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Evidence for a conserved queen-worker genetic toolkit across slave-making ants and their ant hosts

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Abstract

The ecological success of social Hymenoptera (ants, bees, wasps) depends on the division of labour between the queen and workers. Each caste exhibits highly specialized morphology, behaviour, and life-history traits, such as lifespan and fecundity. Despite strong defences against alien intruders, insect societies are vulnerable to social parasites, such as workerless inquilines or slave-making ants. Here, we investigate whether gene expression varies in parallel ways between lifestyles (slave-making versus host ants) across five independent origins of ant slavery in the "*Formicoxenus*-group" of the ant tribe Crematogastrini. As caste differences are often less pronounced in slave-making ants than in nonparasitic ants, we also compare caste-specific gene expression patterns between lifestyles. We demonstrate a substantial overlap in expression differences between queens and workers across taxa, irrespective of lifestyle. Caste affects the transcriptomes much more profoundly than lifestyle, as indicated by 37 times more genes being linked to caste than to lifestyle and by multiple caste-associated modules of coexpressed genes with strong connectivity. However, several genes and one gene module are linked to slave-making across the independent origins of this parasitic lifestyle, pointing to some evolutionary convergence. Finally, we do not find evidence for an interaction between caste and lifestyle, indicating that caste differences in gene expression remain consistent even when species switch to a parasitic lifestyle. Our findings strongly support the existence of a core set of genes whose expression is linked to the queen and worker caste in this ant taxon, as proposed by the "genetic toolkit" hypothesis.

KEYWORDS
caste, dulosis, gene networks, selection, social parasitism, transcriptomes

1 | INTRODUCTION

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

Ten independent origins of slavery in ants are documented (Stoldt & Foitzik, 2021), and at least five convergent origins lie within the "*Formicoxenus*-group" of the myrmicine tribe Crematogastrini (Blaimer et al., 2018). This allows for the investigation into convergent changes in gene expression across these evolutionary transitions to slave-making. There are several morphological, physiological, and behavioural similarities among slave-making species of independent origin. For example, slave-maker workers are often heavily armed with strong mandibles and associated muscles, which leads to enlarged heads (Hölldobler & Wilson, 1990). Given that workers of slave-making species do not take care of the daily duties in the colony, they are more queen-like in their task repertoire. They neither forage for food nor do they engage in brood care (Buschinger, 2009). Moreover, slave-maker workers tend to have increased reproductive potential. While the ovaries of nonparasitic workers have fewer ovarioles than the queen's ovaries and rarely contain mature eggs in the presence of a fertile queen, the ovaries of slave-maker workers often have the same number of ovarioles as the queen (Figure 1; Heinze, 1996a). Slave-maker workers form reproductive hierarchies (Bourke, 1988; Franks & Scovell, 1983; Heinze, 1996b) and frequently lay male-destined eggs even in the queen's presence (Brunner et al., 2005; Foitzik & Herbers, 2001; Suefuji & Heinze, 2014). Gene expression differs strongly between workers and queens of most social insect species, reflecting their divergent function, behaviour, and physiology (Gstöttl et al., 2020; Feldmeyer et al., 2014; Korb et al., 2021; Morandin, Brendel, et al., 2019; Morandin, Hietala, & Helanterä, 2019). For example, queen transcriptomes are characterized by the expression of genes associated with fecundity (e.g., vitellogenins), immunity, DNA repair and response to oxidative stress, functionalities linked to their long lifespan (Stoldt et al., 2021). However, given the described similarities between slave-maker queens and workers, we expected their transcriptomes to differ less than those of queens and workers of nonparasitic species.

Berens et al. (2015) suggested that convergent caste phenotypes may depend on the differential expression of a common "toolkit" of genes across species. We therefore investigated the influence of caste (queen vs. worker) and lifestyle (slave-maker vs. nonparasitic) on gene expression of adult individuals and the interaction between those two parameters, which would indicate that caste is affecting gene expression differently in nonparasitic versus slave-making species. Here, we use a protocol which is aimed at minimizing confounding effects by using all five available and evolutionarily closely related pairs of slave-maker and host in the myrmicine tribe Crematogastrini. For each species, we sequenced the transcriptomes of workers and queens, respectively, taking species as replicates. We investigated gene regulatory network properties according to caste and lifestyle and constructed orthologue clusters to investigate putative parallel selection patterns in genes associated with the slave-maker versus host lifestyle, in comparison to two related nonhost taxa and the distantly related *Cardiocondyla obscurior*.

2 | MATERIALS AND METHODS

2.1 | Sampling and sequencing

Colonies of 17 myrmicine ant species of the "*Formicoxenus*-group" (genera *Harpagoxenus*, *Leptothorax*, and *Temnothorax*), including the previously synonymised genera of slave-making ants *Chalepoxenus*, *Myrmoxenus*, and *Protomognathus* (Ward et al., 2015, but see Seifert et al., 2016) were collected in late spring to early summer between 2016–2018 from various locations across Germany, Italy, Japan and the United States (Table S1, Figure 1). Our transcriptome study included seven slave-making species (*Temnothorax americanus*, *T. pilagens*, *T. duloticus*, *T. ravouxi*, *T. mediterraneus*, *T. muellerianus* and *Harpagoxenus sublaevis*), and the seven host species (*T. longispinosus*, *T. ambiguus*, *T. curvispinosus*, *T. unifasciatus*, *T. recedens*, *Leptothorax acervorum and L. muscorum*). Two nonhost species *T. nylanderi and T. rugatulus* and the distantly related *Cardiocondyla obscurior* were included as outgroups in the selected genes analysis only (Figure 1). For the latter species and for the host *T. unifasciatus*, we sampled two different populations each. The colonies were brought to the laboratory in Regensburg, Mainz or Münster and kept in incubators (day-night cycle 12 h 25°/12 h 16°C) for at least 2 months under standard conditions before sampling. Sampling was done in the morning and on the same day for each slave-maker and host pair and queens and workers alike, so that our sampling regime could not affect lifestyle or caste differences in gene expression. For each host and nonhost species, two workers (one inside, one outside the nest) and one queen were sampled from each of three

