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Brain and antennal transcriptomes of host ants reveal potential links between behaviour and the functioning of socially parasitic colonies

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Abstract

Insect social parasites are characterized by exploiting the hosts' social behaviour. Why exactly hosts direct their caring behaviour towards these parasites and their offspring remains largely unstudied. One hypothesis is that hosts do not perceive their social environment as altered and accept the parasitic colony as their own. We used the ant *Leptothorax acervorum*, host of the dulotic, obligate social parasite *Harpagoxenus sublaevis*, to shed light on molecular mechanisms underlying behavioural exploitation by contrasting tissue-specific transcriptomes in young host workers. Host pupae were experimentally (re-)introduced into fragments of their original, another conspecific, heterospecific or parasitic colony. Brain and antennal mRNA was extracted and sequenced from adult ants after they had lived in the experimental colony for at least 50 days after eclosion. The resulting transcriptomes of *L. acervorum* revealed that ants were indeed affected by their social environment. Host brain transcriptomes were altered by the presence of social parasites, suggesting that the parasitic environment influences brain activity, which may be linked to behavioural changes. Transcriptional activity in the antennae changed most with the presence of unrelated individuals, regardless of whether they were conspecifics or parasites. This suggests early priming of odour perception, which was further supported by sensory perception of odour as an enriched function of differentially expressed genes. Furthermore, gene expression in the antennae, but not in the brain corresponded to ant worker behaviour before sampling. Our study demonstrated that the exploitation of social behaviours by brood parasites correlates with transcriptomic alterations in the central and peripheral nervous systems.

KEYWORDS
dulosis, gene expression, parasite manipulation, social parasitism

1 | INTRODUCTION

In the animal kingdom, a great variety of fascinating social behaviours have evolved ranging from parental care in cichlids, and blood-sharing in vampire bats, to honeybees that signal the location of food sources to their nestmates using a symbolic dance language (Balshine & Abate, 2021; von Frisch, 1967; Wilkinson, 1990, respectively). Social insects, in particular ants, are ideal models for studying behaviour as they exhibit a wide range of behaviours from cooperative behaviours such as mass recruitment to a food source (Chadab & Rettenmeyer, 1975) and sharing of food via trophallaxis (LeBoeuf, 2017) to antagonistic behaviours such as raiding of other ant colonies (Alloway, 1979). Furthermore, they are characterized by a reproductive division of labour in which some individuals reproduce while others seemingly selflessly take care of the brood and their reproductive sisters (Hölldobler & Wilson, 1990). According to Hamilton's rule, altruistic social behaviour of workers should be preferentially directed towards related conspecifics, so it is important that individuals can both signal and perceive information indicating relatedness (Hamilton, 1963).

However, the conditions under which social behaviour is exhibited provide opportunities for potential manipulation of behaviour by parasites, either if the ants' recognition system is compromised in some way or if workers have been selected to care for the closely related brood of the colony in which they hatched. In colonies of socially parasitic ants, exploitation of this social behaviour occurs: Here, young parasitic queens invade the colonies of their host ant species during the foundation phase in order to have themselves and their offspring cared for by the host workers (Buschinger, 2009). Ant social parasites and their hosts are often sister species or at least close relatives, a phylogenetic relationship known as Emery's rule, and similarities in their communication systems and brood requirements may help to exploit the social behaviour of their hosts (Emery, 1909). This exploitation of care behaviours comes with fitness costs to the hosts, resulting in selection acting on defensive traits (Grüter et al., 2018). The first line of defence occurs when host workers prevent a parasite queen from entering the host colony, which includes enhanced enemy recognition and increased aggressiveness of the host (Jongepier et al., 2015; Jongepier & Foitzik, 2016; Kaur et al., 2019). Similar defences have been shown in hosts of avian brood parasites where hosts evolved to better recognize parasitic eggs and reject them (Soler, 2014). In both types of brood parasite systems, defending host individuals may have gained experience with parasites during their lifetime by preventing access to parasitic ant queens or the laying or acceptance of cuckoo eggs. The host's defensive reactions can thus be influenced by individual experiences. In dulotic socially parasitic ants, the parasite workers have to replace and increase their work force by regularly raiding neighbouring host colonies (Buschinger, 2009). During these raids, host pupae and larvae will be stolen and transported back to the parasitic colony, where they will develop into workers who will take over all chores within this foreign colony. For these host workers, a second line of defence to rescue fitness might be to either destroy parasite brood or to reproduce within parasitic nests (Achenbach et al., 2010; Achenbach & Foitzik, 2009; Czechowski & Godzińska, 2015; Foitzik et al., 2001). For these defence strategies to work, host workers need to be able to perceive that their current environment is different from their original colony, in which they had lived only during larval development and never in the adult stage. Contrarily, social parasites are selected to suppress these rebellious instincts of their host workers by preventing them from recognizing the parasitic environment they find themselves in (D'Ettorre et al., 2002). This raises the question of how perception in hosts within parasitic nests is altered compared to their original colonies and what the subsequent consequences are for their behaviour. A cross-fostering experiment in the dulotic ant *Temnothorax americanus* showed that *T. longispinosus* host workers did not differ in their aggressiveness when together with their mother queen or a socially parasitic one (Keiser et al., 2015). This suggests that parasitic queens manage to go undetected for example by carrying fewer recognition cues on their cuticle, also termed chemical insignificance, (Jongepier & Foitzik, 2016; Kaur et al., 2019; Kleeberg et al., 2017) or by using chemical mimicry or chemical camouflage (Johnson et al., 2001; Kleeberg & Foitzik, 2016; Tsuneoka & Akino, 2012). On the other hand, *T. longispinosus* host workers were reported to not only recognize their enemy during an attack on their nest (Kleeberg et al., 2015; Scharf et al., 2011), but also to exhibit divergent behaviours when exploited within a parasitic nest, for instance by killing parasitic pupae, a behaviour described as "rebellion" (Achenbach & Foitzik, 2009; Alloway & Pesado, 1983). This suggests that host workers are in some cases able to recognize the altered social environment and adjust their behaviour accordingly, while others fail to do so.

One way to measure whether and how individuals respond to changes in their social environment is to examine discrete shifts in phenotype that accompany those changes. In ants, for example, such changes occur after the removal of the queen in the form of increased reproduction and lifespan of workers (Almond et al., 2019; Choppin et al., 2021; Heinze et al., 1997; Kohlmeier et al., 2017; Majoe et al., 2021; Negroni et al., 2021) and, in isolated ants, in the form of altered behaviour (Boulay, 1999; Scharf et al., 2021; Seid & Junge, 2016). Over the past decade, with advances in omics technologies, studies were able to not only investigate the molecular basis of discrete phenotypes but also to discover even subtle changes on the molecular level, even with no apparent phenotypic changes. For example, *Polistes dominula* wasps, the host of the socially parasitic wasp *P. sulcifer*, showed shifts in their brain transcriptomes with changes in their social environment, which did not directly correspond to any measured phenotypic changes (Taylor et al., 2021). Thus transcriptomic studies are suited to unravel the molecular underpinnings of changes not (yet) translated into measurable changes on the behavioural, morphological or physiological level.

