Gene expression is stronger associated with behaviour than with age and 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 strongly influenced by age, fertility and nutritional status, with brood carers being younger, more fecund and corpulent. Here, we experimentally disentangle behaviour from age and fertility in *Temnothorax longispinosus* ant workers and analyse how these parameters are linked to whole-body gene expression. Our transcriptome analysis reveals four times more genes associated with behaviour than with age and only few fertility-associated genes. Brood carers exhibited an upregulation of genes involved in lipid biosynthesis, whereas foragers invested in metabolism. Additional simulations revealed that the experimental disassociation of co-varying factors reduces transcriptomic noise, potentially explaining discrepancies between transcriptomic studies on worker behaviour in other social insects. Our study highlights the influence of nutritional status on task choice in ant workers.

Introduction

Division of labour can be found on all levels of biological organization and has arisen independently during several of the major transitions in evolution, for example during the transition from single cell to multicellular organisms or from solitary to social life [1]. The primary driver for the repeated evolution of division of labour is that it allows cells or group members to specialize on few tasks, thus increase in efficiency and allowing for anatomic or physiological adaptations [2–5].

The mechanistic underpinnings regulating specialization are complex and intensively investigated on different levels of biological organisation. A highly developed example of division of labour is found in the societies of ants, bees, wasps and termites. In these colonies of social insects, reproduction is monopolized by a single or very few individuals, the queens (and kings in termites), whereas the majority of the colony members – the workers - remain functionally sterile. All other colony chores are distributed among the workers, which specialize on specific tasks such as brood care, nest defence and foraging [3].

Whether a worker stays inside the nest and cares for the brood or leaves the nest and forages is strongly influenced by age and physiology with brood carers being younger [3], more corpulent [6–14] and more fertile [15,16] than foragers. Due to high external mortality outside the nest [17], this age- and physiology-based non-reproductive division of labour is beneficial, because it ensures that young workers with longest residual life span, the largest amount of stored resources and the highest reproductive potential stay inside the safety of the nest.

Although age and physiology are important regulators of worker task choice in many social insects, few common mechanisms linking age and physiology to behaviour have been found. For example, the switch from brood care to foraging is at least partly regulated by an interaction between age and stable lipid loss [6–14,18,19]. Information on the nutritional status are transported to and in the brain via Target of rapamycin (TOR) and Insulin/Insulin-like signalling (IIS) pathways [12,20–23]. However, transcriptomic patterns and expression biases

of multiple behaviour-associated genes seem to be lineage-specific, despite crosstalk with the highly conserved TOR and IIS pathways. Task-specific signatures of nutritional status and metabolism characterize the brood carer- and forager-specific transcriptomes in bees and wasps [18,22,24–29], but such evidence is lacking in ants [30,31]. Brood caring behaviour in honey bees is associated with low titres of juvenile hormone (JH) and high expression of the gene coding for the yolk protein precursor *Vitellogenin* (*Vg*). Once JH titres rise, *Vg* is downregulated resulting in a reduction in brood care, a forager-like gene expression, alterations in small RNA populations, a mobilization of carbohydrates and precocious foraging [26,32–37]. In other social insects, this linkage between JH, *Vg* and behaviour is similar [23,38–40], absent [41] or reversed [42–44]. Moreover, *Vg* underwent multiple duplications in ants with different orthologs taking over specific functions in the regulation of fertility and behaviour [31,45–49]. In honey bees, the *foraging* gene is highly expressed in the optical lobes and mushroom bodies of forager' brains [50,51], causing an elevated sucrose responsiveness and the onset of foraging behaviour [52,53]. The expression of *foraging* in other social insects is either positively [54–56] or negatively [57–59] linked to foraging behaviour and additionally influenced by age [60], social structure [30], body size [54,58] and time of the day [61]. The manganese transporter *malvolio* is upregulated in foragers, which induces precocious foraging behaviour by increasing sucrose responsiveness in honey bees [62]. Similar to *Vg*, *malvolio* underwent duplication and subfunctionalization in multiple social and subsocial insects, which raises the question as to whether the role of *malvolio* in honey bees can be extended to other insects [63]. The neuromodulators tyramine and its precursor octopamine promote the onset of foraging, are involved into the rewarding system in honey bee foragers and increase gustatory responsiveness [28,64–66], but their role in other species is unknown.

This across-species inconsistency regarding the mechanistic underpinnings of worker task choice may reflect lineage-specific mechanisms regulating behaviour or differences across studies in the experimental design. Gene expression patterns associated with worker behaviour

are typically identified by comparing gene expression of brood carers and foragers. As age and fertility additionally influence gene expression [22,28,67,68], studies that did not experimentally control for age and physiology when comparing gene expression between brood carers and foragers [e.g. 28,31,46,56,57,69–73] might have produced results driven not only by behaviour, but also by age and fertility. Such confounded transcriptomic analyses are not a problem specific to research on insect behaviour but occur across study systems and contexts. For instance, the degree of tissue heterogeneity, i.e. different numbers of cell types present in a tissue, potentially confounds studies comparing gene expression of different tissues [73]. Thus, confounding factors are an important and partly neglected problem when investigating the transcriptomic underpinnings and mechanisms of different traits in general and non-reproductive division of labour in particular.

In this study, we altered the age structure of colonies of the acorn ant *Temnothorax longispinosus* to experimentally create young and old brood carers and foragers respectively. Furthermore, we sampled both fertile and infertile individuals for each combination of behaviour and age. This allowed us to properly assess how behaviour, age, and fertility are associated with gene expression in ant workers. A recent study revealed stronger differences in gene expression between ant queens and workers [68] than between different age-cohorts of the same caste. As we sampled from two clearly distinct age-cohorts differing in age by at least one year, which is estimated to be the mean *T. longispinosus* worker lifespan in the field [74], we expected age to have a stronger impact on gene expression than behaviour. Furthermore, we expected a rather weak association with fertility as we analysed ant workers from queen-right colonies. Nevertheless, a first study on this species revealed differences between fertile and infertile workers [31].

In a second step, we used different subsets of our data to investigate the additional variance introduced to a dataset by not controlling experimentally for confounding factors like age. Transcriptome studies on worker behaviour coupled with such a manipulation have so far

been restricted to honey bees [67,75], where they provided great insights into the interaction between behaviour, age and gene expression.

