 enable future comparisons with native or non-invasive hornet species, paving the way to
567 understand the microbial features that might influence species success and impact on ecosystems.

568 This metagenomic resource is also a promising tool for the development of pest management
569 strategies. Indeed, the understanding of the healthy intestinal microbiome, in terms of diversity
570 and functionality, is the first step for the management approach which uses the manipulation and
571 the exploitation of insect microbiota in order to reduce pest insect-related problems.
572 Understanding the microbial composition and diversity of the pest species is crucial for two
573 reasons (Douglas, 2007; Arora and Douglas, 2017). First, it allows us to understand how to disrupt
574 the microbial community of the pest species by targeting key components, thus reducing insect
575 pest fitness. A similar approach has been recently proposed to control termites by using
576 genetically engineered yeast as a 'Trojan-Horse' that kills the cellulose-digesting protozoa in the
577 termite gut (Sethi et al., 2014). Second, a knowledge of the pest microbial community allows us to
578 understand which microbial components have major impacts on the traits contributing to its pest
579 status (e.g. capacity to vector diseases, natural enemy resistance and host range). Interfering with
580 these components has the potential to reduce the negative impacts of the pest (as it has been
581 shown with the case of the mosquito *Aedes aegypti*, where the experimental transfer of
582 *Wolbachia* suppresses the host vector competence, reviewed in Arora and Douglas, 2017). To our
583 knowledge this approach has never been developed for hornets, and our metagenomic
584 characterization of the gut microbial community in Vv may represent a first step toward this goal.

585 Notwithstanding the somehow limited number of samples and colonies investigated due to
586 the difficulty of working with an alien species under local eradication programs, the results of this
587 study provides a springboard for future assessment of the functional role of gut microbial
588 organisms in hornets. In particular, our microbial characterization indicates specific fungal and

589 bacterial groups associated with specific life stages, thus calling for future assessment of their role
590 in the hornet survival and life-history traits. Moreover, our results represent a first step in the long
591 road to link gut microbiota to the role played by hornets in ecosystems, as well as also enabling a
592 first step toward possible management strategies of this invasive pest. Understanding the
593 microbial composition which are essential to Vv survival has the potential to indicate the target of
594 future microbial control of this pest species.

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604 **Conflicts of interest/Competing interests**

605 The authors declare no conflict of interest/Not applicable

606 **Availability of data and material**

607 Microbial community composition data is available as supplementary material (for bacteria, files:
608 bacteria_counts.csv, bacteria_samples.csv, bacteria_taxa.csv; for fungi, files: fungi_counts.csv,
609 fungi_samples.csv, fungi_taxa.csv). Sample sequences have been archived on the European
610 Nucleotide Archive (ENA), accession number: PRJEB36629.

611 ## Authors' contributions

612 AC, NM, FC, FV, DC, RC: experimental design; AC, FC: sampling; NM, FV, GB: meta-genomic and
613 bioinformatic analyses; AC, NM: MS drafting; all authors: MS reading, discussion and approval.

614

615 ## References

616 Albanese D, Fontana P, De Filippo C, Cavalieri D, Donati C (2015) MICCA: a complete and accurate
617 software for taxonomic profiling of metagenomic data. Sci. Rep. 5:9743. doi:
618 10.1038/srep09743

619 Anderson KE, Ricigliano VA (2017) Honey bee gut dysbiosis: a novel context of disease ecology.
620 Curr. Opin. Insect Sci. 22:125-132. doi: 10.1016/j.cois.2017.05.020

621 Andrews S (2010) FastQC: A Quality Control Tool for high Throughput Sequence data. In:
622 Babraham Bioinformatics (version 0.11.3). Available at: http://www.
623 bioinformatics.babraham.ac.uk/projects/fastqc (accessed March 25, 2015)

624 Arora AK, Douglas AE (2017) Hype or opportunity? Using microbial symbionts in novel strategies
625 for insect pest control. J. Insect Physiol. 103: 10-17. doi: 10.1016/j.jinsphys.2017.09.011