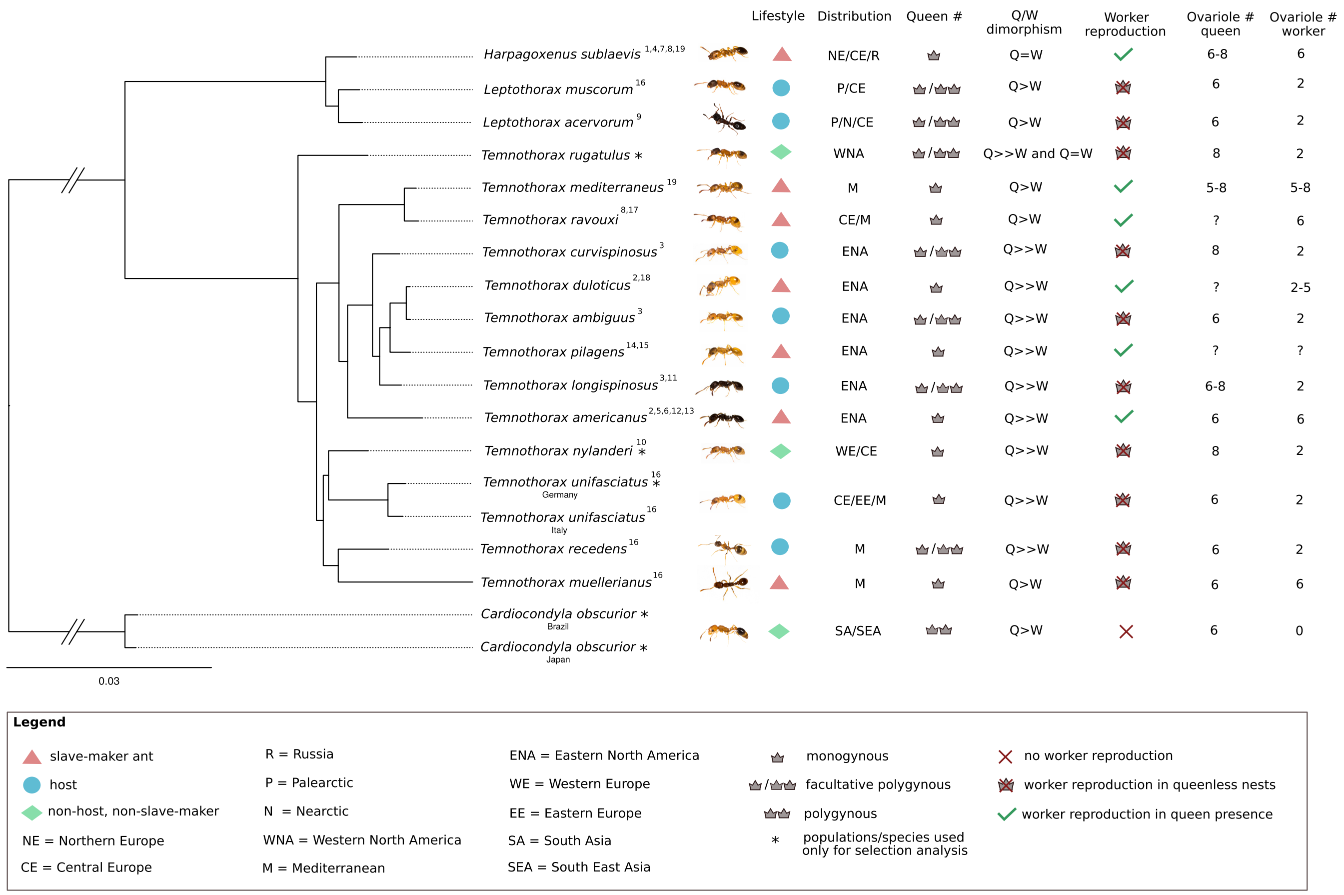

FIGURE 1 A maximum likelihood phylogenetic tree of all species included in this study depicting species relationships as well as information on lifestyle and associated morphological trait and species distribution. Q = W; Q > W: Q ~ 1.1–2.5× the dry weight of workers; Q ≫ W: Q ~ 2.6–5× the dry weight of workers. References referred to in the figure can be found in Table S2

colonies, so in total six workers and three queens were pooled for RNA-extraction, respectively. To get an equivalent amount of RNA for the outgroup species *Cardiocondyla obscurior*, which is considerably smaller than the other species, we took 16 workers (eight inside, eight outside the nest) and eight queens in total. This higher number of samples for the worker pools allowed us to correct for variation in worker behavioural phenotypes, particularly in hosts. For the host species, we carefully chose three workers from the brood pile, and three outside workers. It has been shown before that the spatial position of the workers corresponds to their task, that is, nurse versus forager. Additionally, in the nonparasitic species, as in many other ant species, task is associated with age, as young workers are nurses and old workers are foragers (Kohlmeier et al., 2017). Thus, in the case of the hosts, we collected caste- and age-controlled samples. For the slave-making species, the picture is different. Here the number of workers is generally low and colonies often consist of only one to six workers, even though they can get much larger. Thus, in many cases we chose the workers that were available without being able to control for age. However, slave-maker workers do not perform the "normal" worker tasks such as nursing and foraging. Rather, they usually reside in the nest and go on slave-raids during the raiding season, and we sampled outside that time. Because of the occurrence of "worker-like" wingless queens in *Harpagoxenus sublaevis*, we conducted dissections to identify the reproductive queen by the presence of the spermatheca filled with sperm.

We actively decided to use whole bodies, since we currently do not know which tissue or body part makes a slave-making ant different from a host. Moreover, due to morphological differences, specific tissues and body parts may differ between species. We thus decided to take pools of whole ants. We acknowledge that our sampling scheme, while accounting for the above-mentioned parameters, may have obscured tissue-, task-, or age-specific changes in transcriptional activity, and suggest that future studies should focus on specific tissues of single workers of defined caste and age. For each of the species, RNA was extracted from the pooled workers and pooled queens, respectively, using the Nucleo-Spin Mini kit (Macherey-Nagel). Samples were shipped to StarSEQ (Mainz) for library preparation and 100 bp paired end sequencing on an Illumina HiSeq. In total, we obtained 14 million reads on average per sample (Table S3).

2.2 | Gene expression analyses

Raw reads were quality checked using FastQC version 0.11.8 (Andrews, 2010), and adapters trimmed with Trimmomatic version

2.8.4 (Bolger et al., 2014). HISAT2 version 2.1.0 (Kim et al., 2015) was used to map the reads to the *T. longispinosus* genome version 1 (GenBank accession: GCA_004794745.1_tlon_1.0; Kaur et al., 2019), which was the only available genome at the time, but also of good quality (scaffold N50 = 514 kb; BUSCOs = 92.3%). We chose to use a single species genome as reference for all species to be able to directly compare expression patterns between slave-making ants and their hosts as well as between queens and workers across species, with species as replicates. The count table was created with HTSeq (Anders et al., 2015). To prevent spurious results due to low read counts, we removed genes with less than 10 reads in at least four samples from the counts matrix before the subsequent differential gene expression analysis with the R package DESEQ2 (Love et al., 2014) in R version 4.0.4 (R Core Team, 2017). We started with the full model ~Caste+Lifestyle+Caste:Lifestyle. The interaction turned out to be nonsignificant (no differentially expressed genes for the interaction), and we thus based the follow-up analyses on the simplified additive model. To specifically investigate the effect of caste within lifestyles and lifestyle within castes we ran additional models with single factors only (Tables S4 and S5). All *p*-values were adjusted by false discovery rate (FDR) correction as implemented in DESEQ2. To determine whether the significant slave-maker differentially expressed genes (DEGs) are more numerous than we would expect by chance, we ran 1000 permutations on the DEGs analysis in R.