Material and methods

Sample collection and preparation

We collected 38 monogynous colonies of the ant *T. longispinosus* with an average colony size of 29 ± 1.5 (mean ± sd) workers at the E.N. Huyck Preserve, Rensselearville, 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 one year old (termed "old"). *Temnothorax* queens are singly-mated which reduces the potential genetic effects on worker behaviour [76]. In our laboratory in Mainz, Germany, the ant colonies were transferred to slide glass nests and kept in plastered 3-chambered nest-boxes (Figure S1). We maintained them at a 14h:10h light:dark photoperiod and a +22°C:+18°C temperature regime to facilitate brood development. Ants were fed with crickets and honey three times a week. We randomly marked either all young (upon eclosion in the lab) or all old (field collected) workers with thin metal wires (0.02mm, Elektrisola) in each colony, allowing us to differentiate between the two age cohorts. Disentangling the effects of behaviour and age on gene expression requires a full factorial design regarding behaviour (brood carer, forager) and age (young, old). To achieve this, we manipulated the demography of colonies 28 days after the emergence of a new worker generation (Figure 1). Specifically, we either removed (i) all old workers from the colony to induce foraging behaviour in young workers (n=12), (ii) removed all young workers to force old workers to tend the brood (n=10), or (iii) removed half of each age cohort as a control (n=16). Workers were then given 21 days to adjust their behaviour and social organisation. Six brood carers (observed tending the brood) and six foragers (found outside the nest) were then individually labelled with metal wire and their investment into either brood caring and foraging was observed and recorded ten times a day for three days. In *Temnothorax* ants, a single

observation is sufficient to classify workers into brood carers and foragers [77] (Kohlmeier et al. subm.). 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 foraging (for details on the methods and results of the behavioural observations see Kohlmeier et al. subm.). After the completion of all observations, we dissected these workers on ice to assess their fertility (fertile: at least one oocyte in the ovaries; infertile: no eggs in development). Following this, individuals were homogenized in 100µl Trizol (Invitrogen) and frozen at -80°C until further processing.

Eight brood carers and eight foragers per demography treatment, i.e. a total of 48 workers, were sampled for individual whole-body RNAseq analyses (Figure 1). Muscular activity und consequently behaviour are directly controlled by the brain. However, brain processes are influenced by hormonal activity, nutritional status or ovary development. To gain insights into such up-stream causative factors, we decided to investigate changes in gene expression within the entire worker ant. As *Temnothorax* workers are monomorphic, gene expression differences based on differences in morphology or anatomy are unlikely [78]. As we sampled both, fertile and infertile workers for each combination of behaviour and caste, we were able to investigate gene expression associated with behaviour, age and fertility independent from each other. After defrosting, 100µl Chloroform was added to each sample, after which samples were cautiously inverted for 30s and then centrifuged at 12,000rpm for 15 min at +4°C. The resulting supernatant was collected, and RNA precipitated with 60µl 70% ethanol. Subsequent RNA extraction was conducted with the RNeasy Mini Kit (Qiagen), following the manufacture instructions. Libraries were constructed at GENterprise GmbH Mainz 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 2500 (Table S1). Sequences will be stored at NCBI short read archive.

Gene expression analysis

Quality analyses of raw reads were conducted with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and IIllumina adapters were removed using Trimmomatic [79]. A *de novo* assembly of raw reads was built in two steps: Firstly, remaining raw reads were assembled using CLC Workbench v.7.0.3 (https://www.qiagenbioinformatics.com/; Table S2) and a subsequent meta-assembly was performed in MIRA [80] using CLC Workbench contigs as input. We removed redundant and/or low-confidence contigs from the transcriptome using CD-Hit-Est v.4.6.1 [80].

For the gene expression analysis, reads were aligned to their corresponding contigs using TopHat v2.1.1 [81], in conjunction with Bowtie 2 v2.1.0 (http://bowtie-bio.sourceforge.net/). Read count information was obtained with eXpress v1.5.1 (http://bio.math.berkeley.edu/eXpress/).

Gene expression analysis was performed using *edgeR* v3.6 [82] by running the following three different GLMs to identify genes associated with (i) *behaviour*: brood carers and foragers were compared whereas fertility and age were added as blocking factors, (ii) *age*: old and young workers were compared and fertility and behaviour were added as blocking factors, and (iii) *fertility*: fertile and infertile workers were compared and age and behaviour were added as a blocking factor. 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 versus 8 young and 16 old foragers, figure 1). Contigs that were differentially expressed (FDR < 0.05), but exhibited low expression difference (fold change between groups < 1) were removed. This included 3247 behaviour-, 296 age- and 13 fertility-associated genes. One of the contigs (>philip_contigs_output_c3380) was identified as a chimeric contig containing two similar sized different ORFs (one first ORF was annotated as *Vg*3, the second ORF was annotated as

193 *Vg*1, Kohlmeier et al. subm.). We manually split this contig between both ORFs, remapped and
194 re-counted raw reads and rerun the gene expression analysis. For each of the three factors
195 (behaviour, age, fertility), separate gene onthology (GO) enrichments were performed for both,
196 over- and underexpressed genes, using Blast2GO v4.1.9 [83]. Enriched GO terms were then
197 summarized using ReViGO [84]. A Weighted Gene Coexpression Network Analysis
198 (WGCNA) [85] was performed on the 20,000 contigs exhibiting the strongest variance in
199 expression. Eigengene values were extracted and their association was analysed with a GLMM
200 using *behaviour, age, fertility* and their interactions as explanatory factors and *colony ID* as a
201 random factor.

202 We further assessed the expression of candidate genes, which have previously been
203 identified to be involved in the regulation of division of labour in social insects: *foraging*,
204 *Insulin like growth factor, Insulin receptor 1* and *2, Krueppel homolog 1, Tyramine receptor 2*,
205 all orthologs of *Vitellogenin* (*VgC, MVg2, MVg3, Vg-like A, Vg-like B, Vg-like C, Vg receptor*),
206 *ultraspiracle* [18,24,28,86,87]. To identify *foraging* in our transcriptome, we blasted our
207 transcriptome against a database containing 16 foraging genes from different ant species, honey
208 bee and *Drosophila melanogaster*. We only changed the annotation of a specific contig to
209 *foraging*, if the e-value of *foraging* was lower than the e-value of the previous blast result. This
210 resulted in eleven *foraging* contigs.

211

212 *Characterizing the effect of age on behaviour specific gene expression*

213 Many studies compared gene expression of young brood carers and old foragers to identify
214 genes associated with behaviour [e.g. 25,28,53,54,66–70]. We investigated the effect of not
215 controlling for age by comparing gene expression of young brood carers (n = 8) and old foragers
216 (n = 8) from control colonies (Figure 1) using pairwise comparison (age confounded PWC) in
217 *edgeR* (e.g. used in [31,88–92]). To test whether differences between age confounded PWC and
218 the age controlled GLM (described in the previous section) depend on whether the dataset was

confounded with age or not or on the statistical approach (pairwise comparison *vs.* GLM), we additionally run a GLM on this age confounded dataset including fertility as a blocking factor (age confounded GLM). We compared the number and identity of differentially expressed genes (DEGs) to those, identified with the age controlled GLM. Functional enrichments and WGCNA were performed as described above.

