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Transcriptomic analysis of mosaic brain differentiation underlying complex division of labor in a social insect

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Abstract

Concerted developmental programming may constrain changes in component structures of the brain, thus limiting the ability of selection to form an adaptive mosaic of size-variable brain compartments independent of total brain size or body size. Measuring patterns of gene expression underpinning brain scaling in conjunction with anatomical brain atlases can aid in identifying influences of concerted and/or mosaic evolution. Species exhibiting exceptional size and behavioral polyphenisms provide excellent systems to test predictions of brain evolution models by quantifying brain gene expression. We examined patterns of brain gene expression in a remarkably polymorphic and behaviorally complex social insect, the leafcutter ant *Atta cephalotes*. The majority of significant differential gene expression observed among three morphologically, behaviorally, and neuroanatomically differentiated worker size groups was attributable to body size. However, we also found evidence of differential brain gene expression unexplained by worker morphological variation and transcriptomic analysis identified patterns not linearly correlated with worker size but sometimes mirroring neuropil scaling. Additionally, we identified enriched gene ontology terms associated with nucleic acid regulation, metabolism, neurotransmission, and sensory perception, further supporting a relationship between brain gene expression, brain mosaicism, and worker labor role. These findings demonstrate that differential brain gene expression among polymorphic workers underpins behavioral and neuroanatomical differentiation associated with complex agrarian division of labor in *A. cephalotes*.

KEYWORDS
caste evolution, cognition, polyphenism, RNAseq, sensory ecology, social brain

1 | INTRODUCTION

Understanding the evolutionary and molecular processes that influence brain size and structure in relation to behavior is a central goal in evolutionary neurobiology. A significant aim is to identify if and how social evolution shapes neuroarchitectures that adaptively process information relevant to group living. The selective forces and mechanisms involved in brain evolution are actively debated (DeCasien & Higham, 2019; Dunbar, 1998; Dunbar & Shultz, 2017; Lihoreau et al., 2012; O'Donnell et al., 2015), and contrasting, although not mutually exclusive, hypotheses attempt to explain the adaptive design of neural phenotypes (D'Aniello et al., 2019).

The mosaic brain hypothesis proposes that structural variation in the brain and its constituent functionally specialized compartments—regulated by genetic architectures (Hager et al., 2012; Hibar et al., 2015; Noreikiene et al., 2015; Zwarts et al., 2015) and developmental

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patterning (Sylvester et al., 2010)—may evolve independently of body size. Adaptive brain mosaicism has been identified in eusocial insects in association with reproductive and ergonomic division of labor (Godfrey & Gronenberg, 2019; Gordon & Traniello, 2018; Kamhi et al., 2016; Muscedere & Traniello, 2012; Muscedere et al., 2014; O'Donnell et al., 2013, 2018, 2019), as well as in mice (Hager et al., 2012), fish (Fischer & Jungwirth, 2022; Tamayo et al., 2020; York et al., 2019), and primates (Harrison & Montgomery, 2017). However, brain evolution may be concerted, that is, constrained by developmental processes coupling brain size to body size and/or coordinating the development of different brain centers (Finlay & Darlington, 1995). Moreover, constraint and mosaicism may both influence social brain evolution (D'Aniello et al., 2019; Herculano-Houzel et al., 2014; Hoops et al., 2017; Montgomery et al., 2016; Moore & DeVoogd, 2017).

Genomic data provide a foundation to examine transcriptional signatures of brain evolution in complex societies (Favreau et al., 2018; Qiu et al., 2018; Traniello et al., submitted). In eusocial insects, worker polymorphism—division of labor by physical castes—is regulated by gene networks (Lillico-Ouachour & Abouheif, 2017; Rajakumar et al., 2012, 2018; Trible & Kronauer, 2017, 2021a; b; Abouheif, 2021), as are age-related changes in task performance (Whitfield et al., 2006; Bloch & Grozinger, 2011; Wang et al., 2012; Sinha et al., 2020; Habenstein et al., 2021). Gene expression, including brain gene expression, has been linked to worker behavior, neural phenotype, and experience (Howe et al., 2016; Qiu et al., 2018; Alleman et al., 2019; Kohlmeier et al., 2019; Friedman et al., 2020; Lucas & Ben-Shahar, 2021; Miyazaki et al., 2021), suggesting division of labor is strongly influenced by dynamic gene regulation (Kapheim et al., 2016; Kocher et al., 2018). Several key genes mediating sensory response and task performance differ developmentally in expression (Ben-Shahar et al., 2003; Ingram et al., 2005, 2011; Lucas & Sokolowski, 2009; Oettler et al., 2015; Bockoven et al., 2017; Trible et al., 2017; Yan et al., 2017). Furthermore, regulation of developmental gene expression can be impacted by gene evolution, which in the case of sensory genes can correlate with behavioral differentiation. For example, olfaction-related genes have evolved within the Family Formicidae (the single clade of ants) in a manner reflecting species life histories (Engsontia et al., 2015; Cohanim et al., 2018; Saad et al., 2018; McKenzie et al., 2021). Transcriptomic analyses accentuate the importance of changes in regulatory flexibility associated with social insect evolutionary transitions (Simola et al., 2013; Kapheim et al., 2015; Rubin et al., 2019), and may offer insight into the role of plastic gene expression in generating the phenotypic variation characteristic of advanced division of labor.