In the following study, we investigated the ant *Leptothorax acervorum*, which is a host to the obligate European dulotic ant *Harpagoxenus sublaevis*. This social parasite also exploits a smaller second host species, *L. muscorum*; therefore, mixed colonies with exploited host workers of both species occur regularly (Fischer-Blass

et al., 2006). Even though parasitic colonies are functional, aggressive interactions, such as antennal boxing and biting, frequently occur in *H. sublaevis* colonies (Heinze et al., 1994). In particular, parasite workers show dominant interactions towards their own sisters as well as towards exploited host workers (Bourke, 1988). Moreover, *L. acervorum* and *L. muscorum* host workers act aggressively towards both con- and heterospecific individuals and their social parasites, indicating that they recognize to a certain extent that their social environment has changed (Heinze et al., 1994). Using this study system, we wanted to investigate how the social environment of a parasitic colony influences the molecular underpinnings of social behaviour of exploited workers, as well as their nestmate recognition system. If the latter would be impeded, this might aid in the functioning of parasitic colonies despite chemical dissimilarities between parasites and hosts.

We, therefore, studied the transcriptomic response of *L. acervorum* host workers that emerged inside nests of the social parasite *H. sublaevis* to shed light on the mechanisms underlying the social behaviour of host workers in parasitic nests. By transferring workers as pupae to parasitic nests – as would happen in nature during raids (Buschinger, 1983) – we rule out any transcriptional changes during larval development caused by a different environment and focus on changes during the first weeks of adult life as workers. We then analysed which genes change their activity to gain insights into the molecular regulation of the observed behaviour and the sensory system in the context of social parasitism. If host workers can indeed perceive the social environment as parasitic and distinguish it from a conspecific environment, we would expect gene expression to change accordingly. If, on the other hand, the ability of the exploited hosts to recognize nestmates is somehow impaired by the parasite, we would expect gene expression, especially of genes known to be involved in the perception of the environment, to change less when living in a parasitic nest compared to the original colony. Other differences between parasitic and non-parasitic nests, such as a general increase in aggression in parasitic nests, may nevertheless be transcriptionally detectable, for example, in the expression of stress-related genes. Therefore, we specifically investigated genes related to sensory perception, in particular, odorant receptors, whose expression changes in response to the social environment of ant larvae (Pulliainen et al., 2021) and varies between workers mainly performing tasks inside or outside the nest (Caminer et al., 2023). Odorant receptors, particularly those of the 9-exon gene family, are known to bind to cuticular hydrocarbons, which play an important role in communication in social insects (Pask et al., 2017; Slone et al., 2017). Additionally, by recording the behaviour and spatial position of the ants shortly before sampling, we would like to gain insights into whether and how gene expression in the brain or antenna parallels behavioural changes in the hosts. While many studies focus on transcriptomic changes in the central nervous system, i.e., the brain, which not only processes information from the sensory organs but also directly controls behaviour and thus largely reflects an individual's behavioural phenotype (Robinson et al., 2008), recent work suggests that also the peripheral nervous system, the antennae, play a major role in determining an individual's behavioural phenotype (Caminer et al., 2023; Kennedy et al., 2021). Thus, we decided to sequence both of these tissues to additionally unravel the molecular basis of behaviour. Determining in which tissues gene expression is more strongly correlated with social insect behaviour will ultimately help in designing future experiments to study the molecular basis of social behaviour.

2 | MATERIALS AND METHODS

2.1 | Ant collection and colony maintenance

Colonies of the dulotic ant *Harpagoxenus sublaevis* and its two hosts *Leptothorax acervorum* and *L. muscorum* were collected in July 2020 close to Nuremberg, Germany (49.345592, 11.258971 and 49.413857, 11.026181). At the University of Mainz, ants were transferred to plastered nest boxes containing artificial nest sites consisting of a Plexiglas cavity sandwiched between two microscope slides (7.5 cm × 2.5 cm × 0.5 cm) covered with a red foil and kept at 18°C with a 12:12 light:dark cycle. Ants were fed with crickets and honey every fourth day and provided with water ad libitum.

2.2 | Experimental manipulation of colonies

For our experiment, we set up a total of five replicates, each with four sub-colonies, one sub-colony for each of the four treatments (more details see Appendix S1). For each replicate, one colony of *L. acervorum* was selected and 24 worker pupae were transferred from this colony into a Petri dish. We then prepared sub-colonies from (a) the original *L. acervorum* colony from which the pupae were taken, (b) another unrelated *L. acervorum* colony from the same population, (c) a colony of *L. muscorum*, the second host species of *H. sublaevis*, which sometimes co-occurs with *L. acervorum* in parasitic nests, and (d) a dulotic colony of *H. sublaevis* from the same ant community. We prepared sub-colonies by standardizing the composition of each sub-colony to one queen, 15 small larvae and 30 adult host workers. 15 workers were sampled from inside and 15 from outside the nest to ensure a representative distribution of worker caste and age. These adult host workers were marked with a thin metal wire between the thorax and abdomen (0.02 mm diameter, electrisola, red) to distinguish them from our focal newly hatched workers at the end of the experimental period. We then transferred six of the pupae from the petri dish into each of these sub-colonies referring to the following treatments: (a) "original" as control (a fragment of the original colony from which the pupae originated), (b) "conspecific" (an unrelated *L. acervorum* colony) to investigate the influence of foreign conspecifics, (c) "heterospecific" to analyse the influence of another species (here a *L. muscorum* colony) on the workers, and finally (d) "parasitic" (a colony of the social parasite *H. sublaevis*). In the parasitic treatment, we standardized the number of host workers to 30 as described above, but additionally added all *H. sublaevis*

ants (18.4 ± 13.56 adult females) to have the same number of host workers in each sub-colony. The workers of these obligate social parasites do not perform worker tasks such as brood care and foraging, which are outsourced to the host workers (Buschinger, 1966), so the number of workers actually performing tasks such as foraging and brood care was standardized. Unfortunately, the worker pupae from two of our replicates did not develop into adult workers in sufficient numbers, so we focussed our transcriptome analyses on the workers from the remaining three replicates. In addition, all *L. acervorum* pupae transferred into *L. muscorum* colonies ("heterospecific" treatment) were either killed or rejected from the colony or did not develop into workers (mortality: Kruskal–Wallis $p = .05$; rejection (pupae outside the nest in the first 3 days): Kruskal–Wallis $p < .001$, Wilcoxon Heterospecific-Parasitic $p = .016$, Wilcoxon Heterospecific-Conspecific $p = .012$, Wilcoxon Heterospecific-Original $p = .004$), which is in contrast to an earlier study that suggested that *L. muscorum* accepts *L. acervorum* pupae in its colonies (Schumann & Buschinger, 1991). We could thus not include this heterospecific treatment in our analyses. However, the number of transferred individuals (including pupae) still alive at the end of the experiment did not differ from the other treatments (Kruskal–Wallis: $p = .34$). A summary of the experimental setup showing the final treatments and replicate numbers is depicted in Figure 1.

2.3 | Behavioural observations and sampling for RNA-Seq

About 10 weeks (68–70 days) after the emergence of the first worker of each colony, we started our behavioural observations, at which point the age of the focal workers ranged between 50 and 70 days. We tested whether the individuals from the three colonies differed in their age according to treatment which was not the case (Kruskal–Wallis test: $p = .54$). Moreover, a previous study on the same population, investigating how age affects worker behaviour, found that only resting times and foraging propensity varied between cohorts with an age difference of about 50 days (Kühbandner et al., 2014). Since our variation in age is considerably smaller and workers of a closely related Myrmicine species, *Temnothorax nylanderi*, can get up to 3 years old (Beros et al., 2021; Prebus, 2017), we concluded that the age variation among our workers would likely not cause any behavioural differences.