As differences between both age confounded and age controlled approaches can also be explained by differences in sample size (age confounded dataset: 8 brood carers *vs* 8 foragers; age controlled dataset: 24 brood carers *vs* 24 foragers), we randomly sampled four young brood carers, four old brood carers, four young foragers and four old foragers (two of each fertile and two infertile) from our dataset and identified DEGs with GLM including fertility as a blocking factor using 1000 permutations (Reduced sample size GLM; RSS GLM). The total number of possible combinations of samples was 907,200. No combination of samples was used twice.

Results

DEGs associated with behaviour independent from age and fertility

A total of 448 genes were differentially expressed between the two behavioural castes (226 overexpressed in brood carers, 222 overexpressed in foragers). 54 of these DEGs were also differentially expressed between young and old workers and 32 between fertile and infertile workers (Figure 2).

Among those genes overexpressed in brood carers, we found several copies of *Vitellogenin* including *Vg-like A*, *Myrmicine Vg2* (according to Kohlmeier et al. subm.), *MVg3* and the *Vg-Receptor* with *Vg-like A* being the strongest overexpressed gene in brood carers (FDR = 5.45 x 10^{-18}). The expression of *VgC, Vg-like B*, and *Vg-like C* was independent from behavioural caste (Table 1). The expression of all differentially expressed *Vg* orthologs was positively correlated with each other (Table S3). Especially the expression of *MVg2* and *MVg3* was tightly linked to each other. Moreover, we found *Neuronal acetylcholine receptor subunit*

alpha-3, which binds the neurotransmitter acetylcholine [93], and the *Odorant binding protein 16*, which is part of the olfactory system in honey bees [70]. Enriched GO terms were grouped into eight biological processes, mainly represented by lipid transport and lipid biosynthesis (Figure 3).

Genes overexpressed in foragers include *Insulin-like growth factor-binding protein complex acid labile chain (ILGFBP*), which is associated with binding the Insulin-like growth factor. Furthermore, *beta hexosaminidase subunit beta* and the *circadian clock-controlled protein* were more expressed in foragers than in brood carers. *Tyramine* and *foraging* were not differentially expressed (Table 1). Out of those genes overexpressed in foragers, 20.3% were of viral origin (15.3% *Formica exsecta* virus 2, 2.7% Deformed wing virus, 0.9% *Varroa destructor* virus-1 and Kakugo virus, 0.5% *Nilaparvata lugens* honeydew virus-3). Enriched GO terms were summarized into 13 biological processes largely represented by RNA-templated transcription, pteridine-containing compound catabolism and multiple processes linked to metabolism (Figure 4).

Six modules of co-expressed genes were identified using WGCNA (Table S4). One of these modules was associated with brood care behaviour (GLMM: $\chi^2 = 4.1$ $p = 0.042$), and exhibited a functional enrichment in metabolism, monocarboxylic acid biosynthesis and biosynthesis.

DEGs associated with age independent from behaviour and fertility

102 genes were differentially expressed between the two age classes (27 overexpressed in young, 75 in old; Figure 2). The number of these age-specific DEGs is smaller compared to caste-specific DEGs (χ^2 test: $p < 0.0001$), but higher than fertility specific DEGs (χ^2 test: $p = 0.001$).

Among those genes overexpressed in young workers, we detected 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 and Zhang 2004). Moreover, the expression of *Elongation of very long fatty acids protein*, potentially involved in cuticular hydrocarbon synthesis, *Transposable element P transposase* and multiple copies of the muscle protein *actin*. All genes overexpressed in young workers were combined to a single biological process (single-organism metabolism; Figure S2).

Out of the 75 genes overexpressed in old versus young workers, 36% were of viral origin including *Formica exsecta* virus 2 (28%), Deformed wing virus (4%), Kakugo virus (2.6%) and *Spodoptera frugiperda* rhabdo-virus (1.3%). Ten biological processes including RNA-templated transcription, cellular aromatic compound metabolism, and biosynthesis were linked to old age (Figure S3).

DEGs associated with fertility independent from behaviour and age

A total of 61 genes were differentially expressed between fertile and infertile workers: 49 of them were overexpressed in fertile and 12 in infertile workers (Figure 2). Among those genes overexpressed in fertile workers, we found *MVg2* (FDR = 3.29×10^{-5}), *MVg3* (FDR < 0.003) and *Vitellogenin-receptor* (FDR < 0.018). The expression of *VgC*, *Vg-like A*, *Vg-like B* and *Vg-like C* was independent from fertility status (all FDR > 0.999). Six biological processes including lipid transport and mitochondrial electron transport were overrepresented in fertile compared to infertile workers (Figure S4). In infertile workers, the only overrepresented biological process was L−phenylalanine metabolism (Figure S5).

The combined effect of behaviour and age on gene expression

We used an age confounded subset of the data to characterize the importance of experimentally controlling for worker age. Pairwise comparisons between young brood carers and old foragers only (age confounded PWC) revealed a total of 917 DEGs, significantly more than the number found when controlling for age (χ^2 test: $p < 0.0001$; Figure 5a). Out of these, 565 were

297 overexpressed in brood carers and 352 overexpressed in foragers resulting in more
298 overexpressed genes in brood carers than in foragers (χ^2 test: $p < 0.0001$), which was not found
299 when controlling for age (χ^2 test: $p = 0.841$). In brood carers, the number of biological processes
300 overrepresented was similar to the ones we found when using the age controlled GLM (3 vs. 8;
301 χ^2 test: $p = 0.132$; Figure S6), but fewer biological processes where detected among the DEGs
302 of foragers (2 vs. 13; χ^2 test: $p = 0.001$, Figure S7).

303 The age confounded GLM yielded a total of 764 DEGs between both behavioral castes
304 (Figure 5a): 72.9% more than with the age controlled GLM (χ^2 test: $p < 0.0001$), but 16.7% less
305 compared to the age confounded PWC (χ^2 test: $p = 0.0002$). When applying a GLM on an age
306 controlled dataset with a reduced sample size (RSS GLM) with 1000 permutations, 330 ± 4.3
307 DEGs (mean ± S.E.) were identified, which is not different from the 442 DEGS found using
308 the full age controlled dataset ($p = 0.813$; Figure 5a). The DEGs identified with both age
309 confounded approaches (PWC and GLM) exhibited a lower overlap with those genes identified
310 with the complete age controlled dataset (Brood carer: PWC: 9.2%; GLM: 35.8% figure 5b;
311 Forager: PWC: 8.5%; GLM: 27.3% figure 5c) compared to the age controlled RSS GLM (Brood
312 carer: mean = 65.5%; Forager: mean = 61.7%; figure 5c).

