

18 **Abstract**

19 The ecological success of social Hymenoptera (ants, bees, wasps) depends on the division of labour
20 between the queen and workers. Each caste is highly specialised in its respective function in
21 morphology, behaviour and life-history traits, such as lifespan and fecundity. Despite strong defences
22 against alien intruders, insect societies are vulnerable to social parasites, such as workerless
23 inquilines or slave-making (dulotic) ants. Here, we investigate whether gene expression varies in
24 parallel ways between lifestyles (slave-making versus host ants) across five independent origins of
25 ant slavery in the “*Formicoxenus*-group” of the ant tribe Crematogastrini. As caste differences are
26 often less pronounced in slave-making ants than non-parasitic ants, we also compare the
27 transcriptomes of queens and workers in these species. We demonstrate a substantial overlap in
28 expression differences between queens and workers across taxa, irrespective of lifestyle. Caste
29 affects the transcriptomes much more profoundly than lifestyle, as indicated by 37 times more genes
30 being linked to caste than to lifestyle and by multiple caste-associated gene modules with strong
31 connectivity. However, several genes and one gene module are linked to the slave-making lifestyle
32 across the independent origins, pointing to some evolutionary convergence. Finally, we do not find
33 evidence for an interaction between caste and lifestyle, indicating that caste differences remain
34 consistent even when species switch to a parasitic lifestyle. Our findings are a strong indication for
35 the existence of a core set of genes whose expression is linked to the queen and worker caste in this
36 ant taxon, supporting the “genetic toolkit” hypothesis.

37

38 **Key words:** Social parasitism, caste, transcriptomes, gene networks, slave-making ants, dulosis,
39 selection

40 **Introduction**

41 The ecological success of social insects is based on the efficient division of labour between
42 reproductives and non-reproductives, i.e., in the social Hymenoptera, the queens and workers
43 (Hölldobler & Wilson, 2009; Wilson, 1971). Instead of producing their own offspring, workers help to
44 raise the offspring of their mother or other related queens. The altruism of workers is explained by
45 their relatedness to the recipients of their help, which is maintained by the closure of the society
46 against unrelated freeloaders or parasites (Hamilton, 1964, 1987). Nevertheless, several species have
47 evolved sophisticated ways to infiltrate and usurp social insect colonies (Rabeling, 2020), and among
48 these are the charismatic “slave-making” or “dulotic” ants (Hölldobler & Wilson, 1990; Buschinger,
49 2009; D’Ettorre & Heinze, 2001; Mori et al., 2001; Visicchio et al., 2001). Freshly mated, young
50 queens of slave-making ants invade the nests of closely related, non-parasitic ant species, where they
51 kill or expel the resident queen(s) and often also the adult workers. Host workers emerging from the
52 conquered host brood take care of the slave-maker queen and her offspring, maintain the nest, and
53 forage for food. Slave-maker workers do not engage in normal worker chores (Hölldobler & Wilson
54 1990; Buschinger, 2009) but instead raid neighbouring host nests and pillage their brood, thus
55 replenishing or increasing the host force.

56 Ten independent origins of slavery in ants are documented (Stoldt & Foitzik, 2021), and at
57 least five convergent origins lie within the “*Formicoxenus*-group” of the myrmicine tribe
58 Crematogastrini (Blaimer et al., 2018). This allows investigation of whether convergent changes in
59 gene expression occurred among those evolutionary switches to slave-making. There are several
60 morphological, physiological and behavioural similarities among slave-making species of independent
61 origin. For example, slave-maker workers are often heavily armed with strong mandibles and
62 associated muscles, which leads to enlarged heads (Hölldobler & Wilson, 1990). Given that workers
63 of slave-making species no longer take care of the daily duties in the colony, they have become more
64 queen-like in their task repertoire. They neither forage for food nor do they engage in brood care
65 (Buschinger, 2009). Moreover, slave-maker workers tend to have increased reproductive potential.

66 While the ovaries of non-parasitic workers have fewer ovarioles than the queen's ovaries and rarely
67 contain mature eggs in the presence of a fertile queen, the ovaries of slave-maker workers often
68 have the same number of ovarioles as the queen (Heinze, 1996b). They form reproductive
69 hierarchies (Franks and Scovell, 1983; Bourke, 1988; Heinze, 1996a) and frequently lay male-destined
70 eggs even in the queen's presence (Foitzik & Herbers, 2001; Brunner et al., 2005; Suefuji & Heinze,
71 2014). Gene expression differs strongly between workers and queens of most species, reflecting their
72 divergent function, behaviour, and physiology (Gstöttl et al., 2020; Korb et al., 2021; Morandin et al.,
73 2019a, b; Feldmeyer et al., 2013). For example, queen transcriptomes are characterised by the
74 expression of genes associated with fecundity (e.g. vitellogenins), immunity, DNA repair and
75 response to oxidative stress functionalities linked to their long lifespan (Stoldt et al., 2021). However,
76 given the described similarities between slave-maker queens and workers, we expected their
77 transcriptomes to differ less than those of queens and workers of non-parasitic species.

78 We therefore investigated the influence of caste (queen vs worker) and lifestyle (slave-maker
79 vs non-parasitic) on gene expression of adult individuals and the interaction between those two
80 parameters, which would indicate that caste is affecting gene expression differently in non-parasitic
81 versus slave-making species. Here we use a protocol which is aimed at maximising reproducibility
82 and minimising confounding effects by using all five available and evolutionary closely related pairs
83 of slave-maker and host in the myrmicine tribe Crematogastrini. For each species, we sequenced the
84 transcriptomes of six pooled workers and three pooled queens, respectively, taking slave-making
85 species vs. host species as replicates. We investigated gene regulatory network properties according
86 to caste and lifestyle and constructed orthologue clusters to investigate putative parallel selection
87 patterns in genes associated with the slave-maker versus host lifestyle, including two related non-
88 host taxa and two samples of the distantly related ant *Cardiocondyla obscurior* as outgroup.

