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Gene expression is more strongly associated with behavioural specialization than with age or fertility in ant workers

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Abstract

The ecological success of social insects is based on division of labour, not only between queens and workers, but also among workers. Whether a worker tends the brood or forages is influenced by age, fertility and nutritional status, with brood carers being younger, more fecund and more corpulent. Here, we experimentally disentangle behavioural specialization from age and fertility in *Temnothorax longispinosus* ant workers and analyse how these parameters are linked to whole-body gene expression. A total of 3,644 genes were associated with behavioural specialization which is ten times more than associated with age and 50 times more than associated with fertility. Brood carers were characterized by an upregulation of three *Vitellogenin* (*Vg*) genes, one of which, *Vg-like A*, was the most differentially expressed gene that was recently shown experimentally to control the switch from brood to worker care. The expression of *Conventional Vg* was unlinked to behavioural specialization, age or fertility, which contrasts to studies on bees and some ants. Diversity in *Vg/Vg-like* copy number and expression bias across ants supports subfunctionalization of *Vg* genes and indicates that some regulatory mechanisms of division of labour diverged in different ant lineages. Simulations revealed that our experimental dissociation of co-varying factors reduced transcriptomic noise, suggesting that confounding factors could potentially explain inconsistencies across transcriptomic studies of behavioural specialization in ants. Thus, our study reveals that worker gene expression is mainly linked to the worker's function for the colony and provides novel insights into the evolution of sociality in ants.

1 | INTRODUCTION

Division of labour can be found at multiple levels of biological organization and has arisen independently in several of the major transitions in evolution, for example in the transition from single cell to multicellular organisms or from solitary to social life (Maynard Smith & Szathmáry, 1995). The primary driver for the repeated evolution of division of labour is that it allows cells or group members to specialize in a single or a few tasks, thus increasing efficiency and allowing

for anatomic or physiological adaptations (Robinson, 1992; Smith, 1776; Wahl, 2002; Wilson, 1971).

The proximate mechanisms regulating division of labour and specialization are complex and have intensely been studied especially in social Hymenoptera, such as bees, ants and wasps. In these insect societies, one or a few individuals—the queen(s)—monopolize reproduction, whereas the majority of colony members—the workers—remain functionally sterile. However, in many species, workers can develop their ovaries and lay unfertilized eggs that will develop into males even in the presence of a queen (e.g., Hodson, Siegel, & Lewis, 2012). All non-reproductive colony chores, such as caring for

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the brood, defending the nest or foraging for food, are distributed among workers (Wilson, 1971). Whether a worker stays inside the nest to nurse the brood or goes outside to forage is strongly influenced by age and physiology: brood carers are typically younger (Kohlmeier, Feldmeyer, & Foitzik, 2018; Wilson, 1971), more corpulent (Ament, Wang, & Robinson, 2010; Toth & Robinson, 2005) and more fertile (Hölldobler & Carlin, 1989; Kühbandner, Modlmeier, & Foitzik, 2014) than foragers. Due to high extrinsic mortality outside the nest (Schmid-Hempel & Schmid-Hempel, 1984; Visscher & Dukas, 1997), this age- and physiology-based division of labour is beneficial, as it ensures that young workers with the longest residual lifespan, largest amount of stored resources and highest reproductive potential stay inside the safety of the nest (Amdam & Page, 2005; Ricklefs, 1998).

Although the association between age, physiology and behavioural specialization is well documented and widespread across the social Hymenoptera, a consensus on the underlying transcriptomic signatures and expression biases of individual candidate genes linked to worker specialization is lacking. For instance, while the transition from brood care to foraging is associated with a strong reduction in body fat reserves in multiple species (Ament et al., 2010, 2011; Bernadou, Busch, & Heinze, 2015; Porter & Jorgensen, 1981; Robinson, Feinerman, & Franks, 2009; Schulz, Huang, & Robinson, 1998; Toth, Bilof, Henshaw, Hunt, & Robinson, 2009; Toth, Kantarovich, Meisel, & Robinson, 2005; Toth & Robinson, 2005; Tschinkel, 1987, 1998), behaviour-specific transcriptomic signatures of nutrition-related and metabolic processes were found in some studies but not in others (Alaux et al., 2009; Ament, Corona, Pollock, & Robinson, 2008; Daugherty, Toth, & Robinson, 2011; Feldmeyer, Elsner, & Foitzik, 2014; Khamis et al., 2015; Manfredini et al., 2014; Qiu, Zhao, & He, 2017). Information on nutritional status is conveyed via the target of rapamycin and insulin/insulin-like signalling (IIS) pathways (Ament et al., 2010; Maestro, Cobo, & Bellés, 2009; Morton, Cummings, Baskin, Barsh, & Schwartz, 2006; Nilsen et al., 2011; Rodrigues & Flatt, 2016), which interact with juvenile hormone (JH) and the yolk protein precursor Vitellogenin (Vg) (Libbrecht et al., 2013). In honeybees, brood care behaviour is associated with low JH titres and high Vg expression, and elevation of JH levels decreases Vg expression and results in reduced brood care, a forager-like gene expression, alterations in small RNA populations, mobilization of carbohydrates, precocious foraging and decreased longevity (Amdam, Norberg, Hagen, & Omholt, 2003; Amdam, Norberg, Page, Erber, & Scheiner, 2006; Guidugli et al., 2005; Marco Antonio, Guidugli-Lazzarini, do Nascimento, Simões, & Hartfelder, 2008; Münch, Ihle, Salmela, & Amdam, 2015; Nunes, Ihle, Mutti, Simões, & Amdam, 2013; Seehuus, Norberg, Gimsa, Krekling, & Amdam, 2006; Wang, Brent, Fennern, & Amdam, 2012; Wheeler, Ament, Rodriguez-Zas, Southey, & Robinson, 2015). In other social insects, the link between JH and Vg can be similar (Azevedo, Paula, Zanoncio, Martinez, & Serrão, 2015; Engel et al., 2016; Manfredini, Brown, & Toth, 2018; Robinson & Vargo, 1997; Rodrigues & Flatt, 2016), absent (Amsalem, Malka, Grozinger, & Hefetz, 2014), or