Ant colonies were transferred to 22°C to increase activity and the red foil from their glass nest was removed to allow workers to adapt to light. The next day, the slide nest was transferred to a fluon-treated arena (3 cm × 7.5 cm) and each colony was filmed for 100 min in 4 k using a SONY FDR-AX33 camera under a Leica KL1500 LED light. Directly after filming, all unmarked ants were removed with forceps, frozen in liquid nitrogen, and stored at −80°C until dissection. The antennae and the brain were dissected from each individual and each tissue was separately placed into 1.5 mL tubes containing 75 μL of Trizol and stored at −80°C. Total RNA was extracted using the Qiagen MiniKit following standard instructions. For half of the samples (all from replicate 5 and half from replicate 3) from the antennae, 100 μL instead of 75 μL of Chloroform was added, so the concentration was corrected by adding 50 μL of Ethanol. To ensure this did not influence RNA yield, we tested the difference in the amount of RNA between the two groups, which was no different (Mann–Whitney–*U*: $p = .3$). Afterwards, samples were sent to Novogene (Cambridge, UK) for library construction and sequencing of 150 bp paired-end reads on an Illumina NovaSeq6000. Details on library construction are found in the Appendix S1.

2.4 | Preprocessing of RNA-Seq data

The RNA-Sequencing resulted in at least 30 Mio. reads per sample per read orientation for both the brain and the antennal samples. Bioinformatic analyses started by identifying and removing contaminations using FastQScreen v0.14.0 (see Table S2 for details about databases used for FastQSCreen; Wingett & Andrews, 2018). Afterwards, reads were adapter- and quality-trimmed using Trimmomatic v0.39 (Bolger et al., 2014) leaving at least 26 Mio. paired-end reads per sample after filtering and trimming (mean: 30.47 ± 3.05 Mio.) for the brain data and at least 27 Mio. (mean: 28.70 ± 0.65 Mio.) for the antennal data. The quality of filtered and trimmed reads was assessed and deemed to be sufficient using FastQC v0.11.8 (Andrews et al., 2015). Reads were mapped against the genome assembly of *L. acervorum* (Jongepier et al., 2022) using HISAT2 v2.1.0 (Kim et al., 2015). Backmapping of the filtered and trimmed reads against the genome assembly of *L. acervorum* was above 90% for all samples (brain: mean: 93.29 ± 4.94%; antennae: mean: 95.09 ± 0.36%; details Table S1). After mapping, StringTie v1.3.6 (Pertea et al., 2015) was used to create a genome-guided transcriptome assembly, which consisted of 85,301 transcripts for the brain data and of 86,415 transcripts for the antennal data. These transcript assemblies were then filtered to only retain contigs which contained an open-reading frame (ORF) of more than 149 bp as identified by TransRate v1.0.3 (Smith-Unna et al., 2016). Thus, the final genome-guided assemblies consisted of 50,616 transcripts after filtering for the brain transcriptome and 50,648 transcripts for the antennal transcriptome. Transcripts were translated to their most likely protein sequence using TransDecoder v5.5.0 (Haas et al., 2013) and Gene Ontology terms were assigned to these predicted proteins based on the presence of functional domains identified using InterProScan v5.51–85.0 (Ashburner et al., 2000; Jones et al., 2014). Thereafter, we quantified transcript and gene counts using the script prepDE.py retrieved from https://ccb.jhu.edu/software/stringtie/dl/prepDE.py (Pertea et al., 2016). The following analyses were based on a gene count matrix from StringTie so that "gene" refers to the gene model implemented in StringTie. Since behavioural analysis of the videos revealed that two of the focal workers were injured, possibly biasing their gene expression, we decided to remove them from our analyses, as well as one antennal sample from individual O14, for which not enough input