626 Berlanga M, Paster BJ, Grandcolas P, Guerrero R (2011) Comparison of the gut microbiota from
627 soldier and worker castes of the termite *Reticulitermes grassei*. 14:83-93. Int. Microbiol. doi:
628 10.2436/20.1501.01.138

629 Broderick NA, Lemaitre B (2012) Gut-associated microbes of *Drosophila melanogaster*. Gut
630 Microbes 3(4):307-321. doi: 10.4161/gmic.19896

631 Brune A, Dietrich C (2015) The gut microbiota of termites: digesting the diversity in the light of

632 ecology and evolution. Annu. Rev. Microbiol. 69:145-66. doi: 10.1146/annurev-micro-092412-
633 155715

634 Cappa F, Cini A, Pepiciello I, et al (2019) Female body size, weight and fat storage rather than
635 nestmateship determine male attraction in the invasive yellow-legged hornet *Vespa velutina*
636 *nigrithorax*. Ethol. Ecol. Evol. 31(1):73-85. doi: 10.1080/03949370.2018.1501437

637 Cardoso P, Leather SR (2019) Predicting a global insect apocalypse. Insect Conserv. and Diversity.
638 12(4):263-267. doi: 10.1111/icad.12367

639 Chapman RE, Bourke AFG (2001) The influence of sociality on the conservation biology of social
640 insects. Ecol. Lett. 4(6):650-662 doi: 10.1046/j.1461-0248.2001.00253.x

641 Chugo S, Lizaso MT, Alvarez MJ, et al (2015) *Vespa velutina nigritorax*: A new causative agent in
642 anaphylaxis. J. Investig. Allergol. Clin. Immunol. 25:231–232.

643 Cini A, Cappa F, Petrocelli I, et al (2018) Competition between the native and the introduced
644 hornets *Vespa crabro* and *Vespa velutina*: A comparison of potentially relevant life-history
645 traits. Ecol. Entomol. 43(3):351-362. doi: 10.1111/een.12507

646 Crotti E, Balloi A, Hamdi C, et al (2012) Microbial symbionts: A resource for the management of
647 insect-related problems. Microb. Biotechnol. 5(3):307-17. doi: 10.1111/j.1751-
648 7915.2011.00312.x.

649 Dapporto L, Stefanini I, Rivero D, et al (2016) Social Wasp intestines host the local phenotypic
650 variability of *Saccharomyces* cerevisiae strains. Yeast 33(7):277-87. doi: 10.1002/yea.3173.

651 Diouf M, Roy V, Mora P, et al (2015) Profiling the succession of bacterial communities throughout
652 the life stages of a higher termite *Nasutitermes arborum* (Termitidae, Nasutitermitinae) using
653 16S rRNA gene pyrosequencing. PLoS One. 10(10):e0140014. doi:

654 10.1371/journal.pone.0140014

655 Douglas AE (2007) Symbiotic microorganisms: untapped resources for insect pest control. Trends
656 Biotechnol. 25(8):338-42. doi: 10.1016/j.tibtech.2007.06.003

657 Edgar RC (2016) UNOISE2: improved error-correction for Illumina 16S and ITS amplicon
658 sequencing. bioRxiv. doi.org/10.1101/081257.

659 Engel P, Moran NA (2013) The gut microbiota of insects - diversity in structure and function. FEMS
660 Microbiol. Rev. 37(5):699-735. doi: 10.1111/1574-6976.1202-

661 Fogleman JC, Starmer WT, Heed WB, Url S (2014) Comparisons of yeast florae from natural
662 substrates and larval guts of southwestern *Drosophila*. 52:187–191. doi: 10.1007/BF00363835

663 Forsgren E, Fries I, Paxton RJ, Flaberg E (2012) Symbionts as major modulators of insect health :
664 lactic acid bacteria and honeybees. 7(3):e33188. doi: 10.1371/journal.pone.0033188

665 Granato A, Negrisolo E, Bonomi J, et al (2019) Recent confirmation of a single haplotype in the
666 Italian population of *Vespa velutina.* Biol. Invasions. 21(9):2811-2817. doi: 10.1007/s10530-
667 019-02051-4