To gain a deeper understanding of the functionality of DEGs, we (1) conducted a functional enrichment analysis, (2) inferred pathways in which the DEGs are involved, and (3) used word mining focusing on longevity and fecundity terms. In detail, we ran INTERPROSCAN version 5.39–77.0 (Jones et al., 2014) locally to obtain gene ontology (GO) information using predicted proteins encoded by the *T. longispinosus* genome (GenBank accession: GCA_004794745.1_tlon_1.0; Kaur et al., 2019) as query sequences. The GO enrichment analysis was performed with the R package TopGO (Alexa & Rahnenführer, 2016), using the "parentchild" algorithm and the Fisher's exact test for significance. Furthermore, the proteome was annotated with Kyoto Encyclopedia of Genes and Genomes (KEGG) functional orthologue numbers using the BlastKOALA web utility (last accessed: 11 February 2021; Kanehisa et al., 2016) with "Eukaryotes, Animals" specified as Taxonomy group. KEGG pathway affiliation of genes that were differentially expressed in each of the four groups (queens, workers, slave-makers, and hosts) was assessed using the online utility of KEGG mapper with *Apis mellifera* as the reference species (Kanehisa & Sato, 2020), and visualized including the log2-fold change using pathview (Luo & Brouwer, 2013) in R version 3.6.3 (R Core Team, 2017). KEGG pathway enrichment was assessed with the enrichKEGG function implemented in CLUSTERPROFILER version 3.14.3 (Yu et al., 2012) where the reference set was defined as the total set of *T. longispinosus* protein predictions with a KEGG functional orthologue annotation and organism KEGG orthology (KO). For the text mining approach, gene annotations obtained through a BLASTP search of the predicted proteins encoded by the *T. longispinosus* genome versus the RefSeq invertebrate database were used as query sequences for a UniProt search. More specifically, we extracted the gene function information for each gene with UniProt entries from *C. elegans*, *D. melanogaster* and *A. mellifera* and searched for terms related to fecundity and longevity (Table S6), both traits which we predict to differ between queens and workers (script available from Negroni et al., 2021). We conducted an enrichment analysis by conducting a Fisher's exact test to test whether the proportion of differentially expressed genes with terms related to fecundity or longevity was higher than expected in comparison to the complete proteome. The online tool VENNY version 2.0.2 was used to generate the Venn diagram (https://bioinfogp.cnb.csic.es/tools/venny).

2.3 | Network analysis

To identify networks of coexpressed genes (modules), we conducted a weighted gene coexpression network analysis using the WGCNA (Langfelder & Horvath, 2008) package in R. We used all genes that had passed the quality filtering step for the expression analysis ($N = 8327$), thus not only the differentially expressed genes. Gene counts were normalized using the variance StabilizingTransformation function from DESEQ2 (Love et al., 2014). Following the WGCNA guidelines, we picked a soft-thresholding power of 8 for adjacency calculation. To associate modules to either caste or lifestyle, we first calculated the modules' eigengene using the moduleEigengenes function and tested for module trait correlation using the corPvalueStudent function. The hub gene of each module (i.e., the gene with the highest connectivity within a module) was determined using the chooseTopHubInEachModule function. Moreover, for modules associated with caste or lifestyle, we tested whether the connectivity of genes with caste-specific expression differed from the connectivity of those that were not differentially expressed. We ran linear models with connectivity as the response and caste-specificity (queen, worker, no expression difference) as the explanatory variable in R.

Slave-maker queens generally produce less workers, thus are less fecund, than host queens, and fecundity is often positively correlated with longevity in social insects (Korb & Heinze, 2016; Negroni et al., 2021). To determine the relevance of fecundity- and longevity-associated genes in lifestyle and caste-associated modules, we downloaded a list of 123 genes that were assembled based on information from *Drosophila* and are part of the TI-J-LiFe pathways (TOR/IIS-JH-Lifespan and Fecundity) (Korb et al., 2021). We used the Flybase identifiers to download the corresponding *Drosophila* sequences and used a BLASTX search to identify *T. longispinosus* proteins with a BLAST hit *E*-value < e-10. In cases where we had two or three hits, we selected the protein with the longest match, which mostly corresponded to the lowest E-value. In cases where we had more than three BLAST hits ($N = 29$), we created a sequence alignment using MACSE version 2.03 (Ranwez et al., 2011). A Maximum Likelihood phylogenetic tree with 1000 bootstrap replicates was constructed with RAxML version 8.2.12 with PROTGAMMAWAG as the protein substitution model. Based on the tree topology we

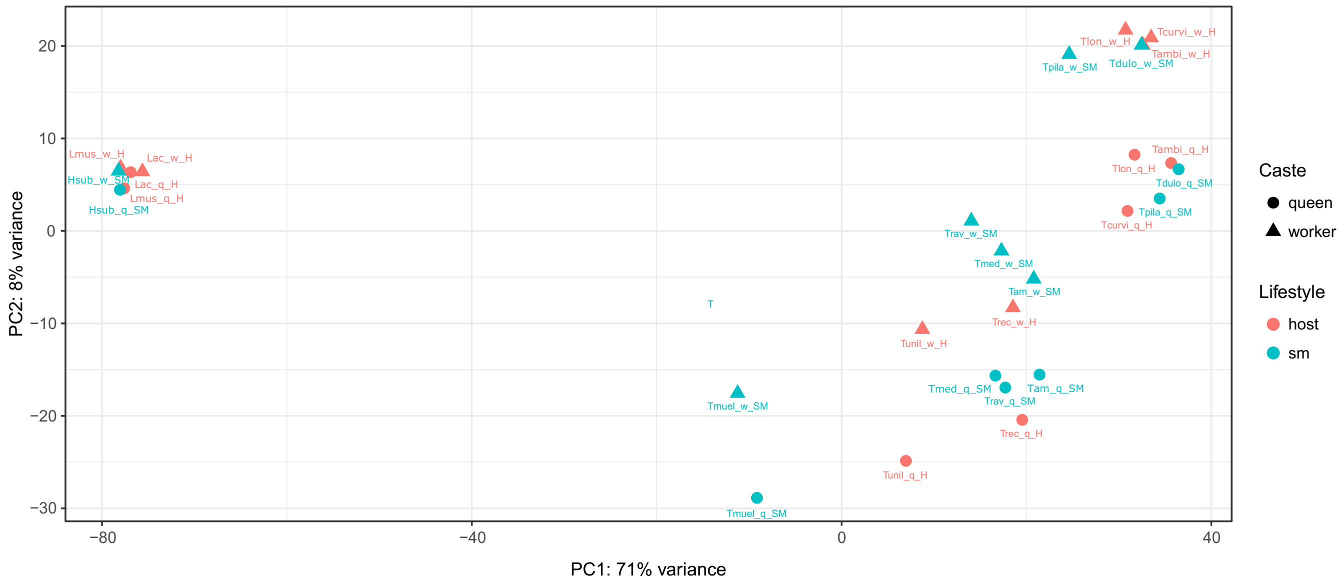

FIGURE 2 Principal component analysis (PCA) depicting sample similarity mainly according to phylogenetic relationship on PC1 and a clear distinction of queens and workers within each species on PC2. The PCA is based on the full read count table

chose the *T. longispinosus* sequence with the closest relationship to the target *Drosophila* sequence.