313 A WGCNA on the age confounded PWC dataset yielded 48 modules. Out of these
314 modules, 14 were associated with brood carers or foragers (Table S5).

315

316 ## Discussion

317 In social insects, behavioural specialization of workers (e.g., on brood care and foraging) is
318 influenced by gene expression. Surprisingly, especially in ants, transcriptomic patterns and
319 expression biases of candidate genes involved in the regulation of behaviour vary between
320 studies. The identification of such patterns and genes is difficult, because behaviour is linked
321 to many other factors such as age and fertility. Thus, transcriptome comparisons of brood carers
322 and foragers are often confounded by these parameters, which potentially explains diverging

results. In this study, we manipulated colonies of the ant *Temnothorax longispinosus* and assessed gene expression patterns associated with behaviour (brood carer *vs* forager), age (young *vs* old) and fertility (fertile *vs* infertile) independently. In a second step, we compared our results to those that would have been obtained if age was confounded with behaviour.

Genes associated with behavioural task independent from age and fertility

Our study sheds new light on the transcriptomic underpinnings of non-reproductive division of labour in ants. Gene expression of *T. longispinosus* brood carers was largely represented by lipid transport and lipid biosynthesis (energy storage; Figure 3). Nutritional status, with corpulent brood carers and food deprived foragers, is one of the most widespread and consistent factors mediating worker behaviour [10,19], and influences gene expression in honey bees [12,18,22,25,26,28] and wasps [29]. In ants however, despite the broad correlative evidence for brood carers harbouring more lipid storages than foragers [6–9,14,94,95], clear transcriptomic signatures of nutritional status have so far not been found [30,31]. Only a single study identified lipid storage and fatty acid metabolism to be enriched functions of genes differentially expressed between brood carers and foragers [96]. In that study however, transcriptomes of young brood carers and old foragers were compared. As lipid storages in experimentally hive-restricted bees are reduced with age and age-dependent reduction in lipid storages has been found in *Drosophila* males [12,97], it remained unclear whether observed nutrition linked gene expression is associated with behaviour or with age. Interestingly, we found an increased investment into lipid biosynthesis in brood carers independent from age. In honey bees, reverted brood carers do not refill their lipid storages [19] and future research should therefore test whether old workers in ants, which return to brood caring duties are able to regain lipid storages.

Among the genes upregulated in brood carers, we found multiple fat body expressed copies of egg yolk precursor and storage protein *Vg* (*Vg-like A, MVg2, MVg3*; Kohlmeier et al. subm.). *Vg-like A* might be of specific importance as its expression was independent from age

and fertility, it is mainly expressed in the fat body, has recently been identified as a key regulator of the behavioural transition from brood care to adult nestmate care and occurs in a large number of social insect genomes (Kohlmeier et al. subm.). Whether the changes in responsiveness are at least partly linked to the upregulation of the *Odorant binding protein 16* has to be answered in future. *Conventional Vg* takes over an important role in the regulation of worker behaviour in honey bees [26,32–37]. However, this *Vg* copy was not differentially expressed in our study (see Kohlmeier et al. in subm. for a *Vg* phylogeny). This indicates that brood caring behaviour in ants is controlled by different pathways than in honey bees. Moreover, brood carers overexpressed the brain expressed *Neuronal acetylcholine receptor subunit alpha-3* binds acetylcholine, a neurotransmitter involved in learning in honey bees [93]. However, the role of the receptor for brood caring behaviour should be investigated in the future.

Foragers exhibited increased investment into catabolism and carbohydrate transport (energy mobilization), as well as multiple metabolic processes (energy usage). Similar to honey bees, these data indicate that foragers rely on carbohydrate as a main source of energy for their foraging trips [10]. For example, among the overexpressed genes was *Insulin-like growth factor-binding protein complex acid labile chain*, which is involved in binding of Insulin-like growth factor (IGF) and part of the IIS pathway [98]. Despite the clear transcriptomic signatures of nutritional status and metabolism, multiple candidate genes that are part of the cross-talk between IIS and TOR (i.e. *malvolio, foraging, Tyramine*) were not differentially expressed. As these genes are mainly expressed in the brain [62,99,100], we might have been unable to detect differences in expression, because we used whole body transcriptomes. Alternatively, our findings might reflect lineage specificity regarding pathway rearrangements, which have been documented in the honey bee. For instance, the negative relationship between Insulin-like peptide titres and abdominal lipids in honey bees are a derived stage and reverse in other insects [12,21]. Potentially, such rearrangements have occurred after bee and ant lineages split. This

375 question needs to be answered in the future, e.g. by age controlled tissue-specific gene
376 expression comparisons.

377 Apart from genes associated with the regulation of behaviour and nutritional
378 physiology, we identified multiple genes that potentially fulfil rather supportive functions for
379 foraging behaviour. Mutations in *Beta hexosaminidase subunit beta*, one of the strongest
380 differentially expressed genes between brood carers and foragers, are linked to the Sandhoff
381 disease in humans causing complex symptoms including a reduced locomotive activity [101].
382 *Clock-controlled protein* is a gene downstream of the circadian clock in *Drosophila*
383 *melanogaster* [102]. Whether these genes for example contribute to maintaining muscle activity
384 (*beta hexosaminidase subunit beta*) or correctly timing foraging trips (*clock-controlled protein*)
385 still needs to be investigated.

386

387 *Genes associated with age independent from behavioural task and fertility*

388 Young age in workers was characterized by the overexpression of several cytochrome *P450*
389 genes including *CYP4AB 1* and *2*, *Elongation of very long chain fatty acids protein*,
390 *transposable element P transposase* and several actin genes. *P450 CYP4AB 1* and *2* were shown
391 to be overexpressed in workers compared to sexuals in the fire ant *Solenopsis invicta* [103].
392 Overexpression of *Elongation of very long chain fatty acids protein* potentially contributes to
393 age-dependent differences in cuticular hydrocarbon profiles, which have been reported for
394 workers of the ant *Diacamma ceylonese* [104]. Old workers in contrast overexpress viral
395 transcripts, indicative of a higher viral load in aged workers, which was previously documented
396 in honey bees, in which a sugar-rich diet further increased the viral load of workers [105]. High
397 viral load might contribute to the increased intrinsic mortality of old compared to young
398 workers as well as foragers compared to brood carers [77] Interestingly, we did not detect any
399 typical aging pathways or genes, such as ROS pathways.