668 Hanshew AS, McDonald BR, Díaz Díaz C, et al (2014) Characterization of Actinobacteria associated
669 with three ant–plant mutualisms. Microb. Ecol. 69(1):192-203. doi: 10.1007/s00248-014-
670 0469-3

671 Haxaire J, Villemant C (2010) Impact sur l'entomofaune des pièges à Frelon asiatique. Insectes
672 159:1-6

673 Hughes DP, Pierce NE, Boomsma JJ (2008) Social insect symbionts: evolution in homeostatic
674 fortresses. Trends Ecol. Evol. 23(12):672-7. doi: 10.1016/j.tree.2008.07.011

675 Joshi NA, Fass JN (2011). Sickle: a Sliding-Window, Adaptive, Quality-Based Trimming Tool for
676 FastQ files (version 1.33) [software].

677 Kaltenpoth M (2009) Actinobacteria as mutualists: general healthcare for insects? Trends
678 Microbiol. 17(12):529-35. doi: 10.1016/j.tim.2009.09.006

679 Kapheim KM, Rao VD, Yeoman CJ, et al (2015) Caste-specific differences in hindgut microbial
680 communities of honey bees (*Apis mellifera*). PLoS One. 10(4):e0123911. doi:
681 10.1371/journal.pone.0123911

682 Kim E, Seo J, Yang SH, et al (2018) Intestine bacterial microbiota of Asian hornet (*Vespa velutina*
683 *nigrithorax*) and honey bee. Korean J. Environ. Agric. 37(2):135-140. doi:
684 10.5338/kjea.2018.37.2.18

685 Koch H, Schmid-Hempel P (2011) Socially transmitted gut microbiota protect bumble bees against
686 an intestinal parasite. Proc. Nat.l Acad. Sci. 108:19288–19292. doi: 10.1073/pnas.1110474108

687 Leather SR (2018) “Ecological Armageddon” – more evidence for the drastic decline in insect
688 numbers. Ann. Appl. Biol. 172:1-3. doi: 10.1111/aab.12410

689 Lemke T, Stingl U, Egert M, et al (2003) Physicochemical conditions and microbial activities in the
690 highly alkaline gut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera:
691 Scarabaeidae). Appl. Environ. Microbiol. 69:6650–6658. doi: 10.1128/AEM.69.11.6650-
692 6658.2003

693 Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersionfor RNA-
694 seq data with DESeq2. Genome Biol. doi: 13059-014-0550-8.

695 Liu Z, Chen S, Zhou Y, et al (2015) Deciphering the venomic transcriptome of killer-wasp *Vespa*
696 *velutina.* Sci. Rep. 5(9454):9454 doi: 10.1038/srep09454

697 Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads.
698 EMBnet. journal 17(1): 10-12.

699 Martinson VG, Moy J, Moran NA (2012) Establishment of characteristic gut Bacteria during
700 development of the honeybee worker. Appl. Environ. Microbiol 78:2830–2840. doi:
701 10.1128/aem.07810-11

702 Matsuura M, Yamane S (1990) Biology of the vespine wasps. Springer Verlag

703 Meriggi N, Di Paola M, Vitali F, et al (2019) *Saccharomyces cerevisiae* induces immune enhancing
704 and shapes gut microbiota in social wasps. Front. Microbiol. 10:2320. doi:
705 10.3389/fmicb.2019.02320

706 Monceau K, Bonnard O, Thiéry D (2014) *Vespa velutina*: a new invasive predator of honeybees in
707 Europe. J Pest Sci. 87:1–16. doi: 10.1007/s10340-013-0537-3

708 Monceau K, Thiéry D (2017) *Vespa velutina* nest distribution at a local scale: An 8-year survey of
709 the invasive honeybee predator. Insect Sci. 24(4):663-674. doi: 10.1111/1744-7917.12331

710 Mutinelli F (2011) The spread of pathogens through trade in honey bees and their products
711 (including queen bees and semen): overview and recent developments. Rev. Sci. Tech.
712 30:257–271