reversed compared to honey bees (Brent & Vargo, 2003; Libbrecht et al., 2013; Pamminer, Treanor, & Hughes, 2016). Moreover, Vg genes underwent multiple duplications in ants with different orthologs taking over specific functions in the regulation of fertility and behavioural specialization (Corona et al., 2013; Feldmeyer et al., 2014; Kohlmeier et al., 2018; Morandin et al., 2014; Oxley et al., 2014; Salmela et al., 2016; Wurm et al., 2011). In honeybees, upregulation of the *foraging* gene in the brain regulates the onset of foraging and causes an elevated sucrose responsiveness (Ben-Shahar, Robichon, Sokolowski, & Robinson, 2002; Heylen et al., 2008; Scheiner, Page, & Erber, 2001; Thamm & Scheiner, 2014). The expression of *foraging* in other social insects can be either positively (Bockoven, Coates, & Eubanks, 2017; Tobback, Mommaerts, Vandersmissen, Smagghe, & Huybrechts, 2011; Toth et al., 2010) or negatively (Ingram, Oefner, & Gordon, 2005; Kodaira, Ohtsuki, Yokoyama, & Kawata, 2009; Lucas & Sokolowski, 2009) correlated with foraging behaviour, and is additionally influenced by age (Oettler, Nachtigal, & Schrader, 2015), social structure (Manfredini et al., 2014), body size (Kodaira et al., 2009; Tobback et al., 2011) and time of the day (Ingram, Kleeman, & Peteru, 2011).

The inconsistency across studies in the association between gene expression and worker task choice may reflect species-specific mechanisms regulating behavioural specialization or differences across studies regarding experimental design. Gene expression patterns associated with behavioural specialization of workers are typically identified by comparing brood carers and foragers that not only differ in behaviour but also in age and fertility (Cardoso-Júnior et al., 2017; Feldmeyer et al., 2014; Ingram et al., 2005; Johnson & Jasper, 2016; Khamis et al., 2015; Manfredini et al., 2014; Qiu et al., 2017; Scheiner, Entler, Barron, Scholl, & Thamm, 2017; Zhao et al., 2013), two factors that are linked to gene expression (Alaux et al., 2009; Feldmeyer et al., 2014; Harrison, Hammond, & Mallon, 2015; Lucas, Romiguier, & Keller, 2017; Nilsen et al., 2011; Whitfield, Cziko, & Robinson, 2003). Not experimentally controlling for age or fertility (in species with potentially fertile workers) when contrasting brood carer and forager transcriptomes could thus lead to confounded data sets and the identification of transcriptomic patterns being linked to any of the three factors or combinations thereof.

In this study, we disentangled the effects of behavioural specialization, age and fertility on gene expression in ant workers. Specifically, we altered the age structure of colonies of the acorn ant *Temnothorax longispinosus* to experimentally produce young and old brood carers, and young and old foragers. Furthermore, we sampled both fertile and infertile individuals for each combination of age and behavioural specialization. Using this experimental design, we (a) explored transcriptomic signatures associated with behavioural specialization, age and fertility, (b) investigated the expression of previously published candidate genes and identified new candidate genes associated with each of the three factors and (c) compared different subsets of our transcriptomic data to test whether inconsistent control for confounding factors across gene expression studies could explain the apparent lineage-specific molecular mechanisms of behavioural specialization in ant workers.

2 | MATERIAL AND METHODS

2.1 | Sample collection and preparation

We collected 38 single-queen colonies of the ant *T. longispinosus* with an average colony size of 29 ± 1.5 (mean \pm SD) workers at the E.N. Huyck Preserve, Rensselaerville, NY, USA in June 2014. Because of the synchronized annual brood emergence in this population around July and August, all field-collected workers were at least 1 year old (termed "old"). *T. longispinosus* queens are singly-mated, which reduces the potential genetic effects on worker behaviour (Foitzik & Herbers, 2001). In our laboratory in Mainz, Germany, we transferred ant colonies to plastered 3-chambered nest-boxes (Supporting Information Figure S1). We maintained colonies under a 14 hr:10 hr light:dark photoperiod with a +22°C:+18°C temperature regime to facilitate brood development. Ants were fed with crickets and honey three times a week. We marked either all young (upon eclosion in the laboratory) or all old (field-collected) workers with thin metal wires (0.02 mm, Elektrisola) in each colony, allowing us to differentiate

between the two age cohorts. Disentangling the effects of behavioural specialization and age on gene expression requires a full factorial design regarding worker behaviour (brood carer, forager) and age (young, old). To achieve this, we manipulated the demography of colonies 28 days after the emergence of the new worker generation was completed (Figure 1). Specifically, we either removed (a) all old workers from the colony to induce foraging behaviour in young workers ($n = 12$ colonies; termed "Only Young"), (b) all young workers to force old workers to tend the brood ($n = 10$; termed "Only Old") or (c) half of each age cohort as a control ($n = 16$; termed "Control"). Workers were given 21 days to adjust their behaviour and social organization. In each colony, we then individually labelled six brood carers (observed tending the brood) and six foragers (found outside the nest) with metal wire and recorded their investment into brood caring and foraging ten times a day for 3 days, that is a total of 30 observations per individual. Behavioural observations revealed a clear age polyethism with young workers focussing on brood care and older workers caring for the adult nestmates and taking over