Age had a much weaker influence on the transcriptome compared to behaviour. This is in line with honey bee age controlled gene expression studies [67,75], although the age difference in honey bee brood carers and foragers is few weeks only, where it is at least one year in our study. Our findings strongly contrast with studies investigating caste (queen *vs.* worker) gene expression across different developmental stages and ages, in which more DEGs were found between different ages or developmental stages than between castes [68,91,106–108]. The larger gene expression differences between brood carers and foragers in combination with no upregulation of pathways associated with longevity indicate strong physiological differences between the two behavioural worker castes, and suggest weak changes with age in the investment in body repair mechanisms [109].

Genes associated with fertility independent from behavioural task and age

Fertility had the weakest effect on overall gene expression. Noteworthy however, *MVg2* and *MVg3* were highly expressed in fertile compared to infertile workers, whereas the expression of *Vg-like A* was independent from fertility status. This supports the hypothesis of a sub-functionalization of *Vg* and *Vg-like* genes. In future, it will be interesting to see whether the Myrmicine-specific *MVg2* and *MVg3* fulfil fertility-linked functions similar to the ancestral *Vg* copies e.g. in *Drosophila.*

The combined effect of behaviour and age on gene expression

We created age confounded subdatasets to compare gene expression between young brood carers and old foragers using pairwise comparison (Age confounded PCW) and a GLM including *fertility* as a blocking factor (Age confounded GLM). This allowed us to investigate the effect of not experimentally controlling for age. Despite few exceptions (*MVg2, clock-controlled protein*), candidate genes involved in the regulation of worker behaviour showed similar expression patterns across all methods (Table 1). Thus, investigations of specific

426 candidate genes seem to be consistent even with confounded data and highlight the potential
427 importance of these genes for the regulation of worker behaviour.

428 However, additional analyses, such as GO enrichment, WGCNA and metabolic pathway
429 comparisons are commonly used and provide valuable insights into broader patterns of gene
430 expression [27,30,31,67,68,75,87,90,96]. Hence, results and conclusions depend on the number
431 and identity of large sets of genes, and partly their FDR values and raw read counts provided
432 as an input. We could show that both age confounded approaches (PWC and GLM) identified
433 more DEGs than the age controlled approaches (RSS and GLM). These findings are likely to
434 be the result of additional variation in gene expression that was introduced by workers not only
435 differing in behaviour but also in age. This additional variation was not a simple combination
436 of those genes found to be differentially expressed between behaviours and age (Figure S8),
437 but seems to be rather random with unpredictable effects on follow-up analyses: Whereas GO
438 term enrichments on the age confounded dataset yielded less enriched biological processes than
439 the age controlled dataset, more co-expressed modules were found in the WGCNA. Thus, our
440 data highlight the necessity of experimentally controlling for confounding factors.

441 The use of age confounded datasets might therefore have contributed to the lack of
442 consensus on clear transcriptomic patterns of worker behaviour, as many studies investigating
443 gene expression in different behavioural castes did not control for age and fertility [e.g.
444 28,31,56,57,69–73]. Similar conclusions were recently drawn from a gene expression
445 comparison between queens and workers, as uncontrolled variation was added by confounding
446 age when comparing gene expression between both castes [68]. Such effects of experimentally
447 introduced variation have not only been described for age but also for tissue choice
448 [68,71,72,78]. We therefore conclude that gene expression analyses aiming at identifying genes
449 associated with worker behaviour benefits from experimentally controlling for confounding
450 factors such as age.

451

Conclusions

Taken together, by disentangling gene expression associated with behaviour, age and fertility, we identified new candidate genes involved in the regulation of non-reproductive behaviour (*Vg-like A*), showed that candidate genes described in honey bees do not play a role in *Temnothorax* ants (*Conventional Vg*), and highlight the importance of nutritional physiology and metabolism in brood carers versus foragers. In respect to expression changes with age, we detected investment in muscles in young workers, and no evidence for age-dependant expression of typical longevity genes. We thus provide evidence that experimentally controlling for confounding factors such as age or behaviour will increase resolution and decrease transcriptomic noise in future RNAseq studies.