89

90 **Material & Methods**

91 *Sampling and sequencing*

92 Colonies of 15 myrmicine ant species of the “*Formicoxenus*-group” (genera *Harpagoxenus*,
93 *Leptothorax*, and *Temnothorax*, including the previously synonymized genera of slave-making ants:
94 *Chalepoxenus*, *Myrmoxenus*, and *Protomognathus* (Ward et al., 2015, but see Seifert et al., 2016)
95 were collected between 2016-2018 from various locations across Germany, Italy, and the US
96 (Supplement_coordinates). Colonies were either brought to the lab in Regensburg, Mainz, or
97 Münster, and kept under standard conditions in incubators (12 h 25°/ 12 h 25°C day-night cycles)
98 before six workers and three queens were pooled per species for RNA extraction. For each species,
99 workers and queens originated from three colonies. We generated one queen and one worker
100 transcriptome for seven slave-making ants and their host species (Supplement Table S1). RNA was
101 extracted using the Nucleo-Spin Mini kit (Macherey-Nagel). Samples were shipped to StarSEQ
102 (Mainz) for library preparation and 100bp paired end sequencing on an Illumina HiSeq. In total, we
103 obtained 14mio reads on average per sample (Supplement Table S2).

104

105 *Gene expression analyses*

106 Raw reads were quality checked using FastQC v.0.11.8 (Andrews, 2010), and adapters trimmed with
107 Trimmomatic v.2.8.4 (Bolger et al., 2014). HiSat2 v.2.1.0 (Kim et al., 2015) was used to map the reads
108 to the *T. longispinosus* genome v.1 (GenBank accession: GCA_004794745.1_tlon_1.0; Kaur et al.,
109 2019). We chose to use a single species genome as reference for all species to be able to later
110 directly compare expression patterns between slave-making ants and their hosts as well as between
111 queens and workers across species, with species as replicates. The counts table was created with
112 HTSeq (Anders et al., 2015). To prevent spurious results due to low read counts, we removed from
113 the counts matrix genes with less than 10 reads in at least four samples before the subsequent
114 differential gene expression analysis with DESeq2 (Love et al., 2014). We started with the full model
115 ~Caste+Lifestyle+Caste:Lifestyle. The interaction turned out to be nonsignificant (no differentially
116 expressed genes for the interaction), and we thus based all follow-up analyses on the two main
117 factors only. All p-values were adjusted by false discovery rate (FDR) correction as implemented in

118 DESeq2. To determine whether the significant slave-maker differentially expressed genes (DEGs) are
119 more numerous than we would expect by chance, we ran 1000 permutations on the DEGs analysis in
120 R.

121 To gain a deeper understanding for the functionality of DEGs, we 1) conducted a functional
122 enrichment analysis, 2) inferred pathways in which the DEGs are involved, and 3) used a word mining
123 approach based on longevity and fecundity terms. In detail, we ran Interproscan v.5.39-77.0 (Jones et
124 al., 2014) locally to obtain GO information using the *T. longispinosus* predicted proteome (GenBank
125 accession: GCA_004794745.1_tlon_1.0; Kaur et al., 2019) as query. The GO enrichment analysis was
126 performed with the R package TopGO (Alexa & Rahnenführer, 2016), using the 'parentchild'
127 algorithm and the Fishers exact test for significance. Furthermore, the proteome was annotated with
128 KEGG functional ortholog numbers using the BlastKOALA web utility (last accessed: 11.02.2021;
129 Kanehisa et al., 2016) with 'Eukaryotes, Animals' specified as Taxonomy group. KEGG pathway
130 affiliation of genes that were differentially expressed in each of the four groups (queens, workers,
131 slave-makers and hosts) was assessed using the online utility of KEGG mapper with *Apis mellifera* as
132 reference species (Kanehisa & Sato, 2020), and visualized including the log₂-fold change using
133 pathview (Luo & Brouwer, 2013) in R v.3.6.3 (R Core team, 2020). KEGG pathway enrichment was
134 assessed with the enrichKEGG function implemented in clusterProfiler v.3.14.3 (Yu et al., 2012)
135 where the universe was defined as the total set of *T. longispinosus* protein predictions with a KEGG
136 functional ortholog annotation and organism 'ko'. For the text mining approach, we used gene
137 annotations based on a BlastP search of the proteome versus the RefSeq invertebrate database, as
138 query for a UniProt search. More specifically, we extracted the gene function information for each
139 gene with entries from *C. elegans*, *D. melanogaster*, *A. mellifera* and searched for terms related to
140 fecundity and longevity (Supplement Table S3), both traits which we assumed to differ between
141 queens and workers (script available from Negroni et al., 2021). We conducted an enrichment
142 analysis by conducting a Fisher's exact test to test whether the number of genes with terms in
143 fecundity or longevity within differentially expressed genes was higher than expected with respect to

144 the complete proteome. The online tool Venny v.2.0.2 was used to generate the Venn diagram
145 (<https://bioinfogp.cnb.csic.es/tools/venny>).

146

147 *Network analysis*

148 To identify networks of co-expressed genes (modules), we constructed a weighted gene co-
149 expression network analysis using the WGCNA (Langenfelder & Horwath, 2008) package in R. We
150 used all genes that had passed the quality filtering step for the expression analysis (N = 8,327), thus
151 not only the differentially expressed genes. Gene counts were normalized using the
152 *varianceStabilizingTransformation* function from DESeq2 (Love et al., 2014). Following the WGCNA
153 guidelines, we picked a soft-thresholding power of 8 for adjacency calculation. To associate modules
154 to either caste or lifestyle, we first calculated the modules' eigengene using the *moduleEigengenes*
155 function and tested for module trait correlation using the *corPvalueStudent* function. The hub gene
156 of each module (i.e., the gene with the highest connectivity within a module) was determined using
157 the *chooseTopHubInEachModule* function. Moreover, for modules associated with caste or lifestyle,
158 we tested whether the connectivity of genes with caste-specific expression and genes differed from
159 the connectivity of those that were not differentially expressed. We ran linear models with
160 connectivity as response and caste-specificity (queen, worker, no expression difference) as
161 explanatory variable in R.