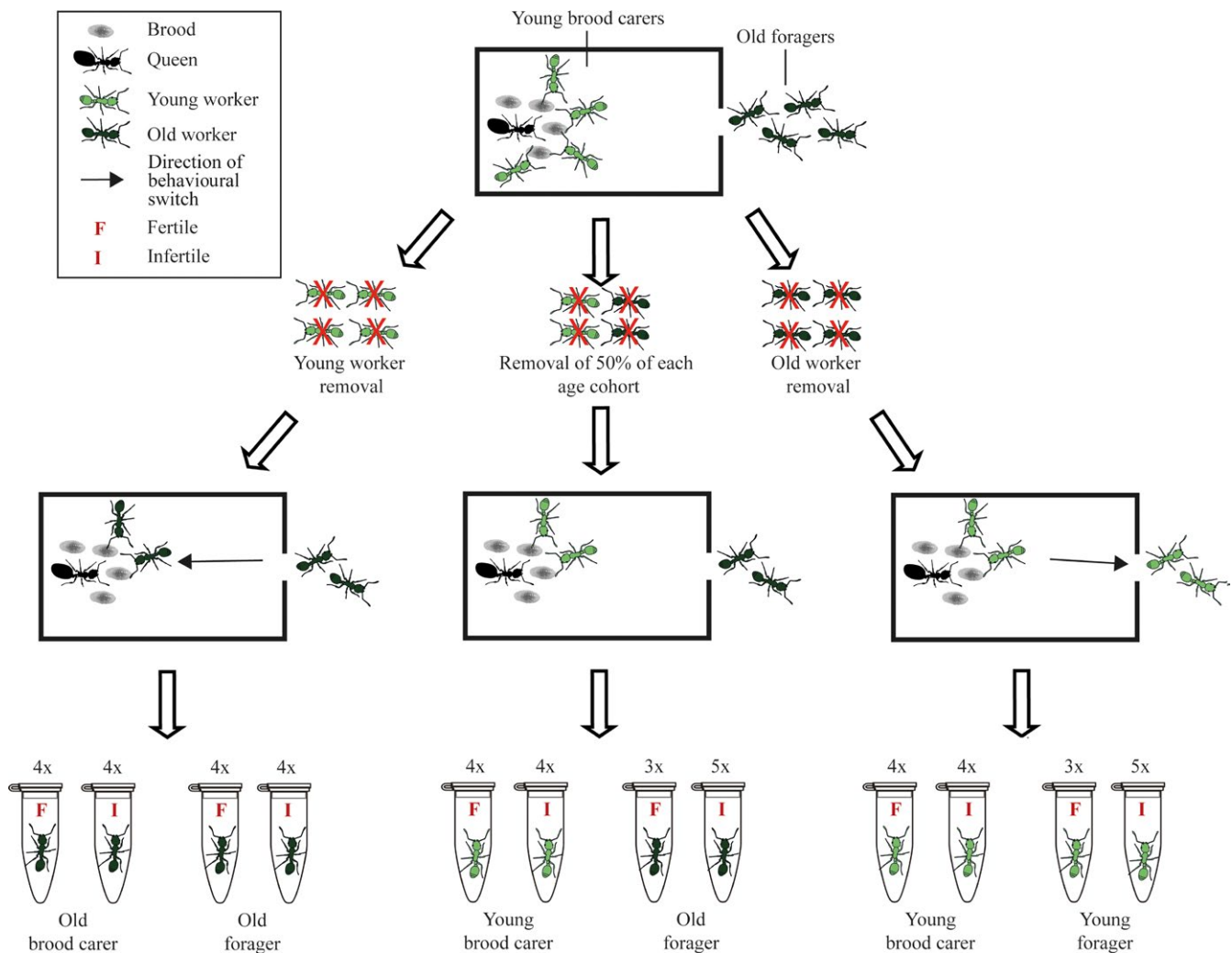


FIGURE 1 Overview of colony demography manipulations. Colony demographics were manipulated to generate foragers and brood carers of both age classes. From each colony, we sampled eight brood carers and eight foragers as well as fertile and unfertile workers. Numbers indicate the total number of replicates [Colour figure can be viewed at wileyonlinelibrary.com]

foraging (for details on the methods and results of the behavioural observations see Kohlmeier et al., 2018). After the completion of all observations, we dissected all individually labelled workers on ice to assess their fertility (fertile: at least one oocyte in the ovaries; infertile: no eggs in development), homogenized them in 100 μ l Trizol and froze them individually at -80°C until further processing. As *T. longispinosus* workers are monomorphic, gene expression differences based on differences in morphology or anatomy are unlikely (Weitekamp, Libbrecht, & Keller, 2017).

To identify genes associated either with behavioural specialization, age or fertility, we selected 48 individual workers for whole-body RNA sequencing (RNA-seq) from the pool of frozen samples, consisting of 16 workers from the “Only Old” colonies (eight old brood carers, eight old foragers), 16 workers from the “Only young” colonies (eight young brood carers, eight young foragers) and 16 workers from the control colonies with both age cohorts present (eight young brood carers, eight old foragers; Figure 1). Behavioural classifications were found to be efficient (Supporting Information Table S1). As some of the sampled brood carers and foragers were fertile, whereas others were infertile, we were able to explore the association between fertility and gene expression independent of behavioural specialization and age (Figure 1). Thus, the 48 individuals represent all combinations of behavioural caste (brood carer, forager), age (young, old) and fertility (fertile, infertile; Supporting Information Table S2).

After defrosting, we added 100 μ l chloroform to each sample, cautiously inverted for 30 s and then centrifuged samples at 11,000 g for 15 min at $+4^{\circ}\text{C}$. We collected the resulting supernatant and precipitated RNA with 60 μ l 70% ethanol. We conducted the subsequent RNA extraction with the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. Libraries were constructed at GENTERprise GmbH Mainz, Germany, following the standard Illumina protocol, and each library was individually indexed. All 48 libraries were pooled and sequenced with 100 bp paired-end equally spread across eight lanes of an Illumina HiSeq 2,500 (Supporting Information Table S2).