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Figures

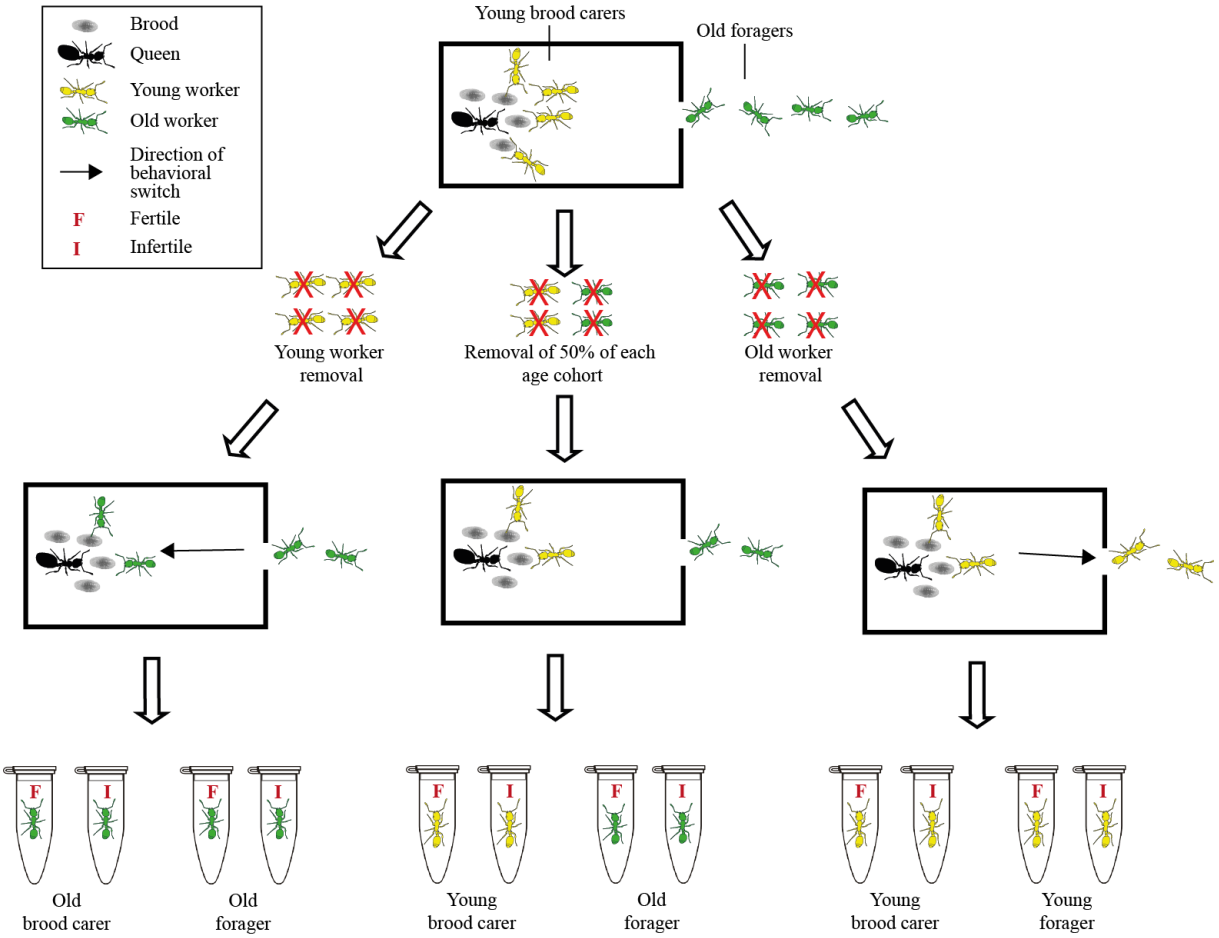


Figure 1: Overview of colony demography manipulations. Colony demographics were manipulated to generate foragers and brood carers of both age classes. From each colony we sampled eight brood carers and eight foragers half of each fertile and half of each unfertile.

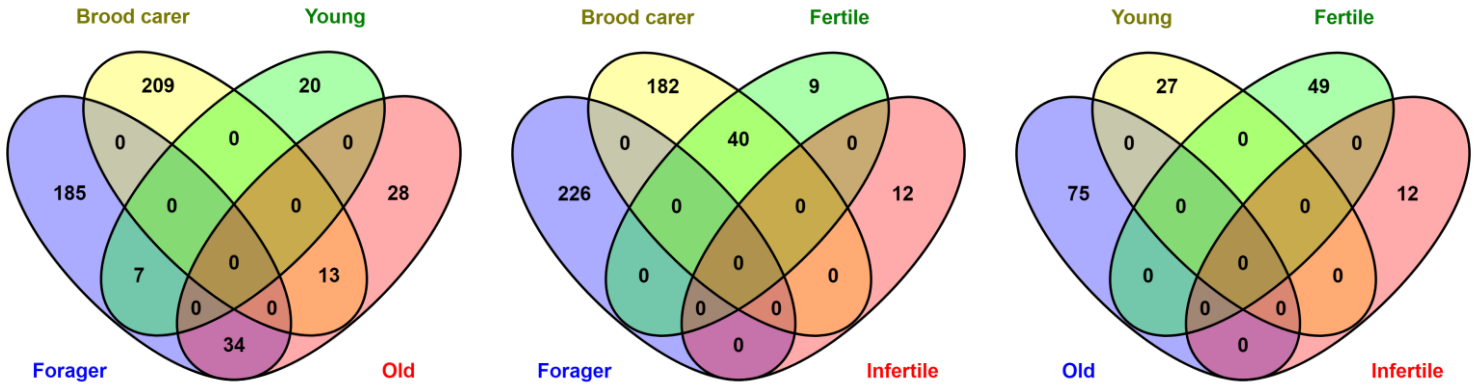
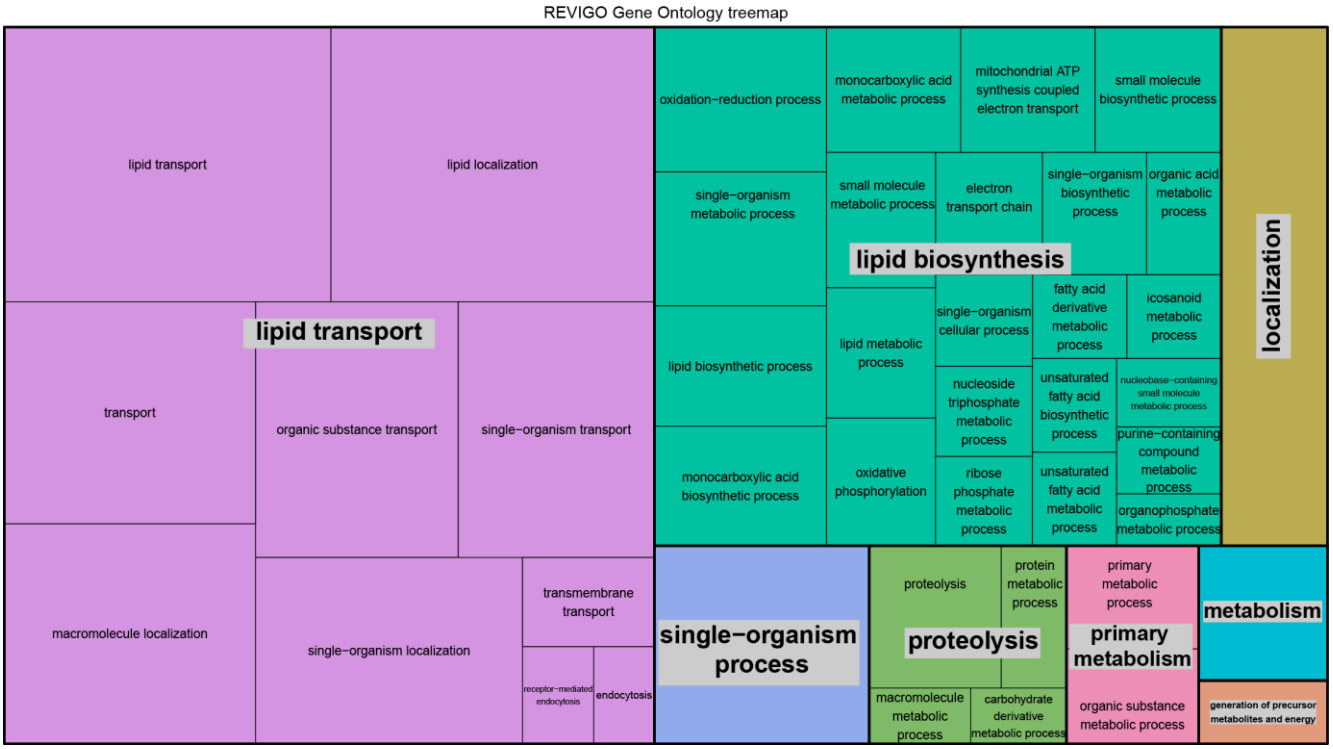


Figure 2: Venn diagrams of genes found to be differentially expressed between brood carers and foragers (left and center) young and old workers (left and right) and fertile and infertile workers (center and right) and their overlap with each other.