Fungus-growing ants (the *Atta* group within the Tribe Pheidolini; Ward et al., 2015) present outstanding opportunities to test theories of brain evolution in light of worker polymorphism, task performance, and neuroanatomy (Muratore & Traniello, 2020). These ants practice fungal agriculture and vary in social structure from small colonies of monomorphic, behaviorally generalized workers to large colonies of extremely polymorphic task-specialized workers (Mehdiabadi & Schultz, 2010; Barrera et al., 2022). Among derived leafcutting ant species, the neoattini (Schultz & Brady, 2008), *Atta cephalotes* displays exceptional worker polymorphism and has evolved a complex system of division of labor to produce a food crop. Recently, significant allometries among functionally specialized brain compartments associated with olfaction and higher-order information processing (Muratore et al., 2022) and visual system evolution across polymorphic workers (Arganda et al., 2020) have been identified. Brain center scaling patterns thus reflect functional differentiation of worker size-related neural phenotypes that have coevolved with variation in behavioral and/or cognitive challenges faced by task-differentiated workers (Muratore et al., 2022). Our knowledge of molecular mechanisms underlying adaptive brain scaling, however, is limited. Genomics and transcriptomics thus provide promising avenues to explore brain evolution in *Atta*. The *A. cephalotes* genome evolution illustrates adaptation to their agricultural life history (Suen et al., 2011) and gene expression differences in some *Atta* species are correlated with synaptic structures (macroglomeruli) in the brains of large workers (Koch et al., 2013). Identifying how differential gene expression contributes to neuroanatomical plasticity among polymorphic workers will provide insight into neuroarchitectural adaptations to worker size-related behavioral demands and evidence for developmental constraint, adaptive mosaicism, or both, in brain evolution.

Atta worker size frequency distributions have been historically described as physical subcastes of minims (~0.6 mm in head width), medias (~1.8 mm in head width), and majors (>3 mm in head width), in order of increasing head width (Wilson, 1980). Minims, the smallest worker size class, are characterized as specialists in within-nest tasks such as fungal gardening and brood care (Wilson, 1980) with occasional extranidal activities (Feener & Moss, 1990; Evison et al., 2008; Griffiths & Hughes, 2010). Medium-size workers (medias) engage in a wider variety of tasks than minims, and specialize on leaf harvesting (Wilson, 1980), behaviors that involve navigation during foraging and selecting leaves based on plant chemistry (Hubbell et al., 1983; Howard, 1987). The largest workers (majors) specialize primarily in defense (Wilson, 1980; Powell & Clark, 2004) and may engage in trail maintenance (Wilson, 1980; Howard, 2001; Evison et al., 2008). Recently, details of morphological evolution in association with behavioral differentiation have been identified in *A. cephalotes*, supporting previously described patterns of division of labor but revealing additional morphological divisions among workers (Muratore et al., 2023, in revision).

Fungus-growing ant social structure and brain size are correlated (Riveros et al., 2012), in that larger colony size is linked to smaller brain size but increased investment in the antennal lobes, the olfactory center of the brain. Worker size-related neural phenotypes have been documented in *A. cephalotes*: brain size and the volumes of most brain compartments increase in terms of absolute size in larger workers, while mushroom bodies and antennal lobes are proportionally largest in mid-sized workers and the central complex takes up the greatest percent of the minim brain (Muratore et al., 2022). Analyses of relative investment patterns in brain centers show the optic lobes are proportionally largest in majors, mushroom bodies and antennal lobes are proportionally largest in medias, and the central complex is proportionally largest in minims (Muratore et al., 2022). *A. cephalotes* brain size and compartmental scaling therefore illustrates mosaic development, although brain size is somewhat constrained by worker size in

that major brains are larger in absolute volume but relatively smaller with respect to body size.

Developmental triggers governing social insect polymorphism have been identified (Abouheif & Wray, 2002; Smith et al., 2008; Trible & Kronauer, 2017; Rajakumar et al., 2018) and debated in regard to the influence of body size (Trible and Kronauer, 2017, 2021b; Abouheif, 2021). However, the genetic underpinnings of neural phenotype differentiation in relation to worker division of labor and its correspondence to mosaic and/or concerted patterns of brain evolution are not well understood. The concerted brain evolution hypothesis predicts a strong correlation between patterns of gene expression and body size, and hence brain size (Hagar et al., 2012). If brain gene expression patterns in *A. cephalotes* are consistent with the concerted brain evolution model, then gene expression should either predictably increase or decrease with worker size. In contrast, the mosaic brain model (e.g., Barton & Harvey, 2000; Hagar et al., 2012; Höglund et al., 2020) posits that individual brain compartments can evolve independently in response to differential selection pressures arising from the need for maximal efficacy and/or efficiency in task performance. This may be mediated by gene expression patterns corresponding directly to the scaling of brain compartments or patterns independent of worker size. In this case, many genes would change in expression across worker groups in a pattern differing from simple increases or decreases as workers and their brains increase in size. However, concerted and mosaic brain evolution may both occur (Herculano-Houzel et al., 2014; Montgomery et al., 2016; Hoops et al., 2017; Moore & DeVoogd, 2017; D'Aniello et al., 2019). To test these hypotheses and understand how transcriptional dynamics influence neural phenotypes (Ben-Shahar, 2005; Kleineidam et al., 2005; Trible et al., 2017; Yan et al., 2017) and behavior (Whitfield et al., 2003; Alleman et al., 2019), we measured brain gene expression in polymorphic *A. cephalotes* workers, examined whether gene expression differences were determined by body size, and identified gene ontology (GO) and coexpression modules correlated with specific workers groups and their patterns of task performance.