2.2 | Gene expression analysis

We conducted quality analyses of raw reads with FASTQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and removed Illumina adapters using TRIMMOMATIC (Bolger, Lohse, & Usadel, 2014). A de novo assembly of raw reads was built in two steps: First, we assembled the remaining raw reads using CLC WORKBENCH v.7.0.3 (<https://www.qiagenbioinformatics.com/>) with word-size = 35, bubble size = automatic and the remaining parameters left at default settings as preliminary test runs with these settings yielded the best results in respect to contig length and number of annotated contigs. We then performed a subsequent meta-assembly in MIRA (Li & Godzik, 2006) using CLC WORKBENCH contigs as input with default settings. We removed redundant and/or low-confidence contigs from the transcriptome using CD-HIT v.4.6.1 (Li & Godzik, 2006). We annotated contigs by conducting a BLASTX search

with an *e*-value cut-off threshold of $e = 10^{-5}$ against the non-redundant database (Nov. 2014). Among these contigs, 413 were annotated as viral origin. To test whether these transcripts are indeed viral or encode for transcription factors, we performed a BLASTX search of these contigs against a uniprot database consisting of all viral sequences and *Drosophila melanogaster* transcription factors. Four of these contigs were identified as transcription factors, whereas the remaining contigs were removed from all downstream analyses.

For the gene expression analysis, we aligned reads to their corresponding contigs using TOPHAT v2.1.1 (Trapnell, Pachter, & Salzberg, 2009), in conjunction with BOWTIE 2 v2.1.0 (<http://bowtie-bio.sourceforge.net/>) with default settings. We obtained read count information with EXPRESS v1.5.1 (<http://bio.math.berkeley.edu/eXpress/>) and manually excluded contigs with read counts for only four or less individuals. We then used EDGER v3.6.6 (Robinson, Feinerman, et al., 2009; Robinson, McCarthy, & Smyth, 2009) to perform gene expression analysis. We ran the following three different generalized linear models (GLMs) to identify genes associated with (a) *behavioural specialization*: brood carers and foragers were compared, and fertility and age were added as blocking factors, (b) *age*: old and young workers were compared, and fertility and behavioural specialization were added as blocking factors, and (c) *fertility*: fertile and infertile workers were compared, and age and behavioural specialization were added as blocking factors. The introduction of blocking factors was necessary as samples were not organized in a symmetric full factorial design resulting in, for example, age biases when comparing brood carers to foragers (16 young and 8 old brood carers vs. 8 young and 16 old foragers, Figure 1). All contigs, also those with the same annotation, went into the differential expression analysis as we do not know whether contigs with the same annotation (a) are artefacts of the de novo assembled transcriptome, (b) represent different splice variants of the same gene that differ in their function or (c) are the transcripts of two genes that underwent recent duplication and exhibit high sequence similarity. One of the contigs (>philip_contigs_output_c3380) was identified as a chimeric contig containing two similar sized ORFs using NCBI ORFfinder. The first ORF was annotated as *Myrmicine Vg3* (MVg3), and the second ORF was annotated as *Conventional Vg* (CVg) (according to Kohlmeier et al., 2018). We manually split this contig between both ORFs, re-mapped and re-counted raw reads, and re-ran the gene expression analysis. For each of the three factors (behavioural specialization, age, fertility), independent gene ontology (GO) enrichments were performed for, both up- and down-regulated genes using BLAST2GO v4.1.9 (Götz et al., 2008). Enriched GO terms were then summarized using REVIGO (Supek, Bošnjak, Škunca, & Šmuc, 2011). For simplification, we will only use the term “contig” when discussing results of the bioinformatic pipeline (e.g., transcriptome size) but otherwise use the term “gene.”

We further assessed the expression of candidate genes associated with the regulation of division of labour in social insects in previous studies: *foraging*, *Insulin-like growth factor*, *Insulin receptor 1* and *2*, *Krüppel homolog 1*, *Tyramine receptor 2*, all orthologs of

Vitellogenin (CVg, MVg2, MVg3, Vg-like A, Vg-like B, Vg-like C), *Vg receptor*, and *ultraspiracle* (Ament et al., 2008, 2011, 2012; Ben-Shahar, Leung, Pak, Sokolowski, & Robinson, 2003; Grozinger, Sharabash, Whitfield, & Robinson, 2003; Scheiner, Entler, et al., 2017; Scheiner, Reim, et al., 2017; Thamm & Scheiner, 2014).

We investigated which of the three factors (behavioural specialization, age and fertility) had the strongest association with gene expression. To this end, we clustered all 48 RNA-seq samples by similarity using `PHEATMAP()` function of the `PHEATMAP` package in R (version 1.0.10). In addition, we assessed the number of differentially expressed genes when comparing gene expression of age-matched workers differing in behaviour (young brood carers vs. young foragers; old brood carers vs. old foragers), and of behaviour-matched workers differing in age (young brood carers vs. old brood carers; young foragers vs. old foragers).

2.3 | Characterizing the effect of age on behaviour-specific gene expression

We investigated the effect of not controlling for age by comparing gene expression of young brood carers ($n = 8$) and old foragers ($n = 8$) from control colonies (Figure 1) using a GLM including fertility as a blocking factor in `EDGER` (termed “age-confounded GLM”). We compared the number and assessed similarity between the DEGs identified with the age-confounded and the complete age-controlled data set. Similarity was estimated as the proportion of genes in the age-confounded data set that were also found in the age-controlled data set.

Differences between the age-confounded and age-controlled GLM could also be explained by the differences in sample size of the underlying data sets (age-confounded data set: eight brood carers vs. eight foragers; age-controlled data set: 24 brood carers vs. 24 foragers). To test this, we randomly sampled four young brood carers, four old brood carers, four young foragers and four old foragers (for each: two fertile and two infertile workers) from the entire data set using 1,000 permutations. We then run GLMs on each of these 1,000 age-controlled subsets of the data including fertility as a blocking factor in `EDGER` (termed “Reduced sample size GLM,” RSS). Finally, we compared the number and identity of DEGs obtained by the 1,000 reduced sample size GLMs with the age-confounded and the complete age-controlled GLM as described above.

3 | RESULTS

3.1 | Transcriptome assembly

The 48 libraries contained between 8.6 and 13.8 million reads, of which between 8.3 and 12.8 million reads remained after trimming (Supporting Information Table S2). CLC assemblies resulted in between 70,520 and 77,246 contigs and a N50 between 1,505 and 1,583 bp. The back-mapping rate was between 92.8% and 93.3% (Supporting Information Table S3). `MIRA` meta-assembly resulted in 72,160 contigs with a N50 contig length of 3,973.