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807 **Figure 3**: Biological processes overrepresented in the list of genes upregulated in brood carers compared

808 to foragers independent from age and fertility.

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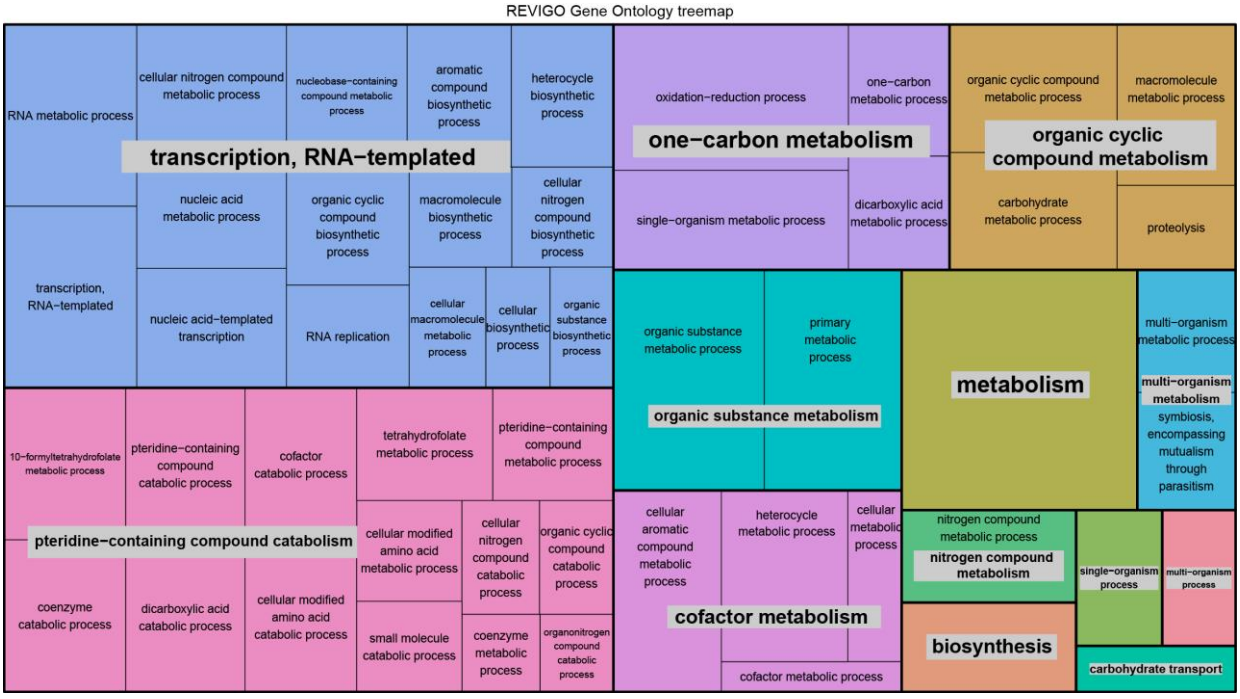

810

811 **Figure 4**: Biological processes overrepresented in the list of genes upregulated in foragers compared to

812 brood carers independent from age and fertility.

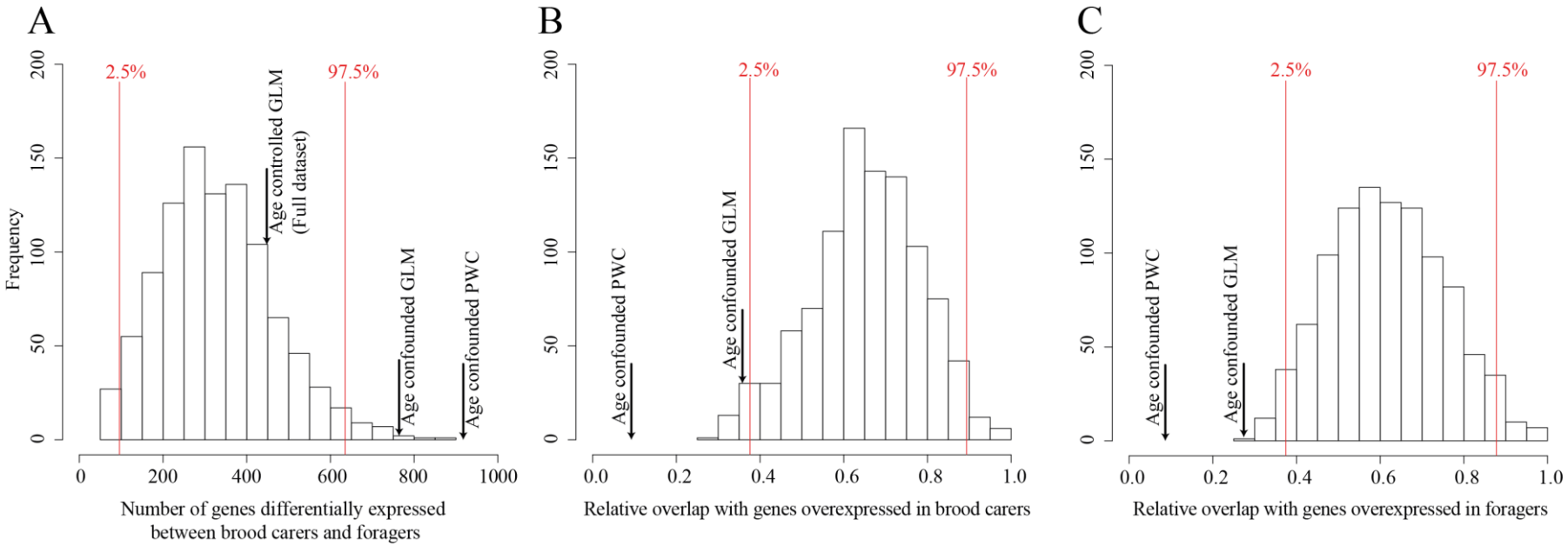

Figure 5: Comparison of age controlled and age confounded gene expression analyses. A) Number of differentially expressed genes identified when comparing brood carers and foragers in an age controlled, reduced sample size GLM (RSS) with 1000 permutations (Bars). B) and C) Relative overlap between the RSS (bars), the age confounded GLM and PWC with the genes found to be upregulated in brood carers (B) and foragers (C) using the age controlled GLM on the full dataset. 5% confidence intervals are given in red. PWC: Pairwise comparison. GLM: Generalized linear model.