2.4 | Selected genes analysis

For this analysis, we added transcriptomes of two taxa from the *Formicoxenus*-group, *Temnothorax nylanderi* and *T. rugatulus*, which are not known to be parasitised by slave-making ants, and which acted as a biological control for comparisons of selection intensity between slave-makers and hosts. Additionally, samples from two populations of *Cardiocondyla obscurior* were included as nonhost outgroups (Table S1, Figure 1). We used Trinity version 2.8.6 (Grabherr et al., 2011) with standard settings to construct species-specific transcriptomes using both worker and queen transcripts. Nucleotide sequences were translated into amino acid sequences with TransDecoder (https://github.com/TransDecoder). As de novo transcriptomes are known to contain many transcript fragments as well as isoforms, we constructed orthogroups across all species with OrthoFinder (Emms & Kelly, 2015), including protein sequences derived from the *Temnothorax longispinosus* genome (Kaur et al., 2019), and retained orthogroups with a genomic orthologue only. After orthogroup construction, the *T. longispinosus* protein sequences derived from the genome were removed from the orthogroups for all downstream summary statistics and analyses. Since we only obtained few single copy orthologues, we used an inhouse script (Supplement_Script) to remove putative paralogues and retain only a single sequence per species. Single copy orthologues are a prerequisite for the identification of selected genes. In short, based on the pairwise BLAST results from OrthoFinder, the sequence with the highest sum of bit scores (i.e., best match to all other sequences) within each orthogroup was chosen as the "centroid". For each species, the sequence with the best match to the centroid was chosen to create the single copy orthogroup. We used Clustal-omega version 1.2.4 (Goujon et al., 2010) to construct sequence alignments for each orthogroup, which were trimmed with TrimAL version 1.4.1 (Capella-Gutiérrez et al., 2009) and the following settings: -gappyout -resoverlap 0.75 -seqoverlap 75 -backtrans. To test for signatures of positive selection, we used the codeml implementation in ete3 version 3.1.1 (Huerta-Cepas et al., 2016) running the branch site test of selection as follows: ete3 evol --models bsA bsA1 --tests bsA, bsA1 --leaves –internals, and used the implemented likelihood ratio test to compare the likelihood of the null model, wherein the value of ω (=dN/dS) in the foreground branch was set to ω<=1, to the likelihood of the alternative model that estimates the proportion of sites with $\omega > 1$. The cluster-specific tree topology as inferred by RAxML version 8.2.12 (Stamatakis, 2014) was used as the input tree, and each species was coded as foreground branch consecutively. Finally, we BLASTed the *T. longispinosus* sequence of each orthologue cluster with signatures of positive selection versus the *T. longispinosus* genome and only retained orthologue clusters with sequences mapping to a single location, as further means of preventing putative paralogues. We further ran a GO enrichment analysis to test for overrepresented functions among the genes under positive selection (details above), and used KEGG annotations to investigate associated pathways.

To construct a phylogeny including all species of this study, we concatenated all above constructed 4792 orthogroups into one long sequence using catfasta2phyml (https://github.com/nylander/catfasta2phyml). We used RAxML version 8.2.12 (Stamatakis, 2014) to infer a maximum-likelihood (ML) phylogeny, by applying rapid bootstrap analysis and searching for the best ML tree in a single run, with the GTRGAMMA model and 1000 bootstraps. The best tree was visualized in FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/).

3 | RESULTS

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

In general, gene expression patterns seem to be most similar among phylogenetically close species. The principal component analysis revealed a very strong phylogenetic effect with 71% of variance explained by PC1, which separated the *Harpagoxenus/Leptothorax* group from the *Temnothorax* species (Figure 2), while PC2, explaining 8% of the variance, separated queens from workers. While this phylogenetic clustering should be kept in mind, our balanced phylogenetic sampling reduces the risk of phylogenetic correlations biasing our results for lifestyle and caste. To determine whether the back-mapping rate affected the observed "phylogenetic clustering" pattern observed in the PCA (Figure 2) we constructed a PCA based on genes expressed by all samples, that is, with read counts above 0 for all samples. This reduced the number of entries from $N = 8327$ to $N = 5452$, but the pattern remained the same. *Harpagoxenus* and *Leptothorax* species clustered to the left, and a slight substructuring of North American and European species on the right (results not shown).

Caste had a much stronger effect on gene expression than lifestyle, as also evident in the heatmap dendrogram, where samples cluster according to caste rather than lifestyle (Figure S1). As our analysis did not reveal an interaction between lifestyle and caste (no DEGs associated with the interaction term), we present the gene expression and gene network results according to the main effects, caste and lifestyle, and finally the results from the selection analysis.

3.1 | Lifestyle

We detected 62 differentially expressed genes (DEGs) between lifestyles (host versus slave-maker species) cumulative across the five origins of slavery (see Table S7). A permutation test with 1000 iterations revealed that this is more than expected by chance ($p = .04$). Six genes were consistently more highly expressed in all seven slave-maker species compared to hosts (Figure S2), of which three were annotated: "protein DVR-1 homologue", plays a role in growth factor activity and response to organic substance (uniprot.org), "sulfotransferase family cytosolic 1B member 1-like", is involved in the sulfation of molecules (uniprot.org), and "ATP-dependent DNA helicase II subunit 1-like", is involved in double strand break repair (uniprot.org). Only a single gene, "NADPH oxidase 5", showed higher expression in all seven host species compared to slave-makers (Figure S2), which seems to play a role in moulting and oviposition (Peng et al., 2020). Various metabolic processes were significantly enriched among the differentially expressed genes between lifestyles (total = 21 processes). Among genes upregulated in slave-makers, 14 terms were enriched, and three in genes upregulated in hosts, including "cell death" (Table S8). In addition to the GO term enrichment, we also investigated whether the genes in the lifestyle DEGs set were overrepresented in specific pathways. Due to the low number of lifestyle-associated DEGs, with only half of these possessing a KEGG annotation, the enrichment analyses for the complete set of lifestyle DEGs, as well as for the separate host and slave-maker gene-sets, did not result in any overrepresented functions.