3.2 | DEGs associated with behavioural specialization independent of age and fertility

A total of 3,644 genes were differentially expressed between behavioural castes (1,917 overexpressed in brood carers, 1,727 overexpressed in foragers). We found 2.3% of the behaviour-associated genes to be differentially expressed also between young and old workers, and 1.5% also between fertile and infertile workers (Supporting Information Figure S2). Behavioural specialization had the strongest association with gene expression as (a) samples were primarily clustered by behaviour, (b) we found ten times more genes to be differentially expressed between brood carers and foragers compared to young and old, and 50 times more compared to fertile and infertile workers, and (c) we detected more DEGs when comparing workers of same age but specialized on a different behaviour than vice versa (Figure 2).

Among those genes more highly expressed in brood carers than in foragers, we found several copies of *Vitellogenin* (Vg) including *Vg-like A*, *Myrmicine Vg2* (MVg2) and *MVg3* (see Kohlmeier et al., 2018 for Vg nomenclature). *Vg-like A* was the most strongly overexpressed gene in brood carers (Supporting Information Table S4). The expression of *Conventional Vg* (CVg), *Vg-like B* and *Vg-like C* was independent of behavioural specialization (Supporting Information Table S4). The expression of all differentially expressed Vg orthologs was positively correlated with each other (Supporting Information Table S5). The expression of MVg2 and MVg3 was tightly linked (Pearson: $p < 0.0001$; $R^2 = 0.999$). Across different ant species, Vg and Vg-like genes differ in their behaviour-associated expression bias (Table 1). GO terms related to DNA integrity (DNA repair, postreplication repair, DNA synthesis involved in DNA repair, translesion synthesis, double-strand repair via homologous recombination, telomere maintenance), oxidative stress response (response to oxidative stress, cellular oxidant detoxification) and lipid processes (lipid transport, lipid localization, lipid metabolic process, lipid biosynthetic process) were enriched among genes more highly expressed in brood carers compared to foragers (Supporting Information Figure S3).

Genes more highly expressed in foragers than brood carers include *Insulin-like growth factor-binding protein complex acid labile chain* (ILGFBP), which is associated with binding the insulin-like growth factor (Leong, Baxter, Camerato, Dai, & Wood, 1992). Moreover, we found *Neuroparsin-A* and *Ras-related and oestrogen-regulated growth inhibitor*, which have recently been identified as potential toolkit genes regulating reproductive division of labour (Qiu et al., 2018), to be more highly expressed in foragers compared to brood carers. Other previously published candidate genes (i.e., *Tyramine*, *foraging*, *Vg receptor*, *ultraspiracle*, *Krueppel homolog 1*) were not differentially expressed (Supporting Information Table S4). Enriched GO terms were largely represented by multiple processes linked to metabolism, catabolism and anion transport (Supporting Information Figure S4). Foragers expressed 48 viral genes more highly than brood carers, whereas brood carers expressed four viral genes more highly than foragers.

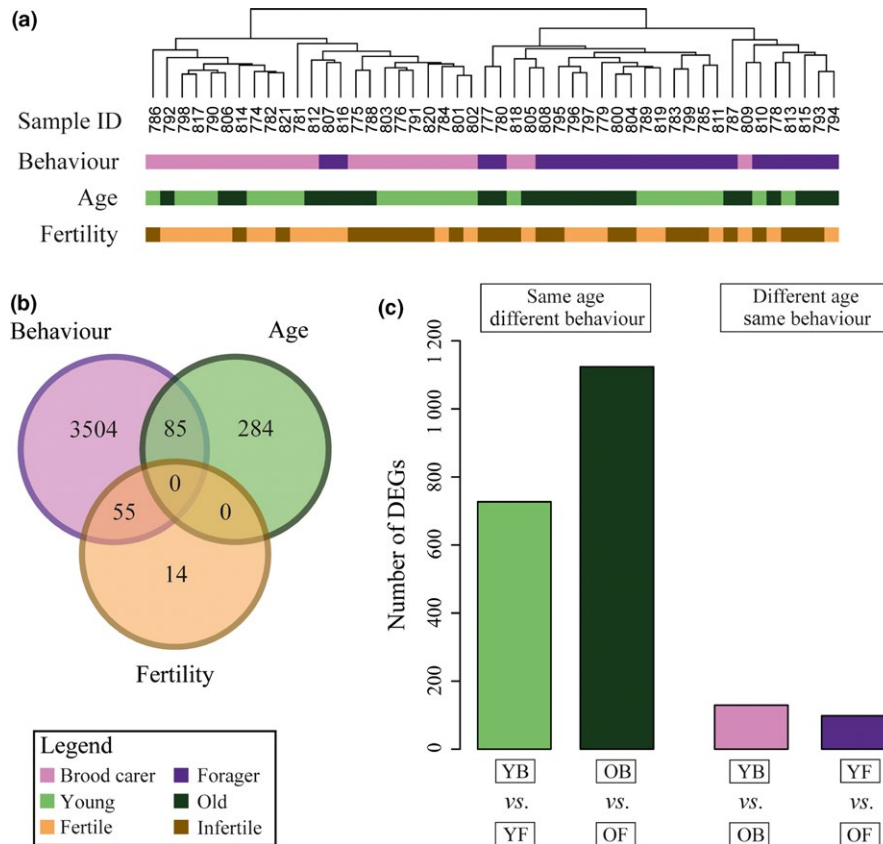


FIGURE 2 Gene expression is most strongly associated with behaviour. (a) Dendrogram analysis revealed two clusters of samples: One mainly represented by brood carers, the other mainly by foragers. (b) Venn diagram depicting the number of differentially expressed genes in each comparison and their overlap. (c) Number of differentially expressed genes detected when comparing workers of same age but different behaviour (light and dark green bars) and when comparing workers of same behaviour but different age (pink and purple bars). OB: old brood carer; OF: old forager; YB: young brood carer; YF: young forager. Colour code: Pink: Brood carer. Purple: Forager. Light green: Young. Dark green: Old. Light brown: Fertile. Dark brown: Infertile [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | DEGs associated with age independent of behavioural specialization and fertility