2 | METHODS: COLONY COLLECTION AND CULTURING

Incipient colonies of *A. cephalotes* were collected in Trinidad and Costa Rica from 2016 to 2018. The colony collected from Trinidad (Ac16) was housed in a Harris environmental chamber with a 12-h light:12-h dark regimen at 25°C and 55% humidity at Boston University, and colonies collected in Costa Rica (M1 and M2) were housed in an environmental chamber with a 12-h light:12-h dark regimen at 20°C and 50% humidity at the Museum of Science, Boston. Colonies were cultured in large plastic bins (30 cm × 46 cm × 28 cm) connected by plastic tubing and containing smaller plastic boxes (11 cm × 18 cm × 13 cm) to serve as fungal chambers. Colonies were provisioned with washed and pesticide-free frisée, arugula, baby spinach, romaine, and oatmeal flakes (primarily during cold seasons), and leaves from rhododendron, rose, beech trees, andromeda, oak, lilac, bramble, and willow (primarily during spring and summer).

2.1 | Brain sampling for gene expression

To obtain whole-brain mRNA sequences to assess patterns of brain gene expression in polymorphic workers, we prepared between nine and 11 samples from each of three worker size groups, composed of three to 10 pooled brains depending on worker size group, distributed across three colonies of origin, for a total of 30 samples (Table S1). Brains of mature, fully sclerotized workers categorized by size group as minims (0.5–0.7 mm in head width), medias (1.7–1.9 mm in head width), and majors (≥3 mm in head width) were sampled from three mature colonies (Ac22, M1, M2). We focused on adult workers because phenotypic gene expression differences persist or increase in adulthood in ants (Morandin et al., 2015), creating the potential to sequence samples with greater distinctiveness in expression profiles and minimize differences more to worker age than to worker subcaste identity. Furthermore, the expression of adult-biased genes show increased evolutionary rates potentially related to caste differentiation (Hunt et al., 2011). We therefore hypothesize that significantly differentially expressed genes (DEGs) identified in adults underpin social role variation among *A. cephalotes* polymorphic workers. We did not assess gene expression in larval and/or pupal brains because sampling immature stages in adequate numbers is prohibitively destructive to the limited number of colonies available.

2.2 | RNAseq sampling and library preparation

Workers were collected and snap-frozen for dissection only during 11:00–13:00 h to minimize potential influences of circadian rhythms on gene expression (Das & Bekker, 2022). Dissections were performed in RNase-away-cleaned petri dishes with dry ice-cooled ethanol (−20°C). First, the head capsule was separated from the body. To facilitate perfusion, dorsal cuticle was removed from the head to expose the brain and head capsules were perfused with RNALater-ice overnight at −20°C to stabilize RNA against freeze–thaw cycles and degradation. After incubation overnight, brains were removed under the same dissection conditions and immediately transferred into RNase-free tubes and stored in dry ice until transfer to −80°C freezer storage before extraction. Individual samples belonging to different worker size groups and colonies were distributed across extraction batches, library batches, and sequence lanes to mitigate confounding batch effects.

Total RNA was extracted from worker brains using a ThermoFisher PicoPure kit and a modified protocol for homogenization. Three to 10 brains were pooled into a single microcentrifuge tube for each sample during the dissection process, 50 μl of extraction buffer was added, brains were pulverized for 1 min using a sterile pestle attached to a Fisher Scientific motorized tissue grinder, an additional 50 μl of extraction buffer was added to rinse residual tissue from the pestle, and brains were incubated at 42°C for 30 min. The remainder of the extraction protocol was performed according to instructions for cell pellet samples. RNA was extracted from isolated brains rather than from whole heads to avoid obscuring brain gene expression profiles from

cephalic muscle and glandular tissue gene profiles. Sample quality and quantity, as well as lack of protein or DNA contaminants, were assessed using a Thermo Scientific Nanodrop spectrophotometer and an Agilent Bioanalyzer 2100, respectively.

Libraries were sequenced by Harvard BioPolymers using a combination of Illumina NextSeq and MiSeq with SE 75 reads. RNAseq unstranded libraries with mRNA poly-A selection were prepared using a KAPA mRNA HyperPrep kit. mRNA sequence libraries were individually barcoded and multiplexed in equal proportions and all libraries were sequenced across four lanes. mRNA rather than total RNA was sequenced because rRNA, if not removed, will dominate gene expression profiles. Furthermore, commonly used rRNA depletion methods, a less restrictive alternative to poly-A selection, are less effective for arthropods (Kumar et al., 2012).

2.3 | Transcriptome assembly

Transcriptome assembly was performed for the purpose of Benchmarking Universal Single-Copy Orthologs (*BUSCO*) quality assessment; differential gene expression was analyzed using pseudoalignment. Transcriptome assembly, annotation, and quality assessment were performed on Boston University's Shared Computing Cluster (100 Gb memory 16 CPU node). Read quality was confirmed using *FastQC* and reads were filtered and trimmed at the first base with mean quality score <20. Three of 30 samples (two from medias and one from majors) produced many fewer than anticipated reads (<1 million each compared with the average read count of 23 million per sample) and were therefore excluded prior to differential expression analysis, GO analysis, and gene coexpression network analysis. *Trimmomatic* (Bolger et al., 2014) was used to remove sequence adapters and remove leading and trailing bases with Phred quality scores below 3. The perl script "no shorts" (Green et al., 2014) was used to remove sequences below 500 bp. *Bowtie2* and *Tophat2* (Haas et al., 2013) were used to create a reference-guided transcriptome assembly, by leveraging the *A. cephalotes* reference genome (Suen et al., 2011), with a max mismatch value of two and a maximum intron length of 1000 base pairs (bp) and a minimum intron length of 20 bp. The resulting assembly was trimmed to remove contigs under 500 bp, and to find and remove residual contaminating rRNA by BLASTing against the SILVA LSU and SSU rRNA databases. Assembly contigs were then annotated using BLAST sequence homology searches against UniProt and Swiss-Prot protein databases. Quality-assessment was done using *BUSCO* with a reference set of highly conserved hymenopteran orthologs to confirm adequate representation of expected sequences (Simão et al., 2015).