162 To determine the relevance of fecundity and longevity associated genes in lifestyle and caste
163 associated modules, we downloaded a list of 123 genes that were assembled based on information
164 from *Drosophila* and are part of the TI-J-LiFe pathways (TOR/IIS-JH-Lifespan and Fecundity) (Korb et
165 al. 2021). We used the Flybase identifiers to download the corresponding *Drosophila* sequences and
166 used a BlastX search to identify *T. longispinosus* proteins with a blast hit e-value < e-10. In cases
167 where we had two or three hits, we selected the protein with the longest match, which mostly
168 corresponded to the lowest e-value. In cases where we had >3 blast hits, we created a sequence
169 alignment using MACSE v.2.03 (Ranwez et al., 2011). A Maximum Likelihood phylogenetic tree with

170 1000 bootstrap replicates was constructed with RAxML with PROTGAMMAWAG as protein
171 substitution model. Based on the tree topology we chose the *T. longispinosus* sequence with the
172 closest relationship to the target *Drosophila* sequence.

173

174 *Selected genes analysis*

175 For this analysis, we added transcriptomes of four taxa that are not known to be parasitized by slave-
176 making ants, which acted as a biological control for comparisons of selection intensity between slave-
177 makers and hosts: as outgroup, two populations of *Cardiocondyla obscurior*, and the two non-host
178 species of the *Formicoxenus* group, *Temnothorax nylanderi* and *T. rugatulus* (Supplement Table S1).
179 We used Trinity v.2.8.6 (Grabherr et al., 2011) with standard settings to construct species-specific
180 transcriptomes using both worker and queen transcripts. Nucleotide sequences were translated into
181 amino acid sequences with TransDecoder (<https://github.com/TransDecoder>). As *de novo*
182 transcriptomes are known to contain many transcript fragments as well as isoforms, we constructed
183 orthogroups across all species with OrthoFinder (Emms & Kelly, 2015), including protein sequences
184 derived from the *Temnothorax longispinosus* genome (Kaur et al., 2019), and retained orthogroups
185 with a tlon-v1 ortholog only. After orthogroup construction, the *T. longispinosus* protein sequences
186 derived from the genome were removed from the orthogroups from all downstream summary
187 statistics and analyses. Since we only obtained few single copy orthologs, we used an inhouse script
188 (Supplement S_script) to retain only a single sequence per species. In short, based on the pair-wise
189 blast results from OrthoFinder the sequence with the highest sum of bit scores (i.e. best match to all
190 other sequences) within each orthogroup was chosen as “centroid”. From each species the sequence
191 with the best match to the centroid was chosen to create the single copy orthogroup. We used
192 Clustal-omega v.1.2.4 (Goujon et al., 2010) to construct sequence alignments for each orthogroup,
193 which were trimmed with TrimAL v.1.4.1 (Capella-Gutierrez et al., 2009) and the following settings: -
194 gappyout -resoverlap 0.75 -seqoverlap 75 -backtrans. To test for signatures of positive selection we
195 used the codeml implementation in ete3 v.3.1.1 (Huerta-Cepas et al., 2016) running the branch site

196 test of selection as follows: ete3 evol --models bsA bsA1 --tests bsA, bsA1 --leaves --internals. The
197 cluster-specific tree topology as inferred by RAxML v.8.2.12 (Stamatakis, 2014) was used as input
198 tree, and each species was coded as foreground branch consecutively. Finally, we blasted the *T.*
199 *longispinosus* sequence of each ortholog cluster with signatures of selection versus the *T.*
200 *longispinosus* genome and only retained ortholog clusters with sequences mapping to a single
201 location, as further means of preventing putative paralogs. We further ran a GO enrichment analysis
202 to test for overrepresented functions among the selected genes (details above), and used the KEGG
203 annotations to investigate the pathways in which genes with signature of selection are involved.

204

205 **Results**

206 We set out to investigate the effect of lifestyle and caste on gene expression patterns across 15
207 different species of ants contrasting slave-making ants with their hosts, and queens with workers.
208 We were mainly interested in determining whether there are common toolsets of genes
209 characteristic for a specific lifestyle or caste. We additionally tested for genes under selection in the
210 above-mentioned slave-maker-host pairs plus four additional non-host outgroup species.

211 In general, gene expression patterns seem to be most similar amongst phylogenetically close
212 species. The principal component analysis revealed a very strong phylogenetic effect with 71% of
213 variance explained by PC1, which separated the *Harpagoxenus* / *Leptothorax* group from the
214 *Temnothorax* species (Supplement Figure S1), while PC2, explaining 8% of the variance, separated
215 queens from workers. Caste had a much stronger effect on gene expression compared to lifestyle, as
216 also evident in the heatmap dendrogram, where samples cluster according to caste rather than
217 lifestyle (Figure 1). As our analysis did not reveal an interaction between lifestyle and caste (0 DEGs
218 associated to the interaction term), we present the gene expression plus gene network results
219 according to the main effects, caste and lifestyle, and finally the results from the selection analysis.

220

221 *Lifestyle*

222 We detected 62 differentially expressed genes (DEGs) between lifestyles (host versus slave-maker
223 species) cumulative across the five origins of slavery (see Supplement Table 1 DEGs). A permutation
224 test with 1000 iterations revealed that this is more than expected by chance ($p = 0.04$). Six genes
225 were consistently higher expressed in all seven slave-maker species compared to hosts, of which
226 three were annotated: “protein DVR-1 homolog”, “sulfotransferase family cytosolic 1B member 1-
227 like”, and “ATP-dependent DNA helicase II subunit 1-like”. Only a single gene, “NADPH oxidase 5”,
228 showed higher expression in all seven host species compared to slave-makers (Figure 2). Various
229 metabolic processes were significantly enriched among the differentially expressed genes between
230 lifestyles (total = 21 processes). Among genes up-regulated in slave-makers, 14 processes were
231 enriched, and three functions were enriched in genes up-regulated in hosts, including “cell death”
232 (Supplement_GO). In addition to the functional level, we also investigated whether the genes in the
233 lifestyle DEGs set were overrepresented in specific pathways. Due to the low number of lifestyle-
234 associated DEGs, and only about half of these with KEGG annotation, the enrichment analyses for the
235 complete set of lifestyle DEGs, and the separate host and slave-maker gene-sets, did not result in any
236 overrepresented functions.