A total of 369 genes were differentially expressed between the two age classes (296 overexpressed in young, 73 in old; Figure 2b

and Supporting Information Figure S2). The proportion of genes affected by age was smaller than the proportion of genes affected by behavioural specialization (χ^2 test: $p < 0.0001$), but higher than fertility-specific DEGs (χ^2 test: $p = 0.001$). Among those genes more highly expressed in young compared to old workers, we detected

TABLE 1 Expression biases of different Vg genes across ant workers

Species	Gene	B vs. F	Reference
<i>Temnothorax longispinosus</i>	CVg	n.s.	This study
	MVg	B	
	Vg-like A	B	
<i>Solenopsis invicta</i>	CVg	n.s.	Hawkings and Tamborindeguy (2018), Qiu et al., (2017), Wurm et al. (2011)
	MVg	n.s.	
	Vg-like A	B	
<i>Pogonomyrmex barbatus</i>	CVg	F	Corona et al. (2013)
	MVg	B	
<i>Monomorium pharaonis</i>	CVg (Vg1/Vg2)	n.s./B	Mikheyev and Linksvayer (2015)
	MVg	B	
	Vg-like A	n.s.	
<i>Harpegnathos saltator</i>	Unclustered Vg	B	Gospocic et al. (2017)

Note. B: brood carer; CVg: conventional Vg; F: forager; MVg: Myrmicine Vg; n.s.: not significant (see Kohlmeier et al., 2018 for Vg nomenclature).

multiple *cytochrome P450* genes including *CYP4AB2* and *CYP4AB1* that were previously found to exhibit a worker-specific expression in the fire ant *Solenopsis invicta* (Liu & Zhang, 2004). Enriched GO terms of genes more highly expressed in young compared to old workers were largely represented by cell cycle arrest, single-organism cellular process and multiple immunity processes (Supporting Information Figure S5). GO term enrichment of the 73 genes more strongly expressed in old than in young workers yielded no enriched functions, which may be the result of only 41% annotation rate of these DEGs. Old workers expressed 27 viral genes more highly than young workers, whereas young workers expressed two viral genes more highly than old workers.

3.4 | DEGs associated with fertility independent of behavioural specialization and age

A total of 69 genes were differentially expressed between fertile and infertile workers: 62 of them were overexpressed in fertile, and seven in infertile workers (Figure 2b and Supporting Information Figure S2). Among those genes more highly expressed in fertile than infertile workers, we found *MVg2*, *MVg3* and *Vitellogenin-receptor*. The expression of *CVg*, *Vg-like A*, *Vg-like B* and *Vg-like C* was independent of the fertility status (Supporting Information Table S4). GO terms linked to lipid transport and mitochondrial electron transport were enriched in the list of genes more highly expressed in fertile compared to infertile workers (Supporting Information Figure S6). No GO terms were over-represented in the genes more highly expressed in infertile compared to fertile workers.

3.5 | The combined effect of behavioural specialization and age on gene expression

The age-confounded GLM in which we compared gene expression between young brood carers ($n = 8$) and old foragers ($n = 8$) yielded a total of 2,672 DEGs between behavioural castes: 26.7% less than with the age-controlled GLM. We then performed a functional enrichment of those genes more highly expressed in young brood carers compared to old foragers and vice versa. We found 52.2% of the GO term superclusters (Supek et al., 2011) that were enriched in young brood carers (Supporting Information Figure S7), and 57.1% of the GO term superclusters that were enriched in old foragers to

be also enriched in brood carers and foragers in the age-controlled GLM (Supporting Information Figure S8).

We then tested whether the outcome of the comparison between the age-controlled and age-confounded analyses was based on sample size differences (age-confounded data set: 16 samples, age-controlled data set: 48 samples) by generating and analysing 1,000 age-controlled sub-data sets with eight brood carers and eight foragers each (Reduced sample size GLM, RSS). This allowed us to compare three different types of data sets: (a) the complete age-controlled data set with a total of 48 samples, (b) the age-confounded data set with a total of 16 samples and (c) the 1,000 age-controlled data sets with a total of 16 samples (Reduced sample size data set, RSS).

The 1,000 RSS GLMs identified on average 418 ± 7.5 (mean \pm SE) genes more highly expressed in brood carers compared to foragers out of which 85.1% were also identified using the complete age-controlled data set and 480 ± 8.6 genes more highly expressed in foragers compared to brood carers out of which 84.3% were also identified using the complete age-controlled data set (Figure 3). This similarity between the RSS and the complete age-controlled data set was higher than the similarity between the RSS and the age-confounded data set (Similarity between age-confounded data set and complete age-controlled data set for: Brood carers: 59.2%; $p < 0.025$; Foragers: 63.6%; $p < 0.025$).

4 | DISCUSSION

In social insects, worker specialization in performing different tasks (e.g., brood care or foraging) is linked to differences in gene expression (Alaux et al., 2009; Feldmeyer et al., 2014; Gospocic et al., 2017; Khamis et al., 2015; Manfredini et al., 2014; Qiu et al., 2017; Whitfield et al., 2003). The identification of gene expression patterns associated with behavioural specialization is difficult, because behavioural specialization is typically confounded with age and fertility in social insect workers. Expression patterns of candidate genes for the regulation of behavioural specialization vary substantially across ant studies, and it is unclear whether this inconsistency reflects lineage-specific mechanisms of division of labour or is merely the result of study-specific experimental design and associated confounded factors. In this study, we manipulated