2.4 | Differential gene expression analysis

Kallisto (Bray et al., 2016) was used to pseudoalign sequenced reads to the available transcriptome and to quantify transcript abundance. The *kallisto* index file was created using the *A. cephalotes* version 1.0 cDNA set accessed through Ensembl Metazoa Genomes (Howe et al., 2020). *DESeq2* (Love et al., 2014) was used to statistically assess the significance of differential gene expression based on transcript abundance counts generated by *kallisto*. Using *DESeq2*, pairwise comparisons for all sequenced genes (Love et al., 2014) were performed using modified and Benjamin Hochberg-corrected T-tests between libraries from different worker size groups to identify DEGs, and to compile data tables of normalized counts, log counts per million, log fold change (LFC), *p* values, and false discovery rate (FDR)-adjusted *p* values (Supplementary Table 2). The estimated FDR for nonadjusted *p* values for each pairwise comparison, determined using *qvalue* (Storey, 2015), was at or below 0.1, the default value used in *DESeq2*. We therefore used the default FDR setting in *DESeq2*. DEGs are defined in this study as genes with at least one adjusted *p* value < .05 in any of the three comparisons between worker size groups. Finally, we controlled for colony-level differences in gene expression by removing colony DEGs from the list of worker size-group DEGs and by including colony identity as a batch effect in the *DESeq2* experimental design (i.e., design = ~batch + condition; where the first term is controlled for and the second term is tested). We used *DESeq2* to calculate Cook's distance for each sample, a measure of the effects of removing a specific observation on regression analysis results (Cook, 1977) (Figure S1). This process did not reveal any outliers. For further discussion of outlier samples see Supplementary Materials (brain RNA sampling).

2.5 | Gene coexpression network analysis

Weighted gene coexpression network analysis (WGNCA) was performed using the *WGCNA* R package (Langfelder & Horvath, 2008). Read counts for all genes were used as input, rather than only DEGs. WCGNA is designed for unsupervised analysis due to its assumption of scale-free topology (Langfelder & Horvath, 2008). Therefore, we did not filter genes according to a significance cutoff for inclusion in this process. We employed the same experimental design structure as was used for differential expression analysis. After initial identification of coexpression modules (Figure S2a), a module merge cut height of 0.25 was applied to consolidate numerous undersized modules with similar expression patterns (Figure S2b). Genes with zero variance or more than four missing samples were excluded from analysis. An unsigned topology overlap matrix was used with a soft power value of four and a minimum module size of 30, which allowed a scale free topology model fit value of 0.8110. Genes are defined as belonging to a given module if their correlation value exceeds 0.8. GO enrichment analysis was performed on genes in the green yellow module using Fisher's exact test, otherwise adhering to the same settings used for overall GO term enrichment as previously described.

2.6 | Gene set enrichment analysis

BiomaRt was used to assign GO categories to all expressed genes in our data set (Smedley et al., 2009). The Mann–Whitney *U*-test-based gene set enrichment analysis protocol (GSEA) (Matz, 2015;

Wright et al., 2015) was used to test for overrepresented GO terms in the set of genes whose expression was measured. Negative log transformed raw *p* values from *DESeq2*, signed according to LFC, were used as the continuous measure of significance. GO MWU is designed to accept raw metrics as input (Matz, 2015). We therefore opted to use nonadjusted *p* values as our metric of significance for this analysis. GO categories were excluded from enrichment analysis if they contained 50% or more of the total genes analyzed. Categories were merged according to a cluster cut height of 0.75. A minimum of thirty was applied for the number of genes belonging to GO categories.

2.7 | Principal component analysis

Principal components analysis (PCA) was performed on all samples using the plotPCA function from the DESeq2 package in R (Love et al., 2014). PCA was calculated using [all expressed genes] and the 500 genes with the highest variance (regardless of significance). Variance stabilizing transformed normalized counts from DESeq2 were used to create a loadings plot via the prcomp function from the stats package in R (The R Core Team, 2016). This plot was limited to the 20 most influential genes for the purpose of data visualization. A PERMANOVA was performed to test for the significance of differences based on the expression of all genes among samples from different worker groups using the adonis function from the vegan package in R (Oksanen et al., 2020).

2.8 | Analysis of size-related directional gene expression

To identify genes whose expression followed worker growth patterns irrespective of statistical significance, we counted those with a higher LFC in medias compared with minims and in majors compared with medias, or for those negatively correlated to worker growth, those with a lower LFC in medias compared with minims and in majors compared with medias. We also removed this gene set from the list of significantly DEGs to observe how many nongrowth-related significant genes remained.