237 Host colonies are generally larger than slave-maker colonies, *i.e.*, host queens are more
238 fecund than slave-maker queens. Moreover, fecundity is often positively correlated with longevity in
239 social insects (Korb & Heinze, 2016; Negroni et al., 2016). We therefore conducted a word mining
240 approach based on UniProt entries to investigate whether genes with known fecundity or longevity
241 functionality were overrepresented in the sets of DEGs between hosts and slave-makers (Table 1).
242 We indeed recovered more DEGs putatively associated with fecundity than expected by chance ($N =$
243 31; Fisher’s exact test, p -value = $6.619e-09$), but not for the longevity associated genes (Table 1).

244 We obtained 10 modules of co-expressed genes (named after colours as given by the *WGCNA*
245 package), of which one module, red, was positively associated with the slave-making lifestyle (Figure
246 5). This module contained 191 genes, of which 19 were up-regulated in slave-makers and none in
247 hosts. Genes associated with this lifestyle module were enriched for “reactive oxygen species

248 metabolic processes”, “oxidation reduction processes”, or “fatty acid biosynthetic process”
249 (Supplement_WGCNA). Genes up-regulated in slave-makers had the highest connectivity in this
250 module associated with lifestyle (Supplement Figure S2).

251

252 *Caste*

253 Caste had a much stronger effect on gene expression than lifestyle ($\chi^2= 2496.8$, $df = 1$, $p\text{-value} < 2.2e\text{-}$
254 16), with 2,321 differentially expressed genes (DEGs) between queens and workers. Around half of all
255 caste-specific DEGs showed consistently higher expression in queens ($N=738$ out of 1,295) or in
256 workers ($N = 450$ out of 1,026) across all species. For example, genes for an *insulin-like growth factor*
257 *2*, *mRNA-binding protein 1*, *maternal protein exuperantia*, *histone deacetylases*, and several
258 *serine/threonine-protein kinases* were more highly expressed in queens of all 15 species, while *pro-*
259 *corazonine like* had higher counts in workers of all but one species. The number of genes with
260 consistent expression across taxa was significantly lower for the lifestyle comparison ($\chi^2= 36.865$, $df =$
261 1 , $p\text{-value} = 1.266e\text{-}09$).

262 As queens and workers strongly differ in fecundity and longevity, we used a word mining
263 approach based on UniProt entries to investigate whether genes with known fecundity or longevity
264 functionality were overrepresented in the sets of caste-specific DEGs (Table 1). Indeed, we detected
265 more fecundity-associated terms ($N = 1076$; Fisher’s exact test, $p\text{-value} = 2.2e\text{-}16$), and more
266 longevity-associated terms ($N = 412$; Fisher’s exact test, $p\text{-value} < 0.0003$) than expected by chance.

267 In the caste-specific DEG set, 39 gene ontology (GO) functions were significantly
268 overrepresented, many of which were associated with metabolic and biosynthetic processes
269 (Supplement_GO). Genes up-regulated in queens were enriched for 60 functions linked to various
270 metabolic processes or stress responses. Among the worker up-regulated genes, 78 functions were
271 significantly enriched, also belonging to metabolic and biosynthetic processes, but also oxidation-
272 reduction. In addition to the functional enrichment, we conducted a KEGG-pathway enrichment
273 analysis. In total, we found 27 pathways enriched amongst the caste-specific DEGs, 12 for genes up-

274 regulated in queens, and 61 genes up-regulated in workers (Supplement_KEGG). Multiple putatively
275 reproduction-associated and repair pathways, such as “meiosis-yeast”, “cell cycle”, “DNA
276 replication”, “RNA transport,” or “ribosome biogenesis,” were enriched in queens, and pathways
277 such as “olfactory transduction” and “longevity regulating pathway” (Figure 3) were enriched in
278 genes up-regulated in workers (Supplement_KEGG).

279 The gene network analysis resulted in five modules associated with caste, three of which
280 were positively associated with queens (yellow, green, magenta), and two with workers (black and
281 blue) (Figure 5). There was a trend for the pink module to also be associated with caste ($p = 0.07$),
282 and it was included in the following analyses (for details see: Supplement_WGCNA). In all three
283 worker associated modules (black, blue, pink), connectivity was highest for worker up-regulated
284 genes, lowest for queen up-regulated genes, and intermediate for genes that were not differentially
285 expressed. The same pattern was observed in queen-specific modules (yellow, green, magenta), in
286 which the queen up-regulated genes had the highest connectivity (Supplement_WGCNA).

287

288 *Comparison to other species*

289 We identified 84 genes associated with one of the 10 WGCNA modules that have also previously
290 been linked to fecundity or longevity in *Drosophila*. We obtained these from the “TI-J-LiFe” list
291 containing 123 candidate genes (Korb et al., 2021). About half of these ($N = 60$) were found in the
292 turquoise module, which was neither associated to caste nor lifestyle, and eleven were found in the
293 blue, caste-linked module (Supplement_WGCNA). To determine whether overrepresented gene
294 functions linked to caste associated modules from our *Formicoxenus*-group data set could be
295 extrapolated to other ants or even termites, we compared our modules to Morandin et al. (2016)
296 and Lin et al. (2021). Only three functions were shared between enriched GO-terms of caste
297 associated modules in our slave-making data set ($N = 46$) and enriched GO-functions in *Formica* caste
298 associated modules ($N = 155$; Morandin et al., 2016), namely “cellular protein modification process”,
299 “protein modification process”, “monovalent inorganic cation transport” (Table 2). Eight terms were

300 shared with caste associated modules in termites (N = 277; Lin et al., 2021) (Table 2). No terms were
301 shared amongst the three studies. However, these results should be taken with caution as most
302 enrichment algorithms take the hierarchical structure of GO terms into account. Thus one may have
303 a lower or higher term in the hierarchy, which does not mean they are essentially different.