TABLE 1 Number of differentially expressed genes with a fecundity or longevity functionality based on a text mining approach using the UniProt database as a reference. Results of Fisher's exact test are given indicating whether the number of genes with either fecundity or longevity association in caste and lifestyle is higher than expected by chance

	Fecundity		Longevity	
Treatment	**N of genes**	***p*-value**	**N of genes**	***p*-value**
Caste	1077	**2.2e-16**	412	**.0003**
Queen	630		262	
Worker	447		150	
Lifestyle	32	**6.619e-09**	13	.39
Host	9		6	
Slave-maker	23		7	

The Significant values ($p < .05$) are indicated in bold.

Host colonies are generally larger than slave-maker colonies, that is, host queens produce more workers than slave-maker queens. Moreover, fecundity is often positively correlated with longevity in social insects (Korb & Heinze, 2016; Negroni et al., 2021). We therefore conducted a word mining approach based on UniProt entries to investigate whether genes with known fecundity or longevity functionality were overrepresented in the sets of DEGs between hosts and slave-makers (Table 1). We indeed recovered more DEGs putatively associated with fecundity than expected by chance ($N = 31$; Fisher's exact test, p-value = 6.619e-09). In contrast, we did not find DEGs to be significantly enriched for involvement in longevity (Table 1).

We obtained 10 modules of coexpressed genes (named after colours as given by the *WGCNA* package), of which one module, red, was positively associated with the slave-making lifestyle (Figure S3). This module contained 191 genes, of which 19 were upregulated in slave-makers and none in hosts. Genes associated with this lifestyle module were enriched for "reactive oxygen species metabolic processes", "oxidation reduction processes", or "fatty acid biosynthetic process" (Table S9). Genes upregulated in slave-makers had the highest connectivity in this module associated with lifestyle (Figure S4; Table S10).

3.2 | Caste

Caste had a much stronger effect on gene expression than lifestyle ($X^2 = 2496.8$, df = 1, p-value <2.2e-16), with 2321 differentially expressed genes (DEGs) between queens and workers. Around half of all caste-specific DEGs ($N = 1188$) showed consistently higher expression in queens ($N = 738$ out of 1295) or in workers ($N = 450$ out of 1026) across all species (Figure S2). For example, genes coding for an *insulin-like growth factor 2*, *mRNA-binding protein 1*, *maternal protein exuperantia*, *histone deacetylases*, and several *serine/threonine-protein kinases* were more highly expressed in queens of all 15 species, while *pro-corazonine like* had higher counts in workers of all but one species. The number of genes with consistent expression across taxa was significantly higher than for the lifestyle comparison ($X^2 = 36.865$, df = 1, p-value = 1.266e-09).

In addition to evaluating the interaction effect between caste and lifestyle, we also performed the differential expression analyses between castes for the two lifestyles separately. This yielded a total of 1124 caste-biased expressed orthologues in hosts and 1048 in slave-making ants. In total, 664 of these orthologues were differentially expressed in both slave-making ants and hosts, which amounted to 59.1% of the host DEG and 63.4% of the slave-making ant DEG. These 664 genes were either consistently upregulated in queens or consistently upregulated in workers of both lifestyles (Table S4). These findings suggest that hosts have a similarly large repertoire of caste-biased genes compared to slave-making ants. This provides further support against the hypothesis that reduced phenotypic differentiation between slave-making queens and workers is accompanied by a decrease in the number of genes that are differentially expressed between the castes in slave-making ants. Furthermore, we assessed the correlation in caste-biased expression between hosts and slave-makers. The rationale is that, if the reduction in phenotypic differentiation between slave-maker castes is reflected in the differential gene expression profiles, then genes with caste-biased expression in hosts are expected to show less caste-biased expression in slave-makers. Genes with caste-biased expression in slave-makers, on the other hand are not expected to have become less caste-biased in hosts, which is why this comparison served as a control. We assessed the correlation in log2 fold change values of orthologous genes differentially expressed between the castes of slave-makers and hosts. Genes with caste-biased expression in hosts are indeed less caste-biased in slave-makers, but the exact same pattern was found for genes with caste-biased expression in slave-makers. Hence, these findings suggest that the transition to slave-maker lifestyle is not accompanied by consistent shifts in gene expression profiles (Figure 3).

In the caste-specific DEG set, 39 gene ontology (GO) functions were significantly overrepresented, many of which were associated with metabolic and biosynthetic processes (Table S8). Genes upregulated in queens were enriched for 60 terms linked to various metabolic processes or stress responses. Among the worker upregulated genes, 78 terms were significantly enriched, again belonging to metabolic and biosynthetic processes, but also oxidation–reduction. In addition to the GO functional enrichment, we conducted a KEGG-pathway enrichment analysis. In total, we found 27 pathways enriched among the caste-specific DEGs, 12 for genes upregulated in