2.9 | Sequencing quality assessment

We sequenced an average of 23 million reads for each library across four lanes, resulting in 181 million reads for libraries from majors, 242 million reads for medias, and 252 million reads for minims. The total sum of all sequences across samples was 1234370832. A merged FASTQ from all files and samples had 40% GC content and an average sequence length of 75. An initial reference-guided assembly had an N50 of 2352. After applying no shorts (Green et al., 2014), 85.63% of sequences survived and the N50 was 2437. After de novo transcriptome assembly, BUSCO analysis showed relatively high levels of completeness for expected genes. With all samples merged into one assembly and using a hymenopteran reference set (containing 4415 expected transcripts), 74.5% (3290) of expected transcripts were complete, with 70.9% (3132) being present in single-copy and 3.6% (158) being duplicated, while 15.5% (683) of expected transcripts were fragmented and 10.0% (442) were missing. Using an Insecta reference set (containing 1658 expected transcripts), 88.9% (1474) of expected transcripts were complete, with 84.3% (1398) being present in single-copy and 4.6% (76) being duplicated, while 7.6% (126) of expected transcripts were fragmented and 3.5% (58) were missing. Kallisto indexing identified a k-mer length of 31 and generated de Bruijn graphs of 29319 contigs and 20823117 k-mers During quantification of read counts there were 11,098 targets, 20,823,117 k-mers, and 15,498 equivalence classes. For each sample library, approximately 27% of reads were successfully annotated through pseudoalignment to the reference cDNA set. Of the 11,098 genes identified in total, 8688 were matched to GO annotations.

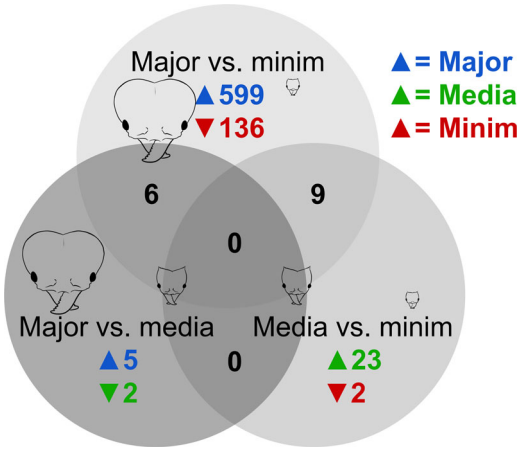


FIGURE 1 Venn diagram illustrating the number of differentially expressed genes (adjusted $p < .05$, colony effects modeled and colony DEGs removed) in pairwise comparisons among worker subcaste brains. DEGs in each pairwise comparison are denoted as upregulated and downregulated where upregulation indicates higher expression in the first worker group listed in the pair (blue = majors, green = medias, red = minims).

2.10 | Antibodies

We did not employ antibodies in this study.

3 | RESULTS

3.1 | Differential brain gene expression among worker size groups

Out of all measured genes (Tables S2), 963 unique genes were found to differ significantly in expression patterns among one or more worker size group pairwise comparisons (Figure 1 and Table S3). The largest number of significantly DEGs (947) was identified between minim and

major workers, with fewer DEGs between medias and majors (8) or minims and medias (45) (Table S4). After removing colony identity associated DEGs and modeling effects from colony identity, a total of 744 unique DEGs remained. The largest number of DEGs was again identified between minims and majors (735), with fewer DEGs distinguishing medias and majors (7) or minims and medias (25) (Figure 1). The number of DEGs identified under different filtering parameters using additional significance criteria and combinations of criteria are listed in Table S5.

3.2 | Significant gene coexpression module

WGCNA revealed five merged gene modules displaying similar patterns of expression within each module among worker groups (Figures 2 and S2b). The green yellow module was significantly negatively correlated with minims and significantly positively correlated with majors (Figure 2a).

3.3 | Enrichment of metabolism and sensory processing-related GO terms

In pairwise comparisons, GO analysis of the complete set of sequenced genes found 40 GO terms were significantly enriched between majors and minims (Figure 3a), 22 between majors and medias (Figure 3b), and 42 between medias and minims (Figure 3c). Notably numerous metabolic process GO terms were enriched among size classes in all three comparisons. Additionally, the *response to stimulus* GO term (GO:0050896) was significantly enriched between majors and minims and between majors and media ($p < .001$), and this GO term was more highly enriched in genes upregulated in both minims and medias compared with majors. The *sensory perception of taste* GO term (GO:0050909) was significantly enriched between majors and media ($p < .01$), being more highly enriched in genes upregulated in medias, and between majors and minims ($p < .01$), being more highly enriched in genes upregulated in minims.