304 As queen and workers differ in fecundity and longevity, we investigated whether the same
305 set of fecundity-longevity associated genes of the “TI-J-LiFe” list (N = 84) which were found in the
306 termites (Lin et al., 2021) could be found in our data set . There were two genes, Ras64B and Kr-h1,
307 associated with a queen-worker co-expression module in termites and in *Crematogastriini*.

308

309 *Selected genes analysis*

310 The transcriptomes of the 19 taxa, including four samples of non-host species, consisted of 67,150-
311 155,629 transcripts with 32,584-51,743 open reading frames and 92-96% DOGMA completeness. In
312 total, we obtained 10,699 ortholog clusters of which 5,826 clusters contained at least one transcript
313 per species. 1,398 clusters remained after trimming and filtering for single copies per species. We
314 identified 660 signatures of selection across all species, that are associated with 424 ortholog
315 clusters, and mapped to a single location on the genome (Supplement_codeml). Between 6-92 genes
316 showed signatures of selection in each species (Supplement_codeml), but there was no difference in
317 the number of selected genes between lifestyles (quasipoisson model, $X^2 = 1.33$, d.f. = 2, $p = 0.51$). In
318 slave-makers, genes with signatures of selection were significantly enriched in functions such as
319 protein modification, demethylation and energy maintenance (Figure 4a, Supplement_codeml). In
320 hosts and non-hosts, several regulatory and metabolic functions are significantly overrepresented in
321 genes with signatures of selection (Figure 4b+c).

322 We identified 114 genes that were both differentially expressed and additionally showed signatures
323 of selection (Supplement_overlap). Of these, a single gene was differentially expressed between
324 castes and lifestyles and positively selected in *T. univasciatus* (TLON_06615-RA; pleckstrin homology
325 domain-containing family F member 2 isoform X1). All remaining 113 were differentially expressed

326 only between castes. 35 of these caste-specific DEGs showed signatures of selection in hosts, 23 in
327 non-hosts, and 57 in slave-makers. Interestingly, many nucleotide metabolism pathways were
328 included in the overlapping gene lists of differentially expressed and selected genes, including purine,
329 alanine, aspartate, valine, and the “mTOR signalling pathway” (Supplement_overlap).

330

331 **Discussion**

332 Eusocial insects are characterised by their sophisticated division of labour, which led to the evolution
333 of different castes. Ant queens are the main reproductives, known for their long life of up to several
334 decades and high fecundity (Keller & Genoud, 1997), while the mostly infertile and short-lived
335 workers take care of the brood, foraging, and nest defence (Hölldobler & Wilson, 1990). In slave-
336 making species, however, workers do not perform the “general” worker chores. They raid host nests
337 in summer and often are permitted to lay male-destined eggs in the presence of the queen. Our
338 study shows that whole-body transcriptomes of seven species pairs of the “*Formicoxenus* group” of
339 Crematogastrini (Blaimer et al., 2018; *Harpagoxenus*, *Leptothorax*, and *Temnothorax*) differ
340 considerably more between castes (queen vs. worker) than with lifestyles (slave-maker vs. non-
341 parasitic species). Caste polyphenism is based on differential gene expression, and our transcriptome
342 analyses show that the expression of 1,188 genes consistently differs between queens and workers
343 of all studied species, regardless of lifestyle. Although the evolution of a parasitic lifestyle is
344 associated with considerable changes in morphology and behaviour, only few transcriptomic shifts
345 from host to slave-making species were consistent across the five independent origins of slave-
346 making.

347 Approximately 40x more genes were differentially expressed between castes than between
348 lifestyles (2,321 vs. 62), and six out of ten co-expression modules were caste-associated in contrast to
349 a single lifestyle module. This difference might in part reflect the single evolutionary origin of caste
350 diphenism in ants, whereas slavery evolved repeatedly (Beibl et al., 2005; Feldmeyer et al., 2017;
351 Prebus, 2017). Nevertheless, as slave-making workers are often fertile and do not take over normal

352 worker chores, we had expected to find a considerable interaction between caste and lifestyle in
353 gene expression as well as in gene connectivity, as caste differences are less pronounced in slave-
354 making species. The lack of the interaction indicates that slave-maker and host queens and workers
355 are rather similar on a molecular level. Furthermore, it corroborates the result of a previous study
356 where caste differences also exceeded differences due to other traits, such as worker sterility, queen
357 number, or invasiveness (Morandin et al., 2016).

358 While in this study, half of the genes that were differentially expressed between queens and
359 workers show the same expression pattern across all species, another study identified only a single
360 gene (the myosin light chain) similarly expressed between queens and workers a set of 16 species of
361 multiple genera (Morandin et al., 2016). The reason for this discrepancy may be explained by the
362 different species relationships as well as the underlying data basis. We studied species within a
363 single, closely related clade and used the genome of a single species as reference. In contrast,
364 Morandin et al. (2016) investigated species from five genera in different subfamilies based on de
365 novo assembled transcriptomes. Additionally, there is evidence for similarities in pathways across
366 different lineages of social insects (ants, bees, wasps) rather than a “common toolkit” of genes
367 responsible for the caste phenotype (Berens et al., 2015). In our phylogenetically more restricted
368 data set however, we identified 1,188 genes representing the “core set” of queen-worker differences
369 across the 15 species. For example, the gene “*corazonin*” has been shown to control social behaviour
370 and caste identity in ants (Gospocic et al., 2017). The gene “*maternal protein exuperantia*”, a
371 maternal effect gene which is needed for proper localisation of the *bicoid* RNA during oocyte
372 formation (de Oliveira et al., 2017; McDonald et al., 1991), but also plays a role in *Drosophila*
373 spermatogenesis (Hazelrigg et al., 1990) was up-regulated in all queens. Also, “G1/S-specific cyclin-
374 D2” (*cycD*) was more strongly expressed in queens compared to workers in all but one species. This
375 gene is involved in cell cycle regulation and part of the “FoxO signalling” and “Wnt signalling
376 pathway”, both important regulators of longevity. It could thus be associated with the lifespan

377 differences between these two castes. More generally, half of the genes differentially expressed
378 between caste were associated with the UniProt functionalities “fecundity” and “longevity.”