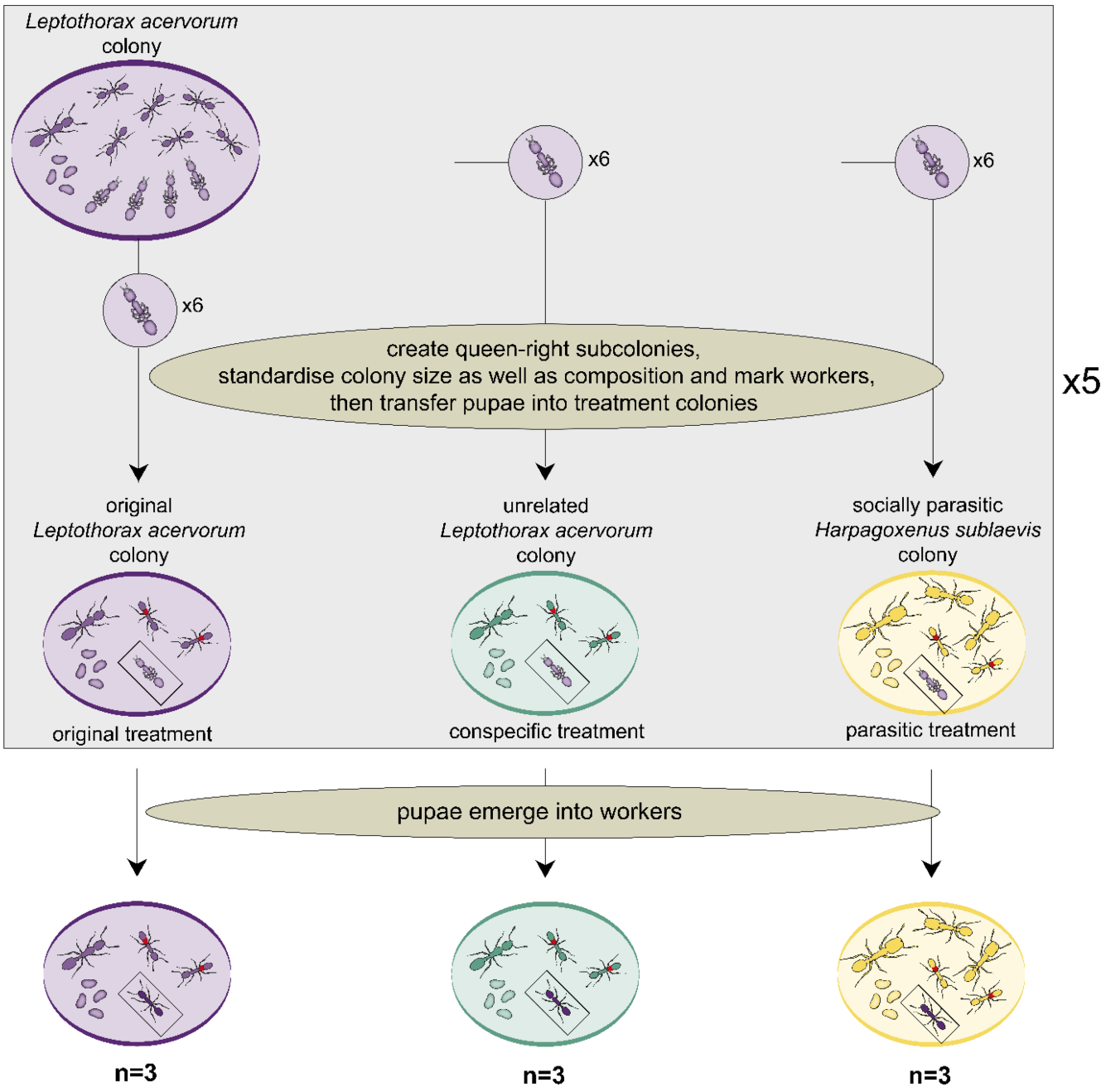

FIGURE 1 Schematic representation of the experimental design. We used five different *Leptothorax acervorum* colonies to extract pupae to be placed into five different replicates of three sub-colonies (setup in grey box). Each replicate consisted of a sub-colony created from (a) the original *L. acervorum* colony the pupae for this array were taken from (on the left) (b) one of five unrelated conspecific *L. acervorum* colonies (in the middle) and (c) one of five parasitic colonies of *Harpagoxenus sublaevis* (on the right). Each of these sub-colonies consisted of 30 host workers, one queen and 15 larvae. The parasitic sub-colonies additionally contained all parasitic workers of the respective *H. sublaevis* colony. To be able to differentiate at the end of the experiment, the workers emerged from the transferred pupae and the other residing *L. acervorum* workers inside the nests, all residing host workers were marked with thin metal wire during sub-colony setup. The transferred pupae (6 per experimental sub-colony) and workers emerged from these are highlighted in boxes. 10 weeks after the emergence of the first worker from the pupae, unmarked workers (in grey boxes) from three of the five manipulated sub-colonies were sampled for transcriptomic analysis in each treatment. Credit: Jenny Fuchs.

material was obtained. Of the remaining 20 individuals, we successfully sequenced the transcriptome of the brain and the antennae of 16 of those individuals while for four we were only able to retrieve the brain transcriptomes. A summary of the experimental set up and the final replicate number is given in Figure 1 (more details in Appendix S1). Our final samples were evenly distributed among the three treatments (antennae: 5–6 samples/treatment; brain: 5–9 samples/treatment; see Table S1). It has to be noted, however, that the parasitic treatment was not evenly composed of individuals from the three source colonies: For both tissues, four

out of six samples of the parasitic treatment were collected from the same replicate of sub-colonies, so that these workers had the same genetic background. In the supplement, we show the expression plots of our candidate genes with colony ID displayed, which illustrates that the patterns are not driven by this one colony. Furthermore, to remove any spurious read counts that might represent noise rather than a true biological signal, we removed genes with fewer than 10 reads in at least four samples, regardless of treatment, from both gene count matrices. The brain data-based gene count matrix contained 17,820 genes and 13,732 after filtering for the minimum read count, while the antenna count matrix contained 21,961 genes and 15,695 after filtering.

2.5 | Analysing gene expression in respect to social environment

We used ComBat-Seq to adjust our gene count matrix for source colony ID as batch effect and treatment as the biological group (Zhang et al., 2020). We decided on this alternative approach instead of using colony ID as batch effect in DESeq2 as source colony ID was not evenly spread across all treatments. As this unbalanced design made further analyses difficult, in the follow-up analyses, such as the GO enrichment analysis, we used the adjusted *p*-values as a quality score for the reliability of differentially expressed genes (DEGs). All further analyses were performed on this adjusted count matrix. We used DESeq2 to model gene expression using treatment as fixed factor. Using the variance stabilizing transform of this adjusted count matrix, created using the function varianceStabilizingTransformation(), a Principal Component Analysis (PCA) based on all genes was performed using the function plotPCA(), both functions implemented in the DESeq2 package (Love et al., 2014). Using the LRT-test, we assessed genes whose expression was more explained by the model incorporating our fixed effect than one only containing the intercept. We considered all genes with an FDR-adjusted *p*-value below .05 as significantly differentially expressed. To detect patterns across those differentially expressed genes, we used the function degPatterns() from the *R* package DEGreport on the filtered rlog-transformed gene count matrix (Pantano, 2017). All transcripts were functionally annotated by using Diamond v2.0.11.149 using the blastx algorithm against the non-redundant invertebrate database (downloaded 10th August 2021) to retrieve similar proteins with an E-value below 10^{-5} (Altschul et al., 1990). DEGs were annotated with their respective best BLAST hit (in respect to bit score) from their longest isoform. Additionally, we searched the respective BLAST hits of each gene for hits in the UniProt database to retrieve additional functional information and based on this specifically searched for genes related to the following functions: Perception, because we were interested in whether or not ants are able to recognize their social environment, behaviour, because in *Temnothorax longispinosus*, a closely related ant and host of a social parasite, destruction of parasitic brood has been reported as a special instance of behaviour; learning to potentially gain insights into the molecular mechanisms of recognition of alterations in the social environment; fecundity because in hosts of a wasp social parasite reproduction of parasitized hosts has been observed (Cini et al., 2014); and ageing because we suspected that the stress of living together with a parasite could impact worker longevity (see Appendix S1).

We used the package topGO to test for the enrichment of GO terms in the lists of DEGs within the same cluster (Alexa & Rahnenfuhrer, 2018). Therefore, we performed a Kolmogorov–Smirnov test using the *p*-value of genes as the gene score and made use of the classic algorithm implemented within topGO and using the genes of the respective filtered count matrix as the universe. Despite the BLAST search, all analyses were performed in R v4.2.1 (R Core Team, 2021). To be able to compare the expression of candidate genes in the respective other tissue, we ran OrthoFinder v2.5.4 to identify orthogroups between the transcriptomes in peptide sequence as identified by TransDecoder before (Emms & Kelly, 2015).

2.6 | Correlating behaviour with gene expression

Behaviour and location of sampled ants were analysed by scan sampling (1 scan every 2 min, 30 scans in total) for the last 60 min of video recording (for ethogram see Table S3, for videos see Appendix S1; Stoldt et al., 2022b) using QuickTime Player 7.6.6. We decided to scan individuals for behaviours that normally occur in the nest, such as walking, grooming, antennation, trophallaxis and brood care, which includes antennation, grooming, carrying or feeding the brood. If an individual showed no movement at all at the time of scanning, this was noted as resting behaviour. Finally, behaviours that did not fall into any of our categories and did not occur frequently enough to fit our ethogram were classified as "other activity". For the location, we focussed on the distance to the brood, since this was shown to be a good predictor of worker task in *Temnothorax unifasciatus* (Sendova-Franks & Franks, 1995). To differentiate between the unmarked individuals, the video was scanned backwards to be able to track the individuals sampled at the end of the observation. The behaviours and the location of sampled individuals recorded were analysed separately using PCA (see Appendix S1; Stoldt et al., 2022a) using the packages FactoMineR, factoextra and missMDA with R version 4.1.2 (Josse & Husson, 2016; Kassambara & Mundt, 2020; Lê et al., 2008). Individual principal components (PCs), which explained at least 10% of the variance, were extracted to be correlated to the transcriptomic data. For the transcriptomic data, we summarized the gene counts into modules of genes with a similar gene expression pattern using a Weighted Gene Co-expression Network Analysis (WGCNA) as implemented within the package WGCNA (Langfelder & Horvath, 2008; Zhang & Horvath, 2005). For a detailed overview of the network construction, see Appendix S1. We then calculated the module eigengene for each module that represents the first principal component of the expression matrix of the respective module. Thus, they can be used to summarize the expression pattern of a

module. Next, we tested whether the module eigengenes correlated with the PCs explaining behaviours and locations recorded during sampling using Pearson correlation.