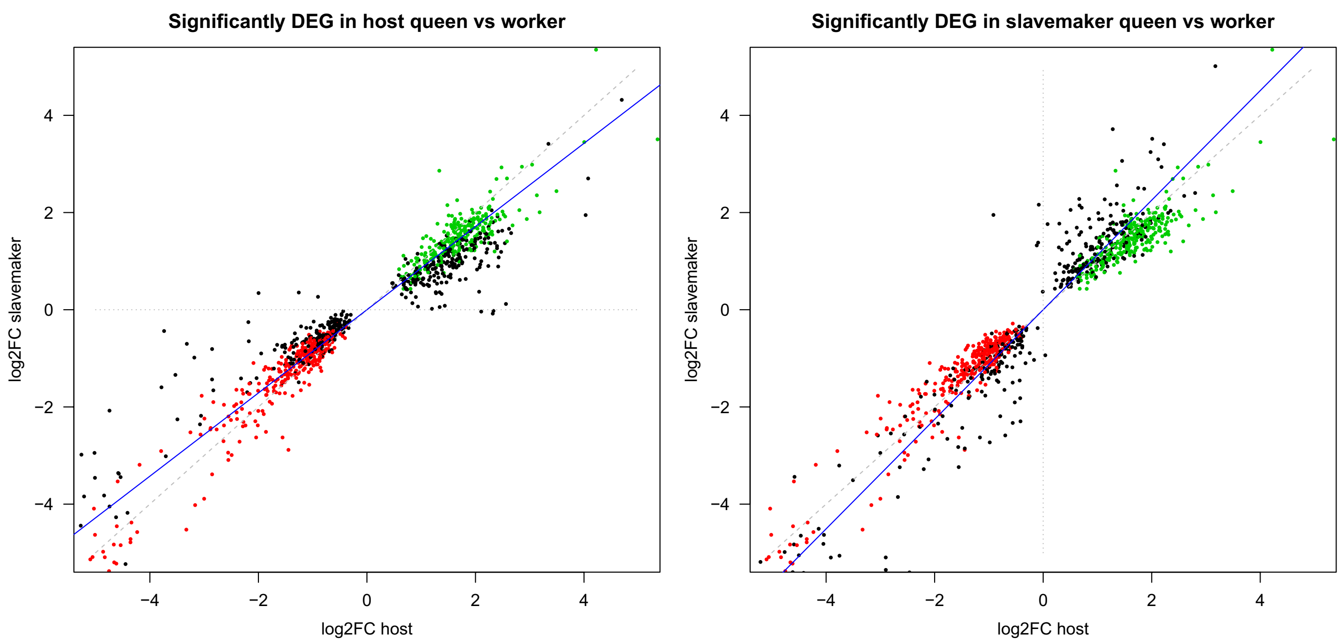

FIGURE 3 Correlation in caste-biased gene expression between host and slave-maker orthologues (based on the single factor model). Genes with caste-biased expression in hosts are less caste-biased in slave-makers, but also caste-biased genes in slave makers are less biased in hosts. This pattern suggests that the transition to a slave-maker lifestyle is not accompanied by consistent shifts in gene expression profiles. Left: 1124 orthologues with caste-biased expression in hosts. Right: 1048 orthologues with caste biased expression in slave-makers. Grey dotted line represents similar levels of caste-biased expression in slavemakers and hosts. The blue line represents the actual correlation between the two lifestyles. Queen-biased genes are coloured in red and the worker-biased genes in green. Log2 fold change values were obtained by running DESeq2 on each of the lifestyles separately, yielding 664 orthologues that were differentially expressed between castes of both slave-making ants and hosts

queens, and 61 genes upregulated in workers (Table S11). Multiple putatively reproduction-associated and repair pathways, such as "meiosis-yeast", "cell cycle", "DNA replication", "RNA transport," or "ribosome biogenesis," were enriched in queens, and pathways, such as "olfactory transduction" and "longevity regulating pathway" (Figure S5), were enriched in genes upregulated in workers (Table S11). As queens and workers strongly differ in fecundity and longevity, we searched for information beyond what is provided through GO and KEGG enrichment analyses. UniProt entries go beyond GO functions and contain some more elaborate information from functional studies. We thus used a word mining approach based on UniProt entries to investigate whether genes with known fecundity or longevity functionality were overrepresented in the sets of caste-specific DEGs (Table 1). Indeed, we detected more fecundity-associated terms ($N = 1076$; Fisher's exact test, p-value = 2.2e-16), and more longevity-associated terms ($N = 412$; Fisher's exact test, p-value < .0003) than expected by chance.

The gene network analysis resulted in five modules associated with caste, three of which were positively associated with queens (yellow, green, magenta), and two with workers (black and blue) (Figure S3; Table S9). There was a trend for the pink module to also be associated with caste ($p = .07$; Figure S3). In all three worker associated modules (black, blue, pink), connectivity was highest for worker upregulated genes, lowest for queen upregulated genes, and intermediate for genes that were not differentially expressed. The same pattern was observed in queen-specific modules (yellow, green, magenta), in which the queen upregulated genes had the highest connectivity (Table S9).

3.3 | Comparison to other species

Of the 123 candidate genes from the "TI-J-LiFe" (TOR/IIS-JH-Lifespan and Fecundity) list, which have previously been linked to fecundity or longevity in *Drosophila* (Korb et al., 2021), 80 were found in modules of our WGCNA network. About half of these ($N = 60$) were found in the turquoise module, which was neither associated to caste nor lifestyle, and eleven were found in the blue, caste-linked module (Table S12). To determine whether overrepresented gene functions linked to caste-associated modules from our *Formicoxenus*-group data set could be extrapolated to other ants or even termites, we compared our modules to those in Morandin et al. (2016) and Lin et al. (2021). Only three terms were shared between enriched GO terms of caste-associated modules in our slave-making data set ($N = 46$) and enriched GO terms in *Formica* caste-associated modules ($N = 155$; Morandin et al., 2016), namely "cellular protein modification process", "protein modification process", "monovalent inorganic cation transport" (Table 2). Eight terms were shared between our *Formicoxenus* data set and caste-associated modules in termites ($N = 277$; Lin et al., 2021) (Table 2). No terms were shared among all three studies. However, these results should be taken with caution as most enrichment algorithms take the hierarchical structure of GO terms into account. Thus, while different studies may not output the exact same terms, their terms may be hierarchically linked, which does not mean they are essentially different.

As queen and workers differ in fecundity and longevity, we investigated whether the same set of fecundity-longevity associated genes of the "TI-J-LiFe" list ($N = 84$) which were found in the termites (Lin et al., 2021) could be found in our data set. There were two genes, *Ras64B* and *Kr-h1*, associated with a queen-worker coexpression module in termites and in our species group.

3.4 | Selected genes analysis

The transcriptomes of the 19 taxa, including four samples of nonhost species, consisted of 67,150–155,629 transcripts with 32,584–51,743 open reading frames and 92%–96% DOGMA completeness. In total, we obtained 10,699 orthologue clusters of which 5826 clusters contained at least one transcript per species. Of these, 1398 clusters remained after trimming and filtering for single copies per species. We identified 660 signatures of positive selection ($dN/dS > 1$) across all species, associated with 424 orthologue clusters, each mapping to a single location on the genome across all species (Table S13). Between 6–92 genes showed signatures of positive selection in each species (Table S13), but there was no difference in the number of positively selected genes between lifestyles (quasipoisson model, $X^2 = 1.33$, df = 2, $p = .51$). In slave-makers, genes with signatures of positive selection were significantly enriched in functions such as protein modification, demethylation, and energy maintenance (Figure S6a, Table S14). In hosts and nonhosts, several regulatory and metabolic functions are significantly overrepresented in genes with signatures of positive selection (Figures S6b,c).

We identified 114 genes that were both differentially expressed and additionally showed signatures of selection (Figure 4; Table S14). Of these, a single gene was differentially expressed between castes and lifestyles and positively selected in *T. unifasciatus* (TLON_06615-RA; pleckstrin homology domain-containing family F member 2 isoform X1). All remaining 113 were differentially expressed only between castes. 35 of these caste-specific DEGs showed signatures of selection in hosts, 23 in nonhosts, and 57 in slave-makers. Interestingly, many nucleotide metabolism pathways were included in the overlapping gene lists of differentially expressed and selected genes, including purine, alanine, aspartate, valine, and the "mTOR signalling pathway" (Table S14).