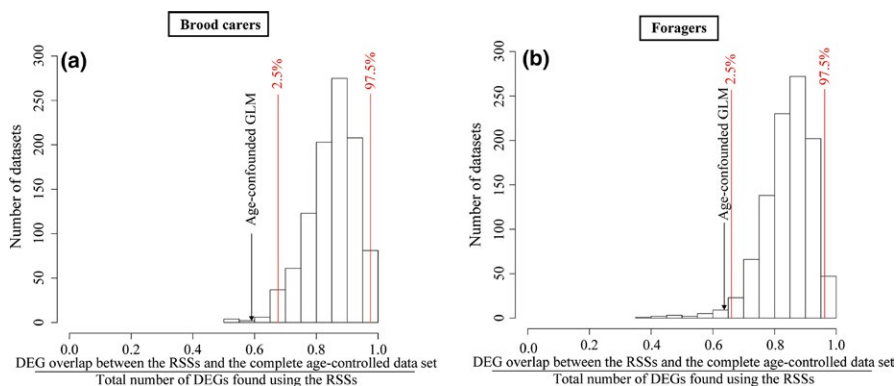


FIGURE 3 Comparison of age-controlled and age-confounded gene expression analyses. Similarity of genes more highly expressed in brood carers (a) and foragers (b) from the age-confounded GLM (arrow) is among bottom 2.5% of the 1,000 age-controlled GLMs with reduced sample size (bars) when compared to the age-controlled GLM on the complete age-controlled data set [Colour figure can be viewed at wileyonlinelibrary.com]

colonies of the ant *T. longispinosus* to independently assess gene expression patterns associated with behavioural specialization (brood carer vs. forager), age (young vs. old) and fertility (fertile vs. infertile).

4.1 | Behaviour- and age-associated gene expression

Worker gene expression was most strongly associated with behavioural specialization, as, for example, illustrated by the finding of ten times more differentially expressed genes (DEGs) between brood carers and foragers than between young and old, or fertile and infertile workers. Gene expression being more strongly associated with behavioural specialization than with age is consistent with previous age-controlled transcriptomic (Alaux et al., 2009; Whitfield et al., 2003) and proteomic (Iovinella et al., 2018) studies on honeybee workers but has so far not been shown in other social insects. In contrast, an age-controlled comparison of queens and workers revealed more DEGs associated with age than with caste in the ant *Lasius niger* (Lucas et al., 2017).

Genes with a higher expression in brood carers compared to foragers and young compared to old workers showed enriched functions related to various repair mechanisms including DNA integrity, oxidative stress response and immunocompetence, indicative of strong investments in a long lifespan (Aamodt, 2009; Bocher, Tirard, & Doums, 2007; Dowling & Simmons, 2009; Helft, Tirard, & Doums, 2012; Kodr k, Bedn rov , Zemanov , & Krishnan, 2015; Selman, Blount, Nussey, & Speakman, 2012; De Verges & Nehring, 2016). As intrinsic mortality is mainly determined by the task a worker performs and not by age, it is not surprising that pathways regulating behavioural specialization and body maintenance are coupled. Brood carers that labour inside the safe nest are selected to upregulate repair processes (Schmid-Hempel & Schmid-Hempel, 1984), whereas this selection diminishes in foragers exposed to high extrinsic mortality (Amdam & Page, 2005; Riecklefs, 1998). In line with this, a previous study on *T. longispinosus* workers revealed a lower intrinsic mortality of young brood carers compared to old foragers (Kohlmeier et al., 2017).

The decreased investment into anti-ageing mechanisms of foragers and old workers supports the classification of the typically old foragers as a disposable caste (Porter & Jorgensen, 1981). This might also explain why we found a higher ratio of viral transcripts among genes more highly expressed in foragers compared to brood carers, and old compared to young workers. These workers probably faced higher viral exposure during their (previous) foraging activity and might have reduced their investment into fighting viral infections.

4.2 | Transcriptomic signatures linked to nutritional physiology

We found multiple GO terms linked to lipid processes to be enriched among the genes more highly expressed in brood carers than in foragers. Differences in nutritional status play an important role in the

regulation of non-reproductive division of labour in social insects, including *Temnothorax albipennis* (Ament et al., 2010; Robinson, Feinerman, et al., 2009; Robinson, McCarthy, et al., 2009; Toth et al., 2005; Toth & Robinson, 2005). In line with this, expression levels and abundance of individual genes and proteins in the fat body are associated with behavioural specialization in workers. For instance, low titres of Tyramine result in high gustatory responsiveness which is linked to elevated foraging activity in honey bees (Scheiner, Entler, et al., 2017; Scheiner, Reim, et al., 2017). In *T. longispinosus*, an RNAi-mediated downregulation of *Vg-like A* in the fat body is associated with the switch from brood- to adult nestmate care by regulating the responsiveness to the cuticular hydrocarbons of brood and adult workers (Kohlmeier et al., 2018). As we compared brood carers to foragers independent of their age, an enrichment of lipid biosynthetic process in genes with a brood carer biased expression might indicate that old foragers switching back to brood care are able to refill their lipid storages, which would contrast with honeybees (Toth & Robinson, 2005). Since we do not know whether the strong signal of lipid processes in brood carer gene expression is based on a larger fat body tissue or indeed a higher expression of genes linked to lipid processes, these findings require experimental validation.

In comparison with brood carers, forager gene expression was mainly characterized by (carbohydrate-) metabolic processes. This suggests that *T. longispinosus* foragers—similar to honeybees (Toth & Robinson, 2005)—rely on carbohydrates rather than lipid stores as a main source of energy for their foraging trips.

4.3 | Candidate genes involved in the regulation of behavioural specialization in workers

Vitellogenin (Vg) genes, which are involved in the reproductive cycle in solitary insects, are associated with the regulation of division of labour in many social Hymenoptera and might have been co-opted early in the evolution of eusociality to additionally mediate behavioural specialization (Amdam, Csondes, Fondrk, & Page, 2006; Amdam, Norberg, et al., 2006; Amdam et al., 2003; Page & Amdam, 2007; Weitekamp et al., 2017). In ants however, *Vg* and *Vg-like* genes underwent numerous lineage-specific duplications (Corona et al., 2013; Kohlmeier et al., 2018; Morandin et al., 2014; Oxley et al., 2014; Wurm et al., 2011) with different copies exhibiting lineage-specific expression patterns between brood carers and foragers (Table 1). For instance, we found in this study that *T. longispinosus* brood carers expressed *Vg-like A*, *Myrmicine Vg2 (MVg)* and *MVg3* more highly than foragers while the expression of *Conventional Vg (CVg)*, *Vg-like B* and *Vg-like C* was independent of behavioural specialization, age and fertility. In contrast, foragers of the harvester ant *Pogonomyrmex barbatus* expressed *CVg* more highly than brood carers, whereas *MVg* was more highly expressed in brood carers compared to foragers (Corona et al., 2013). This suggests that different copies of *Vg* and *Vg-like* genes underwent lineage-specific subfunctionalization with subsequent co-option into novel mechanisms that allow for, for example, a more fine-tuned regulation of behavioural specialization, such as the switch from brood to adult worker care (Kohlmeier et