3 | RESULTS

3.1 | Gene expression changes with the social environment of individuals

3.1.1 | Host brain gene expression changes within the parasitic nest

The PCA revealed that the RNA-Seq samples of individual brains did not cluster by treatment or colony ID, suggesting that the social environment only led to subtle changes in gene expression in adult worker brains (Figure S1A,B). We identified 214 genes in the brain whose expression could be better explained by a model including the social environment of the host workers as a factor. These genes could be classified into four different clusters based on their expression profile (see Figure 2a). Among our treatments, the parasitic treatment showed the strongest influence on gene expression in the brain: the largest cluster of DEGs, cluster 2, which comprised 100 genes, showed higher expression in host workers living in the parasitic environment than in the other two treatments. We identified two interesting candidate genes associated with perception, whose expression we have plotted separately (see Figure S2 for figures with sample names). One of these genes possibly encodes "phosrestin-2-like isoform X2", a protein known to be involved in phototransduction in *Drosophila melanogaster* (Dolph et al., 1993). Another one encoded a "ATP-binding cassette sub-family G member 8", a protein belonging to the ATP-binding cassette sub-family G, which in *D. melanogaster* is not only important for transport of neurotransmitters and bioamines (Borycz et al., 2008; Evans et al., 2008), but members of this family are also involved in eye pigmentation (Mackenzie et al., 1999). When examining GO enrichment of the DEGs, we found several terms enriched with a $p < .05$, including several related to translation, including "translation" itself (Figure 2c). However, we found no enrichment for any of the candidate functions for the DEGs in the brain.

3.1.2 | Antennal gene expression of hosts is affected by the presence of non-related individuals

To gain additional insight into changes in the peripheral nervous system, particularly in relation to odour perception, we analysed the antennal transcriptomes of individual workers from the different social environments. Similar to the brain data, a PCA of the antennal RNA-Seq samples did not show clear clustering by treatment or by colony ID (Figure S1C,D). We identified 85 DEGs between treatments, that could be classified into four distinct clusters (Figure 3a). The largest cluster (cluster 1) comprised 37 genes and exhibited a higher expression in the original treatment, where pupae were placed back into a fragment of their original nest compared to when pupae were placed into foreign nests. Within this cluster, we identified five genes encoding proteins related to behaviour and perception in *D. melanogaster* and *Caenorhabditis elegans*, more than expected by chance (Fisher's exact test: behaviour $p = .032$, perception $p = .03$; Figure 3b). Among

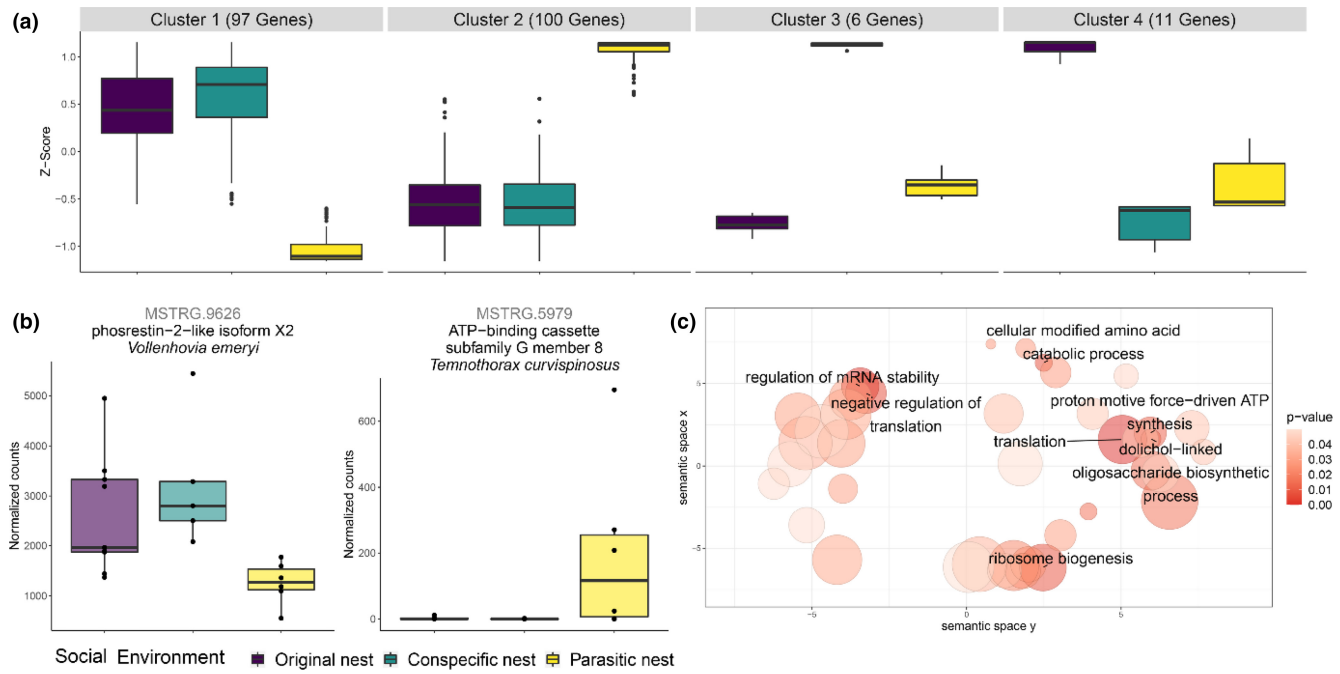

FIGURE 2 Genes affected by the social environment within the brain. (a) Clustering of all differentially expressed genes with adj. $p < 0.05$ according to expression patterns in respect to the three social environments. (b) Expression of two candidate genes related to perception. (c) Gene Ontology terms enriched among the differentially expressed genes with $p < .05$, plotted within semantic space as calculated by Revigo. Size of circles representing broadness of terms while colour indicates *p*-value.