Tables

Table1: Overview of the candidate genes previously described to be associated with worker behaviour and their expression bias using different methods and datasets. A log fold change (logFC) < 0 represents an expression bias towards brood carers (caste), young (age) or fertile (fertility) workers. Expression was only considered as biased if FDR < 0.05 and logFC < -1 or > 1. Significant expression biases are given in bold. Genes labelled with * exhibited different caste expression biases across the different methods. PCW: Pairwise comparison ; RSS: Reduced sample size.

Gene	**Expression bias in age controlled GLM**		**Caste dependent expression bias in age confounded PCW**	**Caste dependent expression bias in age confounded GLM**	**Relative frequency of expression bias in RSS age controlled GLM**
Beta hexosaminidase	**Caste**	**FDR < 0.0001** **logFC = 2.1**	FDR < 0.001 logFC = 4.5	FDR < 0.001 logFC = 2.6	B < F: 99.3 % B > F: 0% B = F: 0.7%
	Age	FDR = 0.850 logFC = 0.2			
	Fertility	FDR = 0.999 logFC = 0.5			
*Clock controlled protein**	**Caste**	**FDR < 0.0001** **logFC = 1.5**	***FDR = 0.048*** ***logFC = -7.9***	**FDR < 0.001** **logFC = 1.6**	B < F: 95.6 % B > F: 0% B = F: 4.4%
	Age	FDR = 0.884 logFC = 0.1			
	Fertility	FDR = 0.999 logFC = 0.5			
Foraging	Caste	FDR = 0.131 logFC = -0.1	FDR = 0.265 logFC = -1.3	FDR = 0.387 logFC = -0.2	B < F: 0.2 % B > F: 3%

	Age	FDR = 0.769 logFC = 0.1			B = F: 96,8%
	Fertility	FDR = 0.999 logFC = -0.2			
ILGFBP	**Caste**	**FDR < 0.0001** **logFC = 1.2**	**FDR < 0.001** **logFC = 3.2**	FDR < 0.0001 logFC = 2.4	B < F: 87.5 % B > F: 0% B = F: 12.5%
	Age	FDR = 0.880 logFC = 0.1			
	Fertility	FDR = 0.381 logFC = 1.0			
Insulin receptor 1	Caste	FDR = 0.001 logFC = -0.4	FDR = 0.505 logFC = -0.7	FDR = 0.008 logFC = 0.7	B < F: 0 % B > F: 0% B = F: 100%
	Age	FDR = 0.989 logFC = 0.4			
	Fertility	FDR = 0.999 logFC = -0.1			
Insulin receptor 2	Caste	FDR = 0.001 logFC = -0.4	FDR = 0.333 logFC = -1.9	FDR = 0.006 logFC = -0.7	B < F: 0 % B > F: 0% B = F: 100%
	Age	FDR = 0.959 logFC = 0.0			
	Fertility	FDR = 0.999 logFC = -0.1			
Krueppel like	Caste	FDR = 0.119	FDR = 0.399	FDR = 0.347	B < F: 0 %

		logFC = 0.2	logFC = -2.2	logFC = 0.266	B > F: 0% B = F: 100%
	Age	FDR = 0.901 logFC = -0.1			
	Fertility	FDR = 0.999 logFC = -0.0			
Tyramine receptor 2	Caste	FDR = 0.305 logFC = 0.2	FDR = 0.492 logFC = 4.1	FDR = 0.280 logFC = 3.7	B < F: 0 % B > F: 0% B = F: 100%
	Age	FDR = 0.989 logFC = -0.0			
	Fertility	FDR = 0.999 logFC = -0.1			
VgC	Caste	FDR = 0.718 logFC = -0.1	FDR = 0.953 logFC = -0.4	FDR = 0.507 logFC = 0.3	B < F: 0 % B > F: 0% B = F: 100%
	Age	FDR = 0.723 logFC = 0.2			
	Fertility	FDR = 0.999 logFC = 0.2			
MVg2	**Caste**	**FDR < 0.0001** **logFC = -4.6**	FDR = 0.063 logFC = -4.9	FDR < 0.0001 logFC = -4.2	B < F: 0 % B > F: 88.8% B = F: 11.2%
	Age	FDR = 0.885 logFC = 0.3			
	Fertility	**FDR < 0.0001**			

		logFC = -2.8			
MVg3	**Caste**	**FDR < 0.0001** **logFC = -4.9**	**FDR = 0.002** **logFC = -12.1**	**FDR < 0.0001** **logFC = -4.5**	B < F: 0 % B > F: 90.5% B = F: 9.5%
	Age	FDR = 0.869 logFC = 0.3			
	Fertility	**FDR < 0.0001** **logFC = -2.7**			
Vg-like A	**Caste**	**FDR < 0.0001** **logFC = -3.7**	**FDR = 0.002** **logFC = -4.5**	**FDR = 0.002** **logFC = -4.0**	B < F: 0 % B > F: 99.5% B = F: 0.5%
	Age	FDR = 0.993 logFC = 0.0			
	Fertility	FDR = 0.999 logFC = -0.5			
Vg-like B	Caste	FDR = 0.233 logFC = -0.1	FDR = 0.913 logFC = -0.5	FDR = 0.543 logFC = 0.1	B < F: 0 % B > F: 0% B = F: 100%
	Age	FDR = 0.536 logFC = -0.1			
	Fertility	FDR = 0.999 logFC = -0.0			
Vg-like C	Caste	FDR = 0.953 logFC = -0.1	FDR = 0.630 logFC = 0.1	FDR = 0.669 logFC = -0.1	B < F: 0 % B > F: 0% B = F: 100%
	Age	FDR = 0.995 logFC = 0.0			

	Fertility	FDR = 0.999 logFC = 0.1			
VgR	**Caste**	**FDR < 0.0001** **logFC = -2.2**	**FDR < 0.0001** **logFC = -9.9**	**FDR < 0.0001** **logFC = -4.0**	B < F: 0 % B > F: 64.4% B = F: 35.6%
	Age	FDR = 0.198 logFC = -0.9			
	Fertility	**FDR = 0.004** **logFC = -1.5**			
ultraspiracle	Caste	FDR = 0.279 logFC = -0.2	FDR = 0.090 logFC = 1.2	FDR = 0.527 logFC = 0.1	B < F: 0 % B > F: 0% B = F: 100%
	Age	FDR = 0.776 logFC = 0.1			
	Fertility	FDR = 0.999 logFC = 0.0			