713 Nilsson RH, Larsson K-H, Taylor AFS, Bengtsson-Palme J, Jeppesen TS, Schigel D, Kennedy P, Picard
714 K, Glöckner FO, Tedersoo L, Saar I, Kõljalg U, Abarenkov K (2018) The UNITE database for
715 molecular identification of fungi: handling dark taxa and parallel taxonomic classifications.
716 Nucleic Acids Research, 8(47):D259-D264. DOI: 10.1093/nar/gky1022

717 Otani S, Zhukova M, Koné NA, et al (2019) Gut microbial compositions mirror caste-specific diets in
718 a major lineage of social insects. Environ. Microbiol. Rep. 11(2):196-205. doi: 10.1111/1758-

719 2229.12728

720 Oksanen J, Blanchet FG, Friendly M, Kindt R et al. (2019) vegan: Community Ecology Package. R
721 package version 2.5-6. https://CRAN.R-project.org/package=vegan

722 Outhwaite CL, Gregory RD, Chandler RE, Collen B, Isaac NJ (2020) Complex long-term biodiversity
723 change among invertebrates, bryophytes and lichens. Nature Ecology & Evolution, 4:384-392.
724 doi: 10.1038/s41559-020-1111-z

725 Pielou EC (1966) The measurement of diversity in different types of biological collections. Journal
726 of Theoretical Biology. 13: 131–144. doi:10.1016/0022-5193(66)90013-0

727 Pinto-Tomás AA, Anderson MA, Suen G, et al (2009) Symbiotic nitrogen fixation in the fungus
728 gardens of leaf-cutter ants. Science 326(5956):1120-3. doi: 10.1126/science.1173036

729 R Core Team (2017) R: A language and environment for statistical computing. R Foundation for
730 Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

731 Regulation (2016) Commission Implementing Regulation (EU) 2016/1141 of 13 July 2016 adopting
732 a list of invasive alien species of Union concern pursuant to Regulation (EU) No 1143/2014 of
733 the European Parliament and of the Council. Official Journal of the European Union, L, 189(4),
734 4-8.

735 Radek R (1999) Flagellates, bacteria, and fungi associated with termites: diversity and function in
736 nutrition–a review. Ecotropica 5:183-196.

737 Requier F, Rome Q, Chiron G, et al (2019) Predation of the invasive Asian hornet affects foraging
738 activity and survival probability of honey bees in Western Europe. J. Pest Sci. 92(2):567-578.
739 doi: 10.1007/s10340-018-1063-0

740 Robinet C, Darrouzet E, Suppo C (2018) Spread modelling: a suitable tool to explore the role of

741 human-mediated dispersal in the range expansion of the yellow-legged hornet in Europe. Int.
742 J. Pest Manag. 65(3): 258-267. doi: 10.1080/09670874.2018.1484529

743 Rosengaus RB, Zecher CN, Schultheis KF, et al (2011) Disruption of the termite gut microbiota and
744 its prolonged consequences for fitness. Appl. Environ. Microbiol. 77(13):4303-12 doi:
745 10.1128/AEM.01886-10

746 Salem H, Bauer E, Strauss AS, et al (2014) Vitamin supplementation by gut symbionts ensures
747 metabolic homeostasis in an insect host. Proc. R. Soc.. B Biol. Sci. 7;281(1796):20141838. doi:
748 10.1098/rspb.2014.1838

749 Salem H, Kreutzer E, Sudakaran S, Kaltenpoth M (2013) Actinobacteria as essential symbionts in
750 firebugs and cotton stainers (Hemiptera, Pyrrhocoridae). Environ. Microbiol. 15:1956–1968.
751 doi: 10.1111/1462-2920.12001

752 Sethi A, Delatte J, Foil L, Husseneder C (2014) Protozoacidal Trojan-Horse: use of a ligand-lytic
753 peptide for selective destruction of symbiotic protozoa within termite guts. PloS one, 9(9):
754 e106199. https://doi.org/10.1371/journal.pone.0106199

755 Seipke RF, Barke J, Ruiz-Gonzalez MX, et al (2012) Fungus-growing *Allomerus* ants are associated
756 with antibiotic-producing actinobacteria. Antonie van Leeuwenhoek, Int J Gen Mol Microbiol.
757 101(2): 443-7. doi: 10.1007/s10482-011-9621-yShannon CE (1948) A mathematical theory of
758 communication. The Bell System Technical Journal, 27.