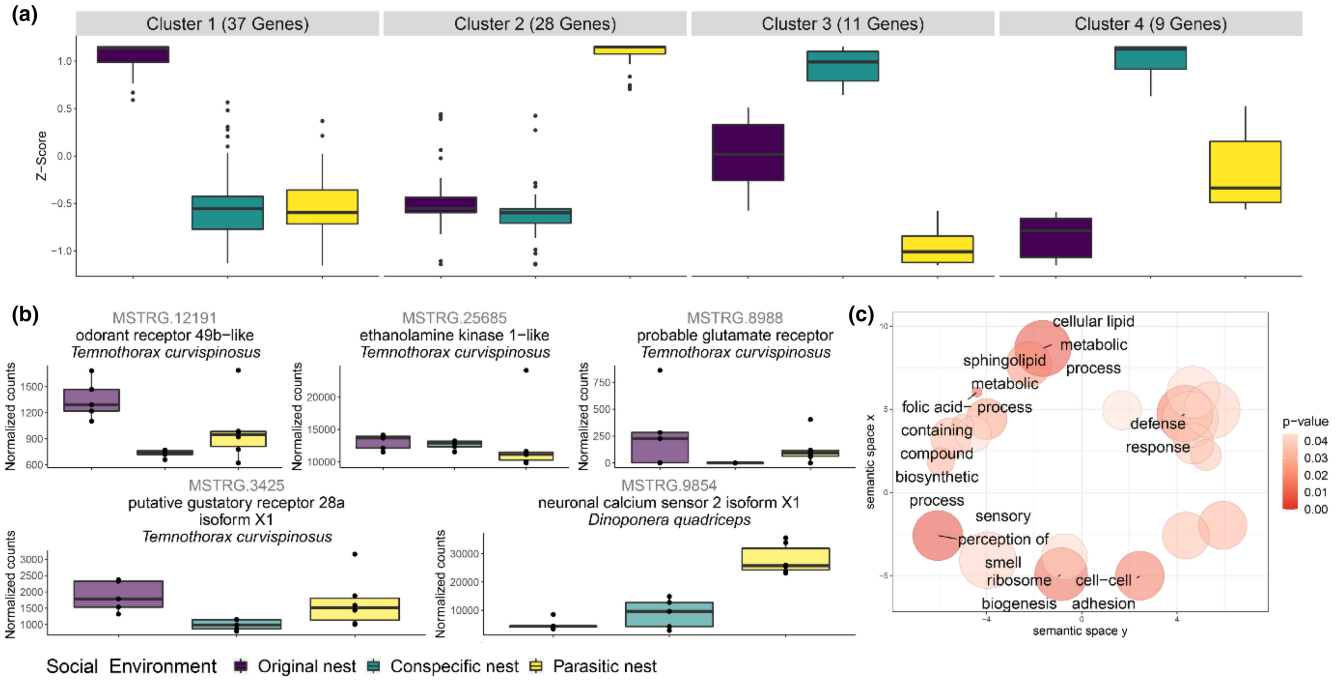

FIGURE 3 Genes affected by the social environment within the antennae. (a) Clustering of all differentially expressed genes with adj. $p < .05$ according to expression patterns in respect to the three social environments. (b) Expression of five candidate genes related to perception. (c) Gene Ontology terms enriched among the differentially expressed genes with $p < .05$, plotted within semantic space as calculated by Revigo. Size of circles representing broadness of terms while colour indicates p-value.

these differentially expressed candidates, we detected one encoding an odorant receptor (OR) and one encoding a gustatory receptor (GR). Both receptors belong to the group of chemoreceptors that bind chemical compounds and play a key role in the perception of taste and smell (Dahanukar et al., 2005). The second largest cluster, cluster 2, consisting of 28 genes, showed higher expression in host workers moved to parasitic nests than in the other two treatments. In addition, genes that were differentially expressed in the antennae were enriched for 'defence response' and metabolic processes as well as 'sensory perception of smell' (Figure 3c). These results led us to further analyse the expression patterns of these two classes of chemoreceptors in the antennae (see Appendix S1 for methods). Of the 127 unique transcripts that could be assigned to at least one odorant receptor, 88 showed a similar expression pattern to cluster 1 (high expression in the original treatment compared to the other two) based on all DEGs (see Figure S3). For GRs, conversely, 24 of the 44 unique transcripts that could be assigned to at least one GR showed an expression pattern with low expression in the conspecific treatment compared to the other two treatments.

3.2 | Gene expression as a predictor of individual behaviour 1 h before sampling

The co-expression network based on the RNA-Seq brain data comprised a total of seven gene modules, none of which were significantly associated with behaviour or spatial location in the hour before sampling (see Figure S4). The co-expression network based on the antennal data consisted of 27 modules, five of which were significantly correlated with at least one of the first five PCs explaining behaviour or with the two PCs explaining the location of individuals (Figure S5).

The three modules with the lowest adjusted p-value were the "green-yellow" module, which correlated positively with PC2 of the location data, the "royalblue" module, which correlated negatively with PC3 of the behavioural data, and module "brown", which correlated negatively with PC5 of the behavioural data (Figure 4). The "green-yellow" module showed higher expression in individuals that had moved away from the brood and was enriched for transcriptional regulation, protein modification and Wnt signalling. The "royalblue" module was enriched in terms related to perception, signal transduction and response to stimuli, and showed high expression in individuals that cared for the brood, and the "brown" module was highly expressed when individuals were cared for by another worker and consisted of genes mainly involved in response to stress and secretion of insulin and arachidonic acid.

To bridge the gap between how gene expression is affected by the social environment of the workers and how genes in general change with the behaviour they exhibit, we also examined the overlap between the DEGs and the identified gene modules. For the modules that consisted of at least ten DEGs, we used Fisher's exact test to see if this overlap was greater than expected by chance. This was the case for three of the four modules with such a high overlap (blue: $p = .052$; brown: $p < .001$; salmon: $p < .001$; yellow: $p < .001$). This included the brown module, which was already correlated with