379 Reflecting the convergent evolution of ant slavery (Beibl et al., 2005; Feldmeyer et al., 2017;
380 Prebus, 2017), the different parasitic species not only show pronounced differences in their
381 morphology, but also in raiding behaviour (Brandt et al., 2006; Johnson, 2008; Kleeberg & Foitzik,
382 2016). Species-specific raiding patterns are mirrored by species-specific gene expression patterns
383 (Alleman et al., 2018) and genes under selection (Feldmeyer et al., 2017). Gene expression
384 differences between pairs of slave-makers and their hosts showed much more variation across
385 species (representing different origins of slaver-making) than the differences between queens and
386 workers, *i.e.*, there are many more idiosyncrasies in how slave-makers and hosts differ. Nevertheless,
387 we found 62 genes that varied with lifestyle, including genes for fatty acid synthases. This could be
388 indicative either of differences in fat synthesis and maybe storage between the two lifestyles, but
389 these synthases may also be involved in the synthesis of cuticular hydrocarbons which are used as
390 communication signals to discriminate species and castes (Leonhard et al., 2016).

391 As slave-makers are the derived lifestyle with species-specific behaviours and traits, we
392 expected to find more genes under selection in slave-makers than in hosts and/or non-host species,
393 however the number of selected genes did not differ between lifestyles. Genes with signatures of
394 selection were significantly enriched in functions such as protein modification, demethylation, and
395 energy maintenance. An interesting candidate among the genes that showed signatures of selection
396 in only one or a few of the species is venom protease-like in *T. muellerianus*. Though at present
397 nothing is known on the composition of the venom used by *T. muellerianus* to kill host ants during
398 slave-raids, many Hymenopteran venoms are proteases (Touchard et al., 2016). On a higher level,
399 many metabolism pathways were included in this overlapping gene list, including purine, alanine,
400 aspartate, valine, to name just a few, and the “mTOR signalling pathway”.

401

402 **Conclusion**

403 Social parasitism represents a derived state with clear differences to host and non-host species from
404 morphology to behaviour. In most social parasites, queens and workers are more similar than in host
405 species. Examining gene expression patterns and gene regulatory networks of 15 different ant
406 species spanning five origins of slave-making, we expected to find an interaction between caste and
407 lifestyle effects on gene expression patterns. Despite the phenotypic differences, between
408 slavemaker and host castes, gene expression profiles were remarkably similar. Our study
409 corroborates previous results indirectly indicating species-specific expression and selection patterns
410 with respect to lifestyle, thus little common difference between host and slave-maker species.
411 However, we observed a very strong and reliable effect of caste on gene expression. Within our
412 broad taxonomic species spectrum, we were able to identify a core-set of 1,188 caste-specific genes,
413 which show a consistent expression pattern across all species irrespective of lifestyle, pointing to a
414 “genetic toolkit” in this set of related ant species.

415

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420

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611

612 **Data Accessibility**

613 All raw sequence data underlying this study have been deposited in the National Centre for
614 Biotechnological Information (NCBI) Sequence Read Archive (SRA) and will be accessible upon
615 publication of this manuscript (BioProject accession number XY will follow in the next version of the
616 manuscript).

617

618 **Author Contributions**

619 The study was conceived by JH, EBB and SF, and was designed by EJ, BF, JH, EBB and SF. DG provided

620 ant samples. BF, CS, JW and EJ conducted the analyses. All authors contributed to writing the paper.

621 Authors declare no conflict of interest.

622 **Table 1:** Number of differentially expressed genes with a fecundity or longevity functionality based
623 on a text mining approach using the UniProt database as a reference. Results of Fisher's exact test
624 are given indicating whether the number of genes with either fecundity or longevity association in
625 caste and lifestyle are more than expected by chance.
626

<i>Treatment</i>	Fecundity		Longevity	
	N of genes	P-Value	N of genes	P-value
<i>Caste</i>	1076	2.2e-16	412	0.0003
<i>Queen</i>	630		262	
<i>Worker</i>	447		150	
<i>Lifestyle</i>	31	6.619e-09	13	0.39
<i>Host</i>	9		6	
<i>Slave-maker</i>	23		7	

627

628

629 **Table 2:** Shared enriched GO terms between queen-worker caste associated modules from this
630 study, a study on 16 ant species from multiple genera (Morandin et al. 2016), and the termite
631 *Cryptotermes secundus* (Lin et al. 2021).
632

Organism(s)	Shared enriched GO-terms with this study	633
Multiple ants	cellular protein modification process	634
Multiple ants	protein modification process	635
Multiple ants	monovalent inorganic cation transport	636
<i>C. secundus</i>	cyclic nucleotide biosynthetic process	637
<i>C. secundus</i>	signal transduction	638
<i>C. secundus</i>	transmembrane transport	639
<i>C. secundus</i>	DNA replication initiation	640
<i>C. secundus</i>	transcription	641
<i>C. secundus</i>	G protein-coupled receptor signaling pathway	642
<i>C. secundus</i>	intracellular protein transport	643
<i>C. secundus</i>	proteolysis	644