4 | DISCUSSION

Eusocial insects are characterized by their sophisticated division of reproductive labour, which led to the evolution of different castes (Wilson, 1971). Ant queens are the main reproductives, known for their long life of up to several decades and high fecundity (Keller & Genoud, 1997), while the mostly infertile and short-lived workers take care of the brood, forage, and defend the nest (Hölldobler & Wilson, 1990). In slave-making species, however, workers do

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

Organism(s)	Shared enriched GO terms with this study
Multiple ants	Cellular protein modification process
Multiple ants	Protein modification process
Multiple ants	Monovalent inorganic cation transport
Cryptotermes secundus	Cyclic nucleotide biosynthetic process
Cryptotermes secundus	Signal transduction
Cryptotermes secundus	Transmembrane transport
Cryptotermes secundus	DNA replication initiation
Cryptotermes secundus	Transcription
Cryptotermes secundus	G protein-coupled receptor signalling pathway
Cryptotermes secundus	Intracellular protein transport
Cryptotermes secundus	Proteolysis

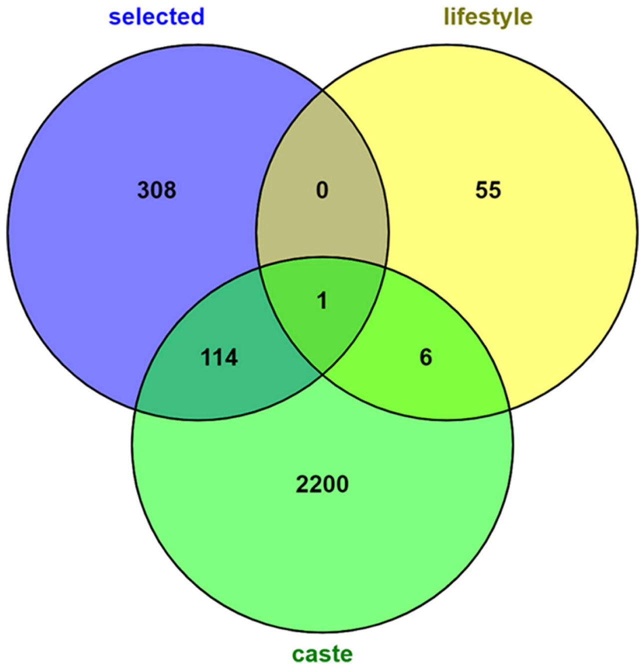

FIGURE 4 Venn diagram depicting the number of genes either specific to or shared between lifestyle or caste differentially expressed genes and positively selected genes. Of the 62 genes with consistent shifts in expression in slave-making versus host ants (that were not also differentially expressed between castes), a single gene showed evidence for positive selection. This gene (pleckstrin homology 411 domain-containing family F member 2 isoform X1) was shared among all three groups. Of the 2321 genes with expression differences among queens versus workers, 114 showed evidence for positive selection. More information on these genes can be found in Table S14

not perform the "general" worker chores. They raid host nests in summer and often are permitted to lay male-destined eggs in the presence of the queen. Our study shows that there is no interaction between the effects of caste and lifestyle on gene expression. However, the whole-body transcriptomes of seven slave-maker/host species pairs of the "*Formicoxenus* group" of Crematogastrini (Blaimer et al., 2018; *Harpagoxenus*, *Leptothorax*, and *Temnothorax*) differ considerably more between castes (queen vs. worker) than between lifestyles (slave-maker vs. nonparasitic species). Caste polyphenism is based on differential gene expression, and our transcriptome analyses show that the expression of 1188 (14%) genes consistently differs between queens and workers of all studied species, regardless of lifestyle. Although the evolution of a parasitic lifestyle is associated with considerable changes in morphology and behaviour, only a few transcriptomic shifts from host to slave-making species were consistent across the five independent origins of slave-making ($N = 6$).

4.1 | Little effect of lifestyle on gene expression

Approximately 40 times more genes were differentially expressed between castes than between lifestyles (2321 vs. 62), and six out of 10 coexpression modules were caste-associated in contrast to a single lifestyle-associated module. This difference might in part reflect the single evolutionary origin of caste diphenism in ants, whereas slavery evolved repeatedly (Beibl et al., 2005; Feldmeyer et al., 2017; Prebus, 2017). Nevertheless, as differences between castes in slave-making species are often less pronounced than in nonparasitic species, we expected to find a considerable interaction between the effects of caste and lifestyle on gene expression as well as on gene connectivity. The lack of an interaction may indicate that each caste, queen and worker, is rather similar on a molecular level between slave-makers and hosts. We specifically chose one replicate per species to investigate general patterns across species with respect to lifestyle and caste. The two traits (lifestyle and caste), however, were replicated across species. Moreover, by including a single sample per species, we circumvented pseudoreplication, for which current statistical frameworks cannot account in a satisfying manner. The small effect of lifestyle found in this study most probably results from the independent origins of slave-making with the putative usage of species-specific slave-making genes, as suggested by previous studies on a subset of species from this study (Alleman et al., 2018; Feldmeyer et al., 2017). To compare gene expression patterns between species, one needs a common reference. For this purpose, we used the genome of the host species *T. longispinosus*. Of course, using the genome of a single species as a reference means that genes specific to other species are not included in the analysis. However, since we were looking for a general and not species-specific pattern, we only considered genes that were expressed in at least four species, that is, about half of the species belonging to a given lifestyle or caste. The strong caste effect corroborates the result of a previous study where caste differences also exceeded differences due to other traits, such as worker sterility, queen number, or invasiveness (Morandin et al., 2016). One caveat to our sampling design which might have obscured putative differences between lifestyles but also castes is the usage of whole body transcriptomes. As gene expression differs largely between tissues, even single cells, tissue specific patterns might cancel out in whole body samples. However, we actively decided to use whole body transcriptomes and pooled nurses and foragers in host, since we currently do not know which tissue or body part discriminates a slave-making ant from a host. Moreover, due to morphological differences, specific tissues and body parts may differ between species. Our sampling scheme may thus have obscured tissue specific differences between lifestyles and also castes, and should be taken into account in future studies.

4.2 | Caste-associated gene expression

The Evo-Devo based "genetic toolkit" hypothesis, based on the observation that conserved gene sets are frequently involved in animal development and morphological innovation (Carroll et al., 2013), has also been applied to the convergent evolution of eusociality (Toth & Robinson 2007; Berens et al. 2015). In social insects, queens and workers are generally produced from the same genome. Thus, the genetic toolkit underlying convergent caste phenotypes may depend on the differential expression of common genes across species (Berens et al., 2015). While in this study, half of the genes ($N = 1188$) that were differentially expressed between queens and workers showed the same expression pattern across all species, another study identified only a single gene (the myosin light chain) showing a consistent differential expression pattern between queens and workers in a set of 16 species of multiple genera (Morandin et al., 2016). The reason for this discrepancy may be explained by the different species relationships as well as the underlying data type. We studied species within a single, closely related clade and used the genome of a single species as a reference. In contrast, Morandin et al. (2016) investigated species from five genera in different subfamilies based on de novo assembled transcriptomes. Additionally, there is evidence for similarities in pathways across different lineages of social insects (ants, bees, wasps) rather than a "common toolkit" of genes responsible for the caste phenotype (Berens et al., 2015). In our phylogenetically more restricted data set, however, we identified 1188 genes representing the "core set" of queen-worker differences across the 15 species. For example, the gene "corazonin", upregulated in workers, has been shown to control social behaviour and caste identity in the ant *Harpegnathos saltator* (Gospocic et al., 2017). The gene "maternal protein exuperantia", a maternal effect gene, which is needed for proper localisation of the *bicoid* RNA during oocyte formation (de Oliveira et al., 2017; Macdonald et al., 1991) but also plays a role in *Drosophila* spermatogenesis (Hazelrigg et al., 1990), was upregulated in all queens. Also, "G1/S-specific cyclin-D2" (*cycD*) was more strongly expressed in queens compared to workers in all but one species. This gene is involved in cell cycle regulation and is part of the "FoxO signalling" and "Wnt signalling" pathways, both important regulators of longevity. It could thus be associated with the lifespan differences between these two castes. Moreover, the "heat shock factor protein 2-like" was more strongly expressed in queens in 12 of 14 pairwise comparisons (exceptions are *H. sublaevis* and *T. ravouxi*). Higher expression of this gene has recently been linked to long lifespan in reproductive *Harpegnathos saltator* ants (Glastad et al., 2022). More generally, half of the genes differentially expressed between castes were associated with the UniProt functionalities "fecundity" and "longevity."