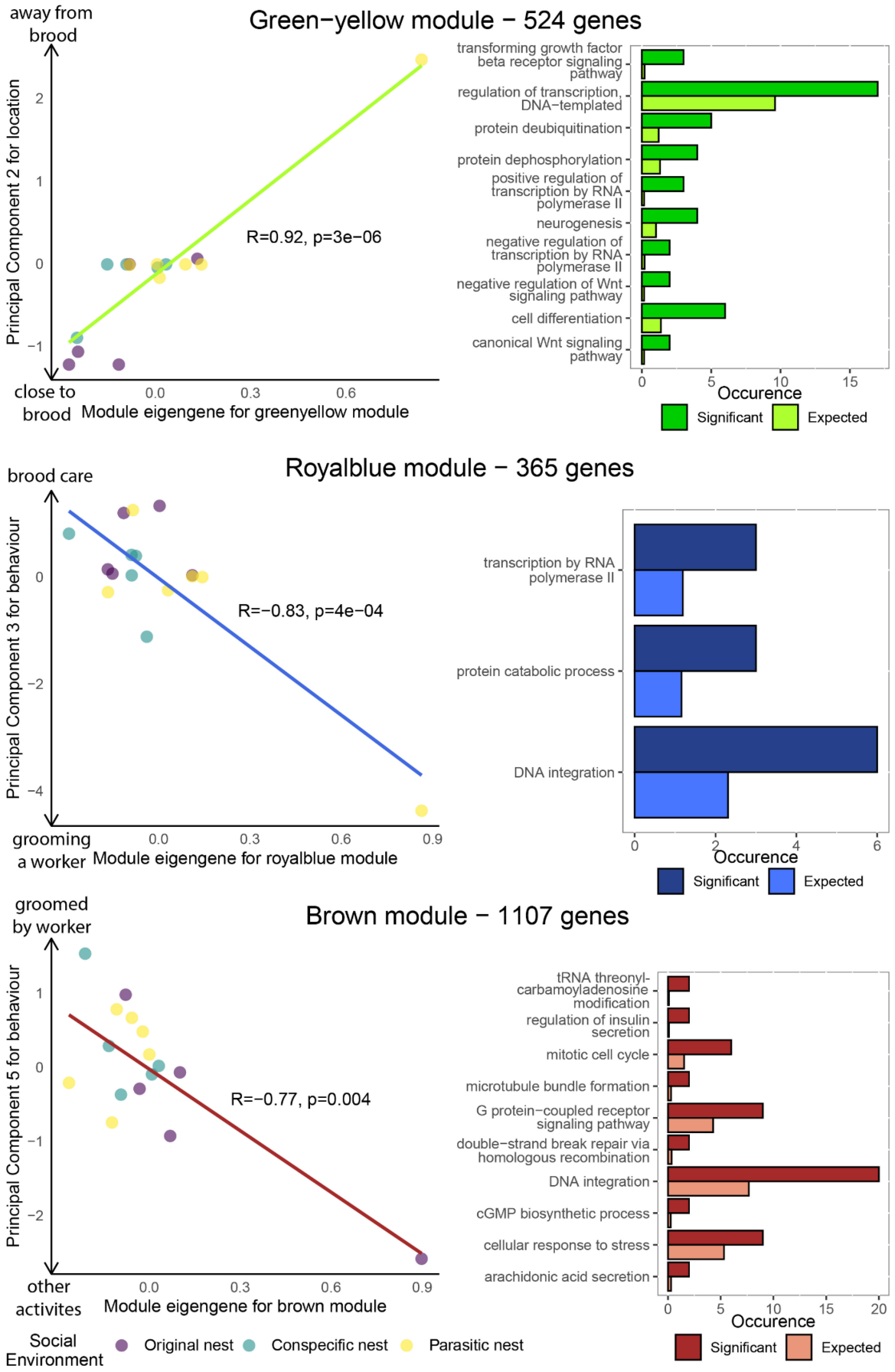

FIGURE 4 Antennal gene networks related to behaviour and location. Module eigengenes for the three significant modules with the lowest *p*-value are plotted against the respective principal component together with R and the *p*-value of the Pearson correlation (left). Since a signed network was constructed, positive eigengene values correspond to higher expression of the genes within this module. End points of *y*-axis indicate the respective behaviours/locations that show the strongest effect on the respective PC. Points are coloured according to social environment. On the right side, the GO enrichment of each module using Fisher's exact test is represented by plotting all terms with $p < 0.05$ that are represented by more than one gene within the module. The number of representing genes within the module is given in the darker colour while the expected number based on the size of the transcriptome is given in the lighter colour.

behaviour, suggesting a possible link between behaviour-regulating genes and differences in the social environment. When testing whether the module eigengenes of these three modules differed between the three social environments, only the module eigengene of the yellow module showed expression changes between the three treatments and was enriched for "DNA integration" (see Figure S6).

4 | DISCUSSION

We combined transcriptome data from brains and antennae of host workers living in different social environments, including parasite nests, with behavioural data to elucidate the molecular mechanisms that facilitate host worker exploitation in social parasite colonies and the gene networks underlying behaviours. We analysed gene expression changes in the brains and antennae of adult workers 10 weeks after emergence in different social environments. Thus, all the changes observed in our experiment indicate plastic responses in the adult workers. By standardizing colony size over the 10 weeks of the experiment and the age of the workers, our study was able to reveal transcriptomic changes in both tissues that were related to the social environment in which the workers lived. We must note that we did not control for the initial colony size, and we cannot exclude that this had an impact on gene expression. However, since we assume that the influence of colony size before the 10 weeks of the experiment is of lesser importance compared to other factors such as relatedness to individuals within the colony or current colony size, this should not change the main conclusions of our experiment. Furthermore, we have identified modules of genes in the antennae whose expression correlates with behaviours exhibited by the workers within their last hour. This allowed us to additionally identify gene networks and their respective functions which shift in workers depending on the behaviours they exhibit and regardless of treatment. However, we cannot rule out that even small differences in age between our focal workers contributed to the observed variation in gene expression.

4.1 | Transcriptomes change with the social environment – more so in the brain than the antennae

Our comparison of brain and antennal gene expression reveals a divergent transcriptome in host ants residing in the three experimental social environments. This mirrors what has been found in other systems where changes in the social environment, especially group size up to complete social isolation, strongly influenced gene expression in the brains of various hymenopterans (Lockett et al., 2016; Manfredini et al., 2021; Pulliainen et al., 2021; Scharf et al., 2021). We found the brain transcriptomes to be more affected by changes in the social environment (as evidenced by the number of DEGs) than the antennal ones. This might partially be explained by differential investment into brain regions in ants living in different social environments resulting in differences in brain morphology. Brain morphology or size has been shown to be affected by multiple factors in ants including age, caste and reproductive behaviour (Gronenberg et al., 1996; Gronenberg & Liebig, 1999; Julian & Gronenberg, 2002; Penick et al., 2021). Such a pattern of induced changes in brain morphology was previously observed in socially isolated ants of *Camponotus floridanus* (Seid & Junge, 2016), and could potentially explain brain gene expression differences. Similarly, in the social wasp *Polistes dominula* the presence of parasites resulted in plastic changes in brain morphology (Gandia et al., 2022). Still, more detailed neuroanatomical experiments are needed to confirm that brain size is plastically adjusted in ants exploited by social parasites.

Another explanation for the more pronounced changes in brain gene expression could be that they underlie changes in behaviours associated with the different environments. Due to our small sample size, we were not confident enough to appropriately test whether individuals raised in different social environments actually differed in their behaviours 1 h before sampling. However, we made sure that individuals were of similar age at the time of sampling, so any differences in behaviour are likely to originate from the change in the social environment since in these ants task is influenced by age (Kühbandner et al., 2014).

We showed that brain gene expression, which underlies the activity of workers living with social parasites, differs from those residing together with related individuals from their original colony. Similarly, changes in brain gene expression have been found in the parasitic wasp-host system of *Polistes sulcifer* and *Polistes dominula*, where transcriptomes of workers living in parasitic colonies differed from those in free-living wasp nests (Cini et al., 2020). Moreover, our experimental design allowed us to show, that expression even differs between hosts that were placed into a parasitic nest compared to a conspecific, unrelated nest, suggesting that these changes are indeed specific to the presence of the parasite and not only the result of being placed into a foreign nest. We observed a similar pattern of expression in the antennae, even though most DEGs differed between the original treatment and the two others, where individuals had been placed into foreign nests, but the second largest cluster again differed between the parasitic treatment and the other two. As antennae and antennal lobes are the main organs involved in nestmate recognition (Ozaki et al., 2005; Stroeymeyt et al., 2010),

our data suggests that the expression of genes linked to recognition abilities is influenced by the presence of non-related individuals followed by the presence of the social parasite. This would mean that individuals, even though they have never experienced the environment within the nest they were laid in as eggs, can differentiate between related individuals and foreign individuals (of another species). One possible explanation would be that they have learned the cues from the original nest or mother queen as larvae (Carlin & Hölldobler, 1983; Morel & Blum, 1988); a second, non-exclusive explanation might be that these ants possess some form of internal template to which they compare, for example, the smell of the nest they reside in (Holmes & Sherman, 1983; Lacy & Sherman, 1983). However, our results suggest that being raised within a foreign colony is not sufficient to fully adapt to the colony odour and be fully adopted without any transcriptional consequences.