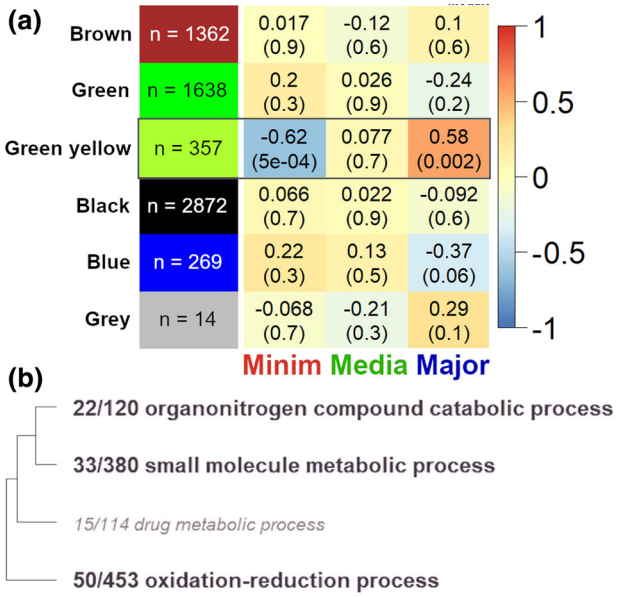


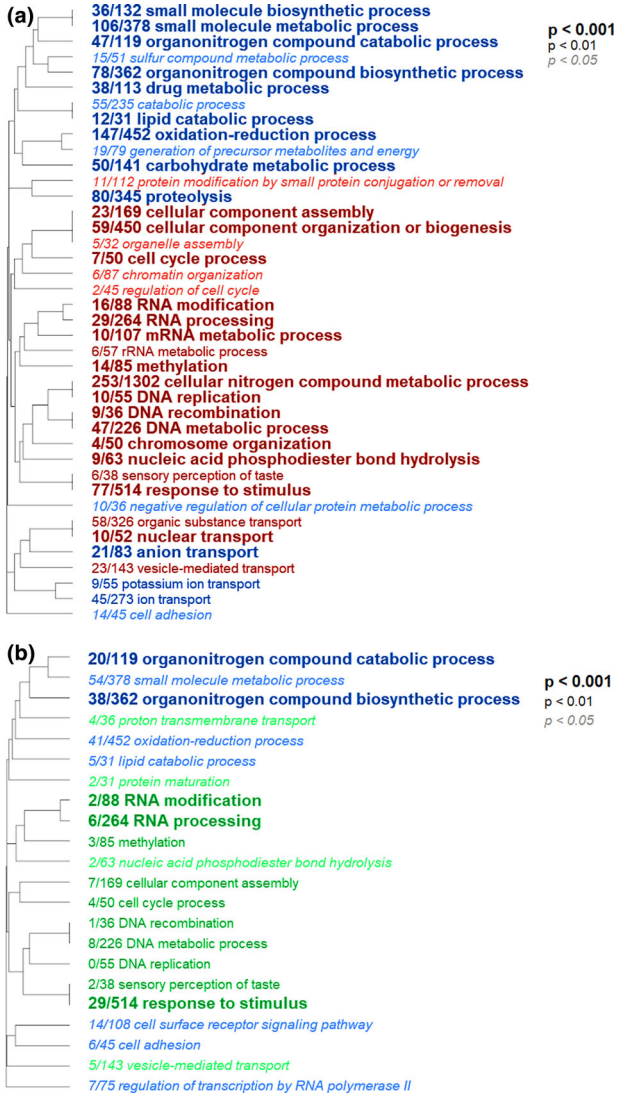
FIGURE 2 (a) WGCNA correlation matrix between identified modules of genes (color blocks) and sample traits of subcastes, with the number of genes in each module. *n* = sample size. Genes are included in the count listed on each block if their absolute correlation value to a module exceeded 0.8. Matrix indicates the signed correlation between traits and gene modules illustrated by color ranging from blue (−1) to red (1), the value indicated in each box. Bottom number indicates *p* value associated with the significance of the correlation of modules and traits. The green yellow module (significantly positively correlated to majors and significantly negatively correlated to minims) is bracketed in dark gray. (b) Comparison of Mann–Whitney *U*-test gene set enrichment analysis using Fisher's exact test for the green yellow gene coexpression module (significantly positively correlated to majors and significantly negatively correlated to minims) using biological process terms in pairwise comparisons. Fractions indicate the number of genes with a green yellow module correlation value 0.8 or greater in an enriched category over the total number of genes in the category. Font style and darkness indicate level of enrichment significance (Mann–Whitney *U*-test). Bold text in black indicates $p < .001$, plain text in black $p < .01$, and italics in gray $p < .05$. Clustering indicates similarity between GO terms.

3.4 | Statistical differentiation of expression profiles

PCA using the 500 genes with the highest variance showed substantial differentiation of samples belonging to different workers groups along PC1, which explained 45% of variance (Figure 4a). While PC1 position was strongly linked to worker size for each sample, indicating the significant influence of worker size on gene expression pattern, PC2, and other PCs, indicate forces influencing gene expression differences unrelated to size. Similarly, PCA of all genes showed strong differentiation of worker groups. In this case differences were highly attributable to PC2, which explained 22% of variance (Figure S3). A PERMANOVA showed a significant difference ($p = .021$) among worker groups. Of the top 20 loadings for the first two principal components, the genes most strongly contributing to PC1 included pheromone-binding protein Gp-9-like (although this did not significantly differ in expression between groups), and those most strongly contributing to PC2 included prohormone-3 and neuroglian (Figure 4b; full list in Table S6).

4 | DISCUSSION

Variation in behavioral performance demands among morphologically differentiated workers is associated with brain compartment allometries in *A. cephalotes* polymorphic workers (Muratore et al., 2022). Here, we found a strong influence of worker size on brain gene expression that may contribute to the differentiation of neural phenotypes.