642



643

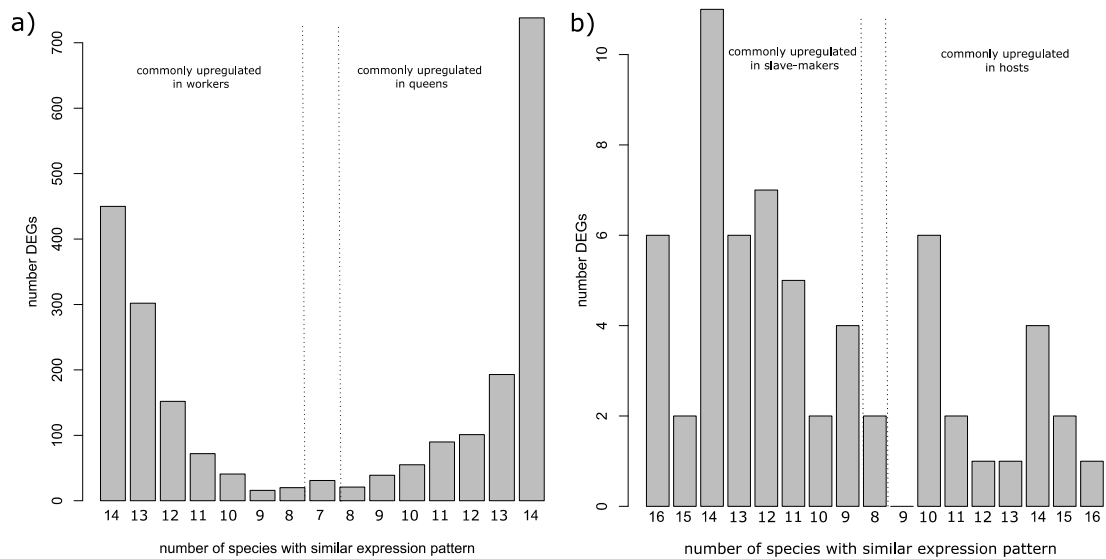
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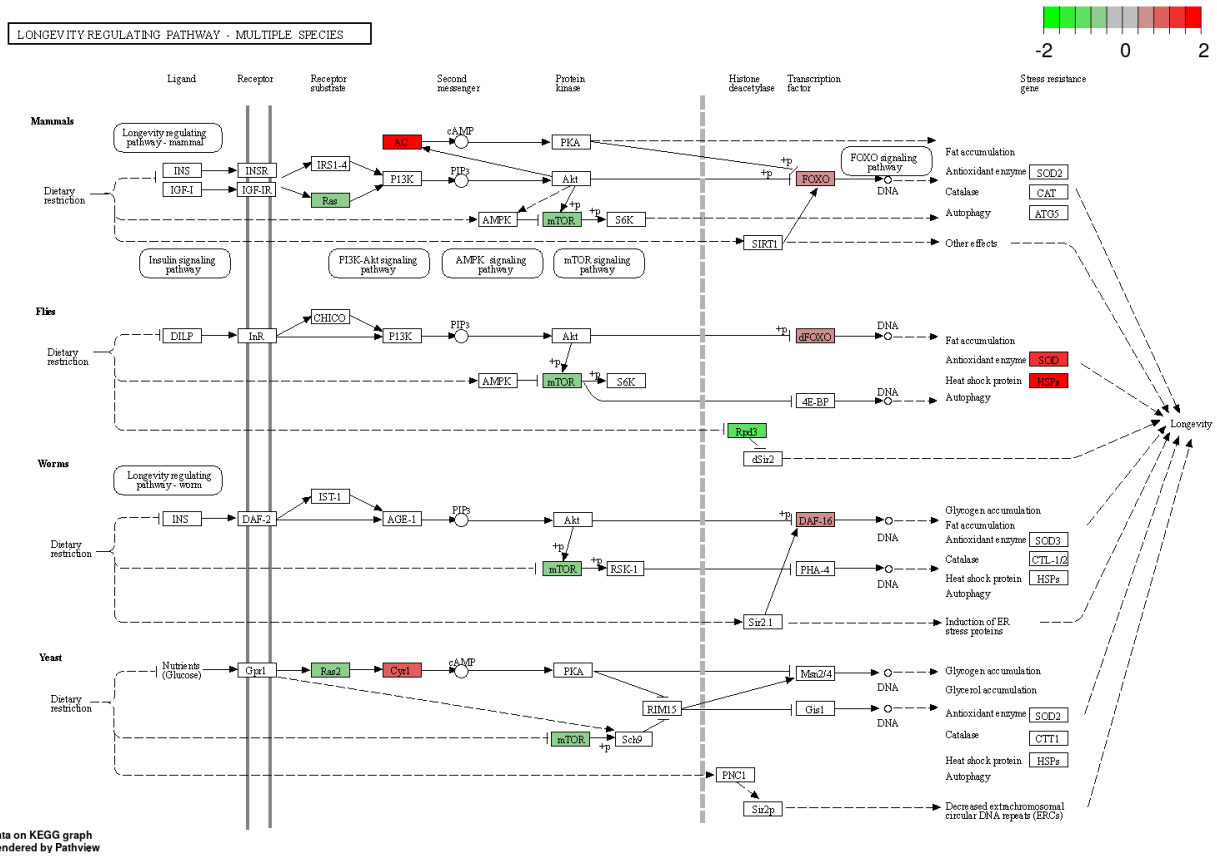
Figure 1: Sample dendrogram and heatmap depicting a strong clustering according to caste and a weaker effect of lifestyle. The heatmap is based on the top 50 highest expressed genes. Slave-making species are highlighted in bold.