4.3 | Lifestyle associated gene expression

Reflecting the independent origins of ant slavery (Beibl et al., 2005; Feldmeyer et al., 2017; Prebus, 2017), the different parasitic species not only show pronounced differences in their morphology, but also in raiding behaviour (Brandt et al., 2006; Kleeberg & Foitzik, 2016; Johnson, 2008). Species-specific raiding patterns are mirrored by species-specific gene expression patterns (Alleman et al., 2018) and genes under selection (Feldmeyer et al., 2017). Gene expression differences between pairs of slave-makers and their hosts showed much more variation across species (representing different origins of slave-making) than the differences between queens and workers, that is, there are many more idiosyncrasies in how slave-makers and hosts differ than in how queens and workers differ (Figure S2). A genome-wide analysis of gene family evolution revealed on a broad scale that genomes of inquilines, another type of specialized and obligate social parasites, are characterized by genome erosion and relaxed selection (Schrader et al., 2021). However, at the gene family level, a convergent pattern was only observed in the loss of odorant receptors in inquiline (Schrader et al., 2021) as well as slave-maker species (Jongepier et al., 2022). We found 62 genes that varied with lifestyle across origins of slave-making, including genes for fatty acid synthases. This could be indicative either of differences in fat synthesis and maybe storage between the two lifestyles, but these synthases may also be involved in the synthesis of cuticular hydrocarbons, which are used as communication signals to discriminate species and castes (Kleeberg et al., 2017; Leonhardt et al., 2016). Five genes related to reproduction were significantly upregulated in the slave-makers compared to the hosts: three genes related to the formation of sperm flagella ("centrosomal protein of 131 kDa-like isoform X1"; Hall et al., 2013), motility ("probable tubulin polyglutamylase TTLL1"; Vogel et al., 2010) and signal transduction ("encurin"; Sutton et al., 2004), a gene required for ovarian follicle maturation ("Chorion peroxidase"; Tootle & Spradling, 2008), and a gene involved in the regulation of reproductive life span and longevity ("tryptophan 2,3-dioxygenase isoform X1"; van der Goot et al., 2012). In hosts, we found two genes upregulated in comparison to slave-makers which are putatively involved in longevity regulation. "Lipase 3-like isoform X1" regulates the metabolism of long-chain fatty acids (Lapierre et al., 2011), and is involved in the activation of autophagy resulting in lifespan extension (O'Rourke et al., 2013). "NADPH oxidase 5" controls oxidative stress response and longevity in *C. elegans* (Ewald et al., 2017), and plays a role in cuticle biogenesis (Edens et al., 2001). In slave-makers we found the "hormone receptor 4 "to be upregulated, which is involved in the sclerotization (tanning) of the insect cuticle and melanisation (Mendive et al., 2005). The frequent involvement in fierce fights during slave raids and the usurpation of host nests (Buschinger, 2009; d'Ettorre & Heinze, 2001; Le Moli et al., 1994; Mori et al., 2000) might indeed select for a more strongly sclerotized cuticula in slave-makers, but at present cuticular thickness has not yet measured in detail.

4.4 | Genes under positive selection

Slave-making is a derived lifestyle with novel behaviours and morphological traits. Slave-makers have thus diverged more strongly in their phenotype from their nonparasitic ancestors than their respective host species, and we expected to find more genes under selection in slave-makers than in hosts and/or nonhost species. On the other hand, we might also expect fewer genes under positive selection because smaller population sizes in slave-makers limit gene flow, and thus decreases the efficiency of selection (Schrader et al., 2021). As the number of selected genes did not differ between lifestyles, we might hypothesize that the two aspects balance each other. It should furthermore be noted that only orthologue clusters including all species are generally used for such an analyses, therefore species-, and thus also slave-maker-specific genes, will be missed. Better sampling and higher-quality genomes will soon allow to overcome this issue and to perform reliable investigations to species-specific genes. Genes with signatures of selection were significantly enriched in functions such as protein modification, demethylation, and energy maintenance. An interesting candidate among the genes that showed signatures of selection in only one or a few of the species is venom protease-like in *T. muellerianus*. Many Hymenopteran venoms are proteases (Touchard et al., 2016), however, nothing is currently known about the composition of the venom that *T. muellerianus* uses to kill host ants during slave-raids. On a higher level, many metabolism pathways were included in this overlapping gene list, including purine, alanine, aspartate, valine, to name just a few, and the "mTOR signalling pathway". The latter plays a major role in cell growth, cell survival and nutrient sensing (Saxton & Sabatin, 2017; Laplante & Sabatini, 2009; Jewell & Guan, 2013).

5 | CONCLUSION

Social parasitism is a derived condition with marked differences between host and nonhost species, from morphology to behaviour. In most social parasites, queens and workers are more similar than in the host species. Studying transcriptome patterns and gene regulatory networks of 15 different ant species, covering five origins of slavery, we expected to find an interaction between the effects of caste and lifestyle on gene expression. Despite phenotypic differences, gene expression profiles of queens and workers were remarkably similar between slave-makers and hosts. Our study confirms previous findings suggesting that expression and selection patterns characterizing the transition to parasitism are species-specific, with little convergence between independently evolving slave-maker/host pairs (Alleman et al., 2018; Feldmeyer et al., 2017). However, we observed a very strong and reliable effect of caste on gene expression. Within our broad taxonomic species range, we were able to identify a core set of 1188 caste-specific genes that show a consistent expression pattern across species regardless of lifestyle, suggesting a "genetic toolbox" in this taxonomic group of related ant species.

AUTHOR CONTRIBUTIONS

The study was conceived by J.H., E.B.-B. and S.F., and was designed by E.J., B.F., C.G., J.H., E.B.-B. and S.F. Ants were collected by C.G., and D.A.G. provided ant samples. B.F., C.G., J.W. and E.J. conducted the analyses. All authors contributed to writing the manuscript.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All raw sequence data underlying this study have been deposited in the National Centre for Biotechnological Information (NCBI) Sequence Read Archive (SRA) (BioProject: PRJNA783286).

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