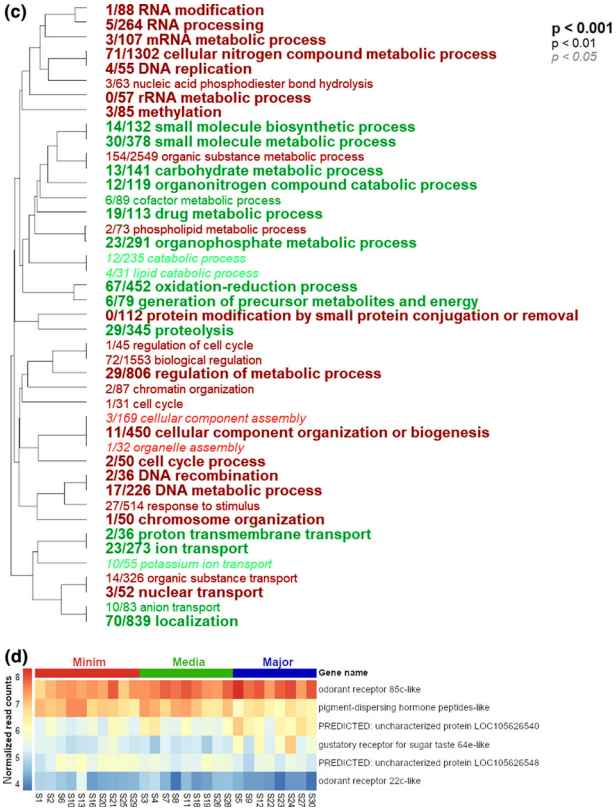


FIGURE 3 Gene set enrichment analysis using biological process terms in pairwise comparisons between (a) majors and minims, (b) majors and medias, and (c) medias and minims. Fractions indicate the number of genes with a nonadjusted $p < .05$ (raw rather than adjusted p values are used as input for the continuous measure of significance in this protocol) in an enriched category/total number of genes in the category. Font style and color intensity indicate level and direction of enrichment significance (Mann–Whitney U-test). Light red and dark red terms are upregulated in minims, light green and dark green terms are upregulated in medias, and light blue and dark blue terms are upregulated in majors, respectively, relative to the other worker subcastes in the pairwise comparison. Bold text in dark red, dark green, or dark red indicates $p < .001$, plain text in dark red, dark green, or dark red indicates $p < .01$, and italics in light red, light green, or light blue $p < .05$. Clustering indicates similarity between GO terms. (d) Expression heatmap normalized read counts of significant DEGs (excluding significant colony-related DEGs) with sensory-related GO terms (taste, smell, vision) with columns ordered by worker subcaste to illustrate differences. Color indicates the normalized read count of a given gene in each sample sequenced.

FIGURE 3 Continued

However, some DEGs did not show common patterns of increased expression in larger workers or common patterns of decreased expression in larger workers. Differential gene expression identified in our study could thus not be wholly explained by variation in body size, as we would expect if size determined the pattern of investment in individual neuropils in a purely concerted manner. Our results instead support a role for mosaic development mediated by parallel patterns of gene expression. Patterns of adaptive brain scaling in *A. cephalotes* correlated with portions of significant differences in brain gene expression among worker size groups. Our transcriptomic results thus support roles for both mosaic and size-constrained evolution in the differentiation and regulation of worker neural phenotypes and characterize molecular processes associated with division of labor based on physical castes.

Despite the uniquely high proportional investment in brain compartments such as the mushroom bodies in media workers relative to minims and majors (Muratore et al., 2022), medias were more similar to majors *and* minims in brain gene expression than either of these two more behaviorally specialized groups was to each other. This finding contrasts with patterns of neuropil investment across polymorphic workers. Interestingly, our results indicate that the plasticity involved in the maintenance and function of adult worker brains is influenced by differential expression specifically in genes that relate to differences in response to behavioral challenges driving selection for worker task specialization. This suggests that influences from brain developmental processes may be linked to specific behavioral and/or cognitive demands.

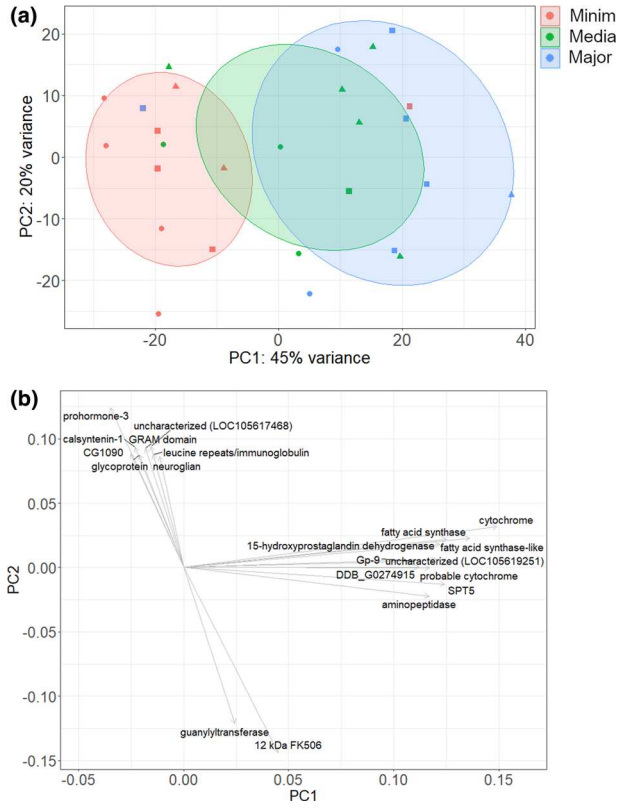


FIGURE 4 (a) Principal component analysis of gene expression using the top 500 genes with the highest variance (regardless of significance). Samples with exceptionally low read counts excluded ($n = 27$). Color indicates subcaste identity (red = minim, green = media, blue = major). Symbols indicate colony of origin (circle, square, and triangle correspond to three different colonies). Ellipses illustrate the 65% confidence interval for each worker group. PERMANOVA test of difference among worker groups $p = .021$. (b) Loading plot showing genes with the highest loading values (limited for readability) for principal components 1 and 2 (PC1, PC2) and the strength and directions of their effects.

Analysis of gene expression identified enriched GO terms containing DEGs related to sensory processing and aspects of metabolism, among other GO terms. Highly significant DEGs include genes related to neural development and sensory processing (see "Discussion of candidate genes of interest related to sensory processing and findings from other studies" in Supplementary Materials), suggesting transcriptomic regulation of brain compartment allometries among worker groups (Muratore et al., 2022). Our DEG set overlaps with several genes linked to behaviorally and morphologically differentiated worker subcastes and positive selection among *Atta*-group ants, potentially facilitating neuroanatomical specialization (Nygaard et al., 2016). Specific patterns of gene expression are discussed below.