759 Simpson EH (1949) Measurement of diversity. Nature. 163 (4148): 688

760 Spradbery JP (1973) Wasps. An account of the biology and natural history of social and solitary
761 wasps, with particular reference to those of the British Isles.

762 Starr CK (2019) 15. The nest as the locus of social life. In: The Social Biology of Wasps. Pp. 520-539

763 in: In: K.G. Ross & R.W. Matthews (eds.), *The Social Biology of Wasps.* Ithaca: Cornell Univ. Press.

764 Stefanini I (2018) Yeast-insect associations: It takes guts. Yeast 315–330. doi: 10.1002/yea.3309

765 Stefanini I, Dapporto L, Legras J-L, et al (2012) Role of social wasps in *Saccharomyces cerevisiae*
766 ecology and evolution. Proc. Natl. Acad. Sc.i 109:13398–13403 10.1073/pnas.1208362109

767 Suen G, Scott JJ, Aylward FO, et al (2010) An insect herbivore microbiome with high plant biomass-
768 degrading capacity. PLoS Genet 6:e1001129. 10.1371/journal.pgen.1001129.

769 Takahashi S, Tomita J, Nishioka K, Hisada T, Nishijima M (2014) Development of a prokaryotic
770 universal primer for simultaneous analysis of Bacteria and Archaea using next-generation
771 sequencing. PloS one, 9(8):e105592. doi: 10.1371/journal.pone.0105592

772 Vadkertiová R, Molnárová J, Vránová D, Sláviková E (2012) Yeasts and yeast-like organisms
773 associated with fruits and blossoms of different fruit trees. Can. J. Microbiol. 58(12):1344-52.
774 doi: 10.1139/cjm-2012-0468

775 Villemant C, Barbet-Massin M, Perrard A, et al (2011a) Predicting the invasion risk by the alien
776 bee-hawking Yellow-legged hornet *Vespa velutina nigrithorax* across Europe and other
777 continents with niche models. Biol. Conserv. 144:2142–2150. doi:
778 10.1016/j.biocon.2011.04.009

779 Villemant C, Muller F, Haubois S (2011b) Bilan des travaux (MNHN et IRBI) sur l'invasion en France
780 de *Vespa velutina*, le frelon asiatique prédateur d'abeilles. Proceedings of the Journée
781 Scientifique Apicole–11 February, 3-12.

782 Visser AA, Nobre T, Currie CR, et al (2012) Exploring the Potential for Actinobacteria as Defensive
783 Symbionts in Fungus-Growing Termites. Microb. Ecol. 63(4):975-85. doi: 10.1007/s00248-011-
784 9987-4

785 Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naïve Bayesian classifier for rapid
786 assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ.
787 Microbiol.73(16):5261-5267. doi: 10.1128/AEM.00062-07.

788 Wagner DL (2019) Insect declines in the Anthropocene. Ann. Rev. Entomol. 65. 7;65:457-480. doi:
789 10.1146/annurev-ento-011019-025151.

790 Weiss M, Bauer R, Sampaio JP, Oberwinkler F (2014) Tremellomycetes and Related Groups. In
791 Systematics and evolution (pp. 331-355). Springer, Berlin, Heidelberg.

792 White TJ, Bruns T, Lee SJWT, Taylor J (1990) Amplification and direct sequencing of fungal
793 ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications,
794 18:315-322.

795 Zientz E, Feldhaar H, Stoll S, Gross R (2005) Insights into the microbial world associated with ants.
796 Arch. Microbiol. 151(7): 513-9. doi:10.1007/s00203-005-0041-0