al., 2018) or specializing in the caring for larvae of specific developmental stages (Walsh, Warner, Kase, Cushing, & Linksvayer, 2018). We thus propose that some of the molecular mechanisms regulating non-reproductive division of labour may result from gene duplication (different copy numbers in different species), subfunctionalization of these genes and most likely differential gene regulation, as the directionality of expression is species-dependent (Table 1). However, it is possible that previous studies might have overlooked expression biases of *Vg* or *Vg-like* genes, because these genes might not have been annotated as *Vitellogenin* genes at the time.

Among the genes more highly expressed in foragers than in brood carers were *ILGFBP* and *Insulin receptor 1* and *2*, which are part of the Insulin/Insulin-signalling pathway and involved in signalling the nutritional status and potentially link nutritional stress to foraging behaviour (Ament et al., 2008; Ben-Shahar et al., 2002; Leong et al., 1992; Toth & Robinson, 2007). Other candidate genes involved in the regulation of behavioural specialization by influencing sucrose responsiveness, for example *foraging*, (Thamm & Scheiner, 2014) and gustatory responsiveness, for example *Tyramine* (Scheiner, Entler, et al., 2017; Scheiner, Reim, et al., 2017) and *malvolio* (Ben-Shahar, Dudek, & Robinson, 2004), were not differentially expressed. As these genes are mainly expressed in the brain (Ben-Shahar et al., 2004, 2003; Wagener-Hulme, Kuehn, Schulz, & Robinson, 1999), we might have been unable to detect differences in expression due to our whole-body transcriptome approach.

It is interesting to note that some of the genes we found to be differentially expressed between brood carers and foragers, such as *Vg* and *Vg-like* genes, genes involved in insulin signalling, *Neuroparsin-A*, and *Ras-related and oestrogen-regulated growth inhibitor*, have also been found to be differentially expressed between brains of queens and workers in multiple ant species (Chandra et al., 2018; Libbrecht, Oxley, & Kronauer, 2018; Okada, Watanabe, Tin, Tsuji, & Mikheyev, 2017; Qiu et al., 2018).

4.4 | The combined effect of behavioural specialization and age on gene expression

We created an age-confounded subset of the data to compare gene expression between young brood carers and old foragers using a GLM including fertility as a blocking factor (Age-confounded GLM). This allowed us to investigate how age confounded the outcome of behavioural studies. Less DEGs were identified using the age-confounded GLM, and only ~50% of the DEGs identified by both methods were similar. Thus, the additional variation introduced by workers that not only differ in the behaviour but also in age leads to a substantial amount of (a) false negatives and (b) wrongly identified DEGs when conducting confounded gene expression analyses. Moreover, downstream analyses (e.g., GO term enrichment, gene network analyses and metabolic pathway comparisons) will also be affected, possibly leading to wrong conclusions. The use of age-confounded data sets might therefore have contributed to the lack of consensus on clear transcriptomic patterns of worker specialized on different behaviours, as many

studies investigating gene expression in different behavioural castes did not control for age and fertility (Cardoso-Júnior et al., 2017; Feldmeyer et al., 2014; Ingram et al., 2005; Johnson & Jasper, 2016; Khamis et al., 2015; Manfredini et al., 2014; Qiu et al., 2017; Scheiner, Entler, et al., 2017; Scheiner, Reim, et al., 2017; Zhao et al., 2013). Similar conclusions were recently drawn from a gene expression comparison between queens and workers, as uncontrolled variation was added by confounding age when comparing gene expression between castes (Lucas et al., 2017). Our data highlight the necessity of experimentally controlling for age in studies of behavioural specialization in workers, and for confounding factors in general (Johnson & Jasper, 2016; Lucas et al., 2017; Weitekamp et al., 2017), which becomes increasingly relevant in gene expression studies.

5 | CONCLUSIONS

Our experimental design allowed us to disentangle gene expression associated with behavioural specialization, age and fertility to show that behavioural specialization is more strongly associated with gene expression than age and fertility. Our analyses identified potential behaviour- and age-specific pathways playing a role in reduced intrinsic mortality in ant workers. Moreover, we found that worker behavioural specialization in *T. longispinosus*, in contrast to honeybees, is independent of the expression of *CVg*, and identified new candidate genes involved in the regulation of non-reproductive division of labour, such as *Vg-like A*, *MVg2* and *MVg3*. *Vg* genes have frequently been found to be involved in the regulation division of labour and behavioural specialization in many social Hymenoptera. In ants however, *Vg* and *Vg-like* genes underwent lineage-specific duplications with different copies exhibiting lineage-specific expression patterns. This indicates that derived regulatory mechanisms of division of labour, which mediate, for example, the switch from caring for young larvae to caring for old larvae, evolved through duplications and subsequent subfunctionalizations of *Vg* and/or *Vg-like* genes. Thus, the evolution of non-reproductive division of labour in ants is based on both already existing pathways and novel genes. Finally, we provide evidence that future RNA-seq studies of insect behaviour should experimentally control for confounding factors such as age.

DATA ACCESSIBILITY

Raw reads are available on the NCBI sequence read archive ID PRJNA487993. Contig sequences and results of the gene expression analyses are available on Dryad (10.5061/dryad.n0v6d91).

AUTHOR CONTRIBUTIONS

P.K., S.F. and B.F. collected the samples, planned and designed the experiment. P.K. performed the experiment. P.K., A.A., S.F. and B.F.

analysed the data. P.K., A.A., R.L., S.F. and B.F. wrote and revised the manuscript.

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