4.2 | Candidate genes changing in response to the social environment

We expected individuals to recognize their environment to be different from their original colony which is why we specifically looked for DEGs related to sensory perception. Among the DEGs in both tissues we were able to identify a handful of genes related to perception, not surprisingly more so in the antennae than in the brain, including odorant and gustatory receptors. Moreover, the DEGs within the antennae were enriched for functions including "sensory perception of smell". This corroborates our earlier conclusion that even though these ants had never experienced their original colony as adults before, they still sense a difference when placed into a nest with non-related individuals. Similarly, in the ant *Formica fusca*, larvae were shown to be already able to distinguish between foreign and parasitic eggs and similarly showed shifts in sensory gene expression in response to their social environment (Pulliainen et al., 2019, 2021; Schultner et al., 2013).

One candidate we identified among the genes differing between the ants transferred into the original treatment or a colony containing unrelated individuals and/or social parasites was a homologue to the odorant receptor 49b-like in *Temnothorax curvispinosus* and most similar to lace_OR205 from the manual annotations of ORs from our focal species (Appendix S1; Jongepier et al., 2022). We additionally used blastn to find the most similar sequence among the ORs previously identified in a closely related species, *Temnothorax longispinosus* (Jongepier et al., 2022), which has been identified to be from subfamily I3 (Caminer et al., 2023). This subfamily is predicted to have an especially important role since it only occurs once in the genome of many species including *L. acervorum* according to our BLAST results (Engsontia et al., 2015). Some OR subfamilies, including the 9-exon ORs, have massively expanded in ants and are expected to be important for nestmate recognition using CHCs (Engsontia et al., 2015; Jongepier et al., 2022; Zhou et al., 2012). A previous study using machine learning suggested that those expanded ORs show higher binding residue conservation, probably to detect and differentiate between very similar odours, such as CHCs (Mier et al., 2022). The OR we identified to be differentially expressed between individuals of the original treatment and those from the conspecific and parasitic treatment, however, is predicted to have lower conservation in its binding residues, potentially to make sure to identify a certain odour even in more complex mixtures. Whether inhibition of this receptor indeed significantly influences acceptance of individuals into foreign colonies needs to be validated for example using RNAi knockdown and functional studies should also investigate what this receptor exactly binds to. Since we identified two chemoreceptors among the candidate DEGs in the antennae, we also investigated the overall expression pattern of these receptors in the antennal transcriptomes. The majority showed a pattern similar to most of the DEGs with the original treatment differing from the other two. This suggests that alterations in the social environment can impact the activity of these chemoreceptors but that this is not specific to the presence of social parasites. However, the largest cluster of gustatory receptors showed expression that did not differ between the original and parasitic treatment. The inability of hosts to perceive the difference between their original and the parasitic nest could potentially explain the functioning of socially parasitic colonies in the field. Another possibility could be that certain tastes do not exist within parasitic colonies, which could also explain why the host *L. acervorum* showed a significant number of gustatory receptor duplications compared to its social parasite *H. sublaevis* (Jongepier et al., 2022).

4.3 | Antennal gene expression as a predictor of individual behaviour 1 h before sampling

Behavioural observations shortly before sampling allowed us to connect PCs of behaviour and location to the brain and antennal gene expression of our focal workers. While brain transcriptomes showed no association to behaviour and spatial position, we identified correlations to several gene modules of the antennal expression data. Furthermore, one of these modules was significantly enriched in DEGs, bridging the results of the differential gene expression and the network analysis.

Most studies investigating the correlation between behaviour and gene expression have used brain, a tissue which has been implicated to directly control behaviour and thus best explain an individuals behavioural phenotype (Robinson et al., 2008). However, recent studies suggest that (at least in the case of social insects) antennal transcriptomes might explain behaviours such as task or learning behaviour more accurately (Caminer et al., 2023; Kennedy et al., 2021). This is consistent with our observation that individual behaviours correlated well with the expression of hundreds of genes 1 h before sampling within the antennae, whereas such patterns were absent from the brain data. A possible explanation for this might be that odorant receptor expression happens mainly within the antennae, in concordance with Caminer et al. (2023), who have shown that almost half of these receptors

are differentially expressed between ants that work outside the nest and those that work inside. However, we could not detect more of the genes encoding for these receptors within the modules that showed a correlation to behaviour.

The brown module, which is negatively correlated with the behaviour of being groomed by another worker and therefore might be associated with social interactions between individuals, comprised genes with functions related to "DNA integration" but also to "cellular response to stress" and "G protein-coupled receptor signaling pathway". Since DNA integration is often linked to the activity of transposable elements (TE), this suggests an increased expression of transposable elements in individuals which show high expression of genes inside the brown module. Previous work in the social termites suggested that TEs are upregulated especially in older workers while reproductive individuals including queens show low TE activity (Elsner et al., 2018) possibly due to differences in transposon regulation (Post et al., 2023). As the DEGs in the brown module showed low expression in the parasitic treatment and conspecific treatment, this would suggest that social interactions occur more often in colonies consisting of non-related individuals irrespective of whether they are from the same or a different species and that this potentially has an influence on individual transposon regulation. In support of this, among the DEGs, we identified a candidate gene not only related to fecundity but to transposon regulation, encoding a HBS1 protein, in cluster 1 with low expression in the conspecific and parasitic treatment (Yang et al., 2015). However, due to our low sample size we were not able to investigate differences in social interactions while controlling for source colony ID. Whether indeed host workers show a higher number of social interactions within foreign nests and whether this is key to how parasitic colonies still manage to function properly thus remains to be investigated.

5 | OUTLOOK

Our results suggest that antennal odorant perception and information processing in the brain of host workers inside parasitic nests are likely altered in a way that facilitates coexistence and exploitation of their social behaviours. Our experimental design did not allow us to disentangle whether these changes are due to a manipulation by the parasite or whether they are the result of different social dynamics in parasitic nests. Likewise, we cannot rule out that differences in behaviour underlie the observed changes in hosts, even though we expected individuals to perform similar tasks as we controlled for age and standardized colony size. We initially predicted that host workers within parasite nests might show transcriptional signs of reduced lifespan due to stress or increased fecundity as a form of rebellion, similar to findings in the parasitized wasp *P. dominula* (Cini et al., 2014). However, we did not find any signs that, during our experimental period, host workers were especially impacted in their life-history traits when living together with their parasites. Nevertheless, it would be of interest to investigate long-term changes in longevity and fecundity of exploited host workers to gain more insights into fitness costs for individual host workers and their role within the coevolutionary arms race between parasite and host (Cini et al., 2020).

AUTHOR CONTRIBUTIONS

M.S., E.C. and S.F. designed the experimental set up and collected the ant colonies. M.S. and E.C. set up the experimental colonies and sampled workers. E.C. and M.N.M. performed dissections and E.C. performed RNA extractions. E.C. analysed the behavioural data. M.S. with the assistance of E.C. analysed the gene expression data. M.S. wrote a first draft of the manuscript and all authors revised it.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT AND BENEFIT-SHARING

The following supplementary material is available under open access: The video material for the behaviour and location of individuals under 10.5281/zenodo.6977368 (Stoldt et al., 2022b). The gene counts matrices, lists of DEGs, GO enrichment results, WGCNA and PCA results as well as more detailed information on materials and methods and scripts which are available via the following link: https://data.mendeley.com/datasets/32kr9b2bk7/1 https://data.mendeley.com/datasets/32kr9b2bk7/draft?a=2d4fdbfe-c387-4240-aaca-c7f51b6311c7 (Stoldt et al., 2022a). Raw sequence data was uploaded to the Sequence Read Archive (SRA) and can be accessed under BioProject ID PRJNA865882.

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