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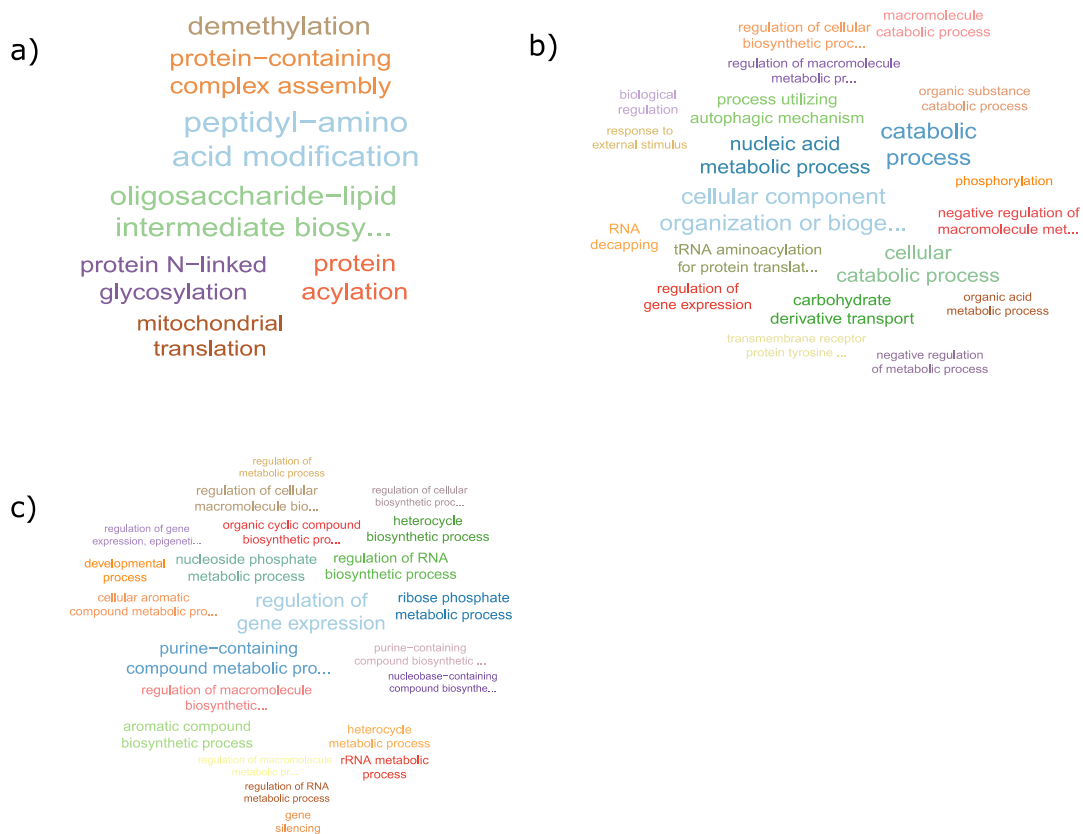
649 **Figure 2:** Number of differentially expressed genes (DEGs) which showed shared expression patterns
650 across a majority of study species during the pairwise comparisons of queens vs workers or slave-
651 makers vs hosts. a) Number of DEGs upregulated in workers (left) or upregulated in queens (right)
652 across more than half the study species. b) Number of DEGs upregulated in slave-makers (left) or
653 upregulated in hosts (right) across more than half the study species. The vertical lines delimit the
654 start of a majority of species in either direction, with the bar between the lines showing DEGs that
655 showed a common expression pattern in one direction in half the species, and in the other direction
656 in half the species - e.g. in graph a), DEGs which were upregulated in queens in 7 species, and
657 upregulated in workers in the other 7 species. Please note that the host-slave-maker pairs add up to
658 16 comparisons instead of 14, since *Harpagoxenus sublaevis* parasitizes two host species: *L.*
659 *acervorum* and *L. muscorum*.

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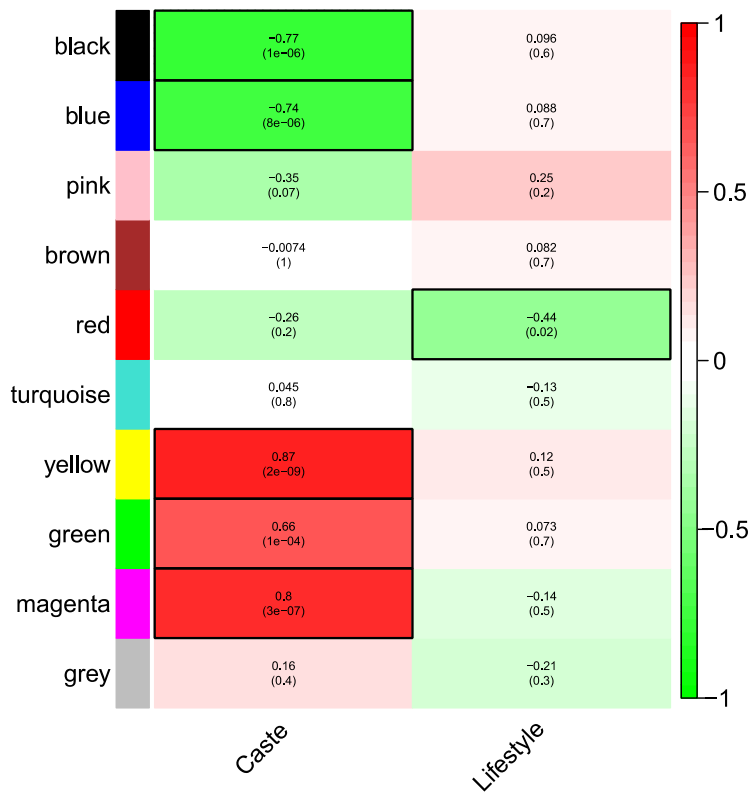
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Figure 3: KEGG map depicting the “longevity regulating pathway” with up-regulated genes in queens (negative logFC; green) versus up-regulated in workers (positive logFC; red).



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 668 **Figure 4:** Word clouds of enriched GO functions based on genes with signature of selection in a)
 669 slave-makers, b) hosts, c) non-hosts.
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Figure 5: Module trait relationship of the 10 gene co-expression clusters with caste and lifestyle. The strength of the correlation is given in the upper numbers and significance levels in parentheses below. Significant modules are highlighted by black boxes. Modules are labelled by colours according to standard WGCNA output. Green indicates modules of genes overexpressed in workers / slave-makers, red indicates those with genes more expressed in queens / hosts.