4.1 | Gene expression patterns in relation to worker size

We observed a large difference in gene expression between minims and majors as well as many genes that increased in expression with worker size. However, removing these genes revealed that many significant DEGs remained, indicating that while a worker growth-related pattern describes the expression of a significant proportion of genes in this set, it is insufficient to explain the presence of differential brain gene expression among workers. The group of genes that increased in expression with worker size might be related in part to the worker size-related increase in optic lobe size (Arganda et al., 2020). The genes more highly expressed in medias relative to majors and minims, may be correlated with mushroom body and antennal lobe size, which are allometrically enlarged in *A. cephalotes* medias (Muratore et al., 2022). The small number of genes showing a downward trend in expression could correspond to the trend seen in the central complex, a brain compartment proportionally the largest in minims (Muratore et al., 2022).

4.2 | Weighted gene coexpression analysis

Further exploration of our gene expression data using WGCNA identified one coexpression module (green yellow), which showed a trend of correlation with worker size (Figure 2b) that was significantly positive in majors and significantly negative in minims. Enriched GO terms in this module related to metabolic processes (Figure 2b), consistent with our GO enrichment results, and contained several genes related to neurotransmission, steroid hormone activity, as well as nucleic acid binding and regulation. Therefore, it appears likely that metabolic processes scale in a size-determined manner, consistent with other findings (Coto & Traniello, 2022).

4.3 | Gene set enrichment

Our GO analysis of the data set as a whole revealed that two highly represented GO term categories, responsiveness to stimuli and metabolism, were significantly enriched, suggesting both of these gene functions likely are important in the differentiation of subcaste neural phenotypes. Metabolism-associated genes have previously been implicated in ant behavior and caste identity (Feldmeyer et al., 2014; Chandra et al., 2018; Friedman et al., 2018; Liutkevičiūte et al., 2018), honey bee worker age-related task transitions (Whitfield et al., 2003; Ament et al., 2008), and responsiveness to stimuli (Ben-Shahar et al., 2003; Ingram et al., 2005, 2011; Lucas & Sokolowski, 2009; Oettler et al., 2015; Bockoven et al., 2017; Trible & Kronauer, 2017; Yan et al., 2017). Metabolic processes appear to have changed with the transition to higher agriculture in the neoattini, for example, the loss of arginine biosynthesis (Suen et al., 2011) and up-and-down shifts in colony metabolism related to fungal-garden energy storage (Shik et al., 2014). The direction of expression with respect to subcaste varies across metabolic GO terms, although DNA metabolism in general was most enriched in genes upregulated in minims whereas carbohydrate metabolism was typically more enriched in genes upregulated in majors and medias. We also found several enriched terms related to regulation of DNA/RNA, epigenetics, and cell developmental organization

(Figure 3d). The *response to stimulus* GO term (GO:0050896) was significantly enriched between majors and minims and between majors and media ($p < .001$) and was more highly enriched in genes upregulated in both minims and medias as compared with majors. A more narrowly defined stimulus response GO term—*sensory perception of taste* (GO:0050909)—was significantly enriched between majors and medias (more highly enriched in genes upregulated in medias), and between majors and minims (more highly enriched in genes upregulated in minims). Such chemosensory processes appear to be more significant to the work performed by minims and medias, which includes interpreting cues from the fungal garden (Green & Kooij, 2018) and selecting appropriate plant substrates (Howard, 1987), respectively, than the defensive behavior of majors.

4.4 | Principal components linked to worker size

Samples from different worker groups were well differentiated by PCA (Figures 4a and S3). Genes most strongly contributing to PC1 and PC2 included some related to metabolic processes, hormone transmission, transcription, and chemical communication (Table S6). This latter category included pheromone-binding protein Gp-9-like, likely a homolog of a gene important in the regulation of social organization in fire ants (Ross & Keller, 1998; Krieger & Ross, 2002). Interestingly, pheromone-binding protein Gp-9-like increases in expression with worker size, which may regulate intruder recognition and defense by large workers (Krieger & Ross, 2002). Neuroglian, a gene that regulates mushroom body axon development in flies (Goossens et al., 2011; Yang et al., 2019), also contributed strongly to PC2. This gene may therefore regulate allometric scaling in *A. cephalotes* mushroom bodies. Furthermore, several DEGs (*carboxypeptidase B-like*, *G-protein coupled receptor moody*, *sialin*) also overlapped with those identified as undergoing positive selection in *Atta*-group ants (Nygaard et al., 2016). These genes may serve a novel role in regulating brain plasticity associating with the proliferation of polymorphic workers.

4.5 | Transcriptomic versus neuroanatomical reaction norms

The overall pattern of increasing gene expression with worker size suggests an association of intermediate doses of certain genes with novel features, including broader task repertoires and the disproportionate investment in antennal lobes and mushroom bodies identified in medias (Muratore et al., 2022). If allometric scaling of these neuropils is determined by expression levels of brain genes, then some genes seem capable of influencing the largest proportional investment in these tissues when expressed at intermediate levels. Similarly, the enlargement of the central complex in minims could result either from a low dose of certain genes catalyzing investment in this compartment or from the action of a small portion of the genes that were more highly expressed in minims than in majors or medias. However, given the significant worker size-correlated expression patterns in our gene set, the possibility of an indirect translation between a gene's expression level and its impact on neuropil size deserves more study.

5 | CONCLUSION

We identified strong differential gene expression among behaviorally and neuroanatomically variable *A. cephalotes* polymorphic workers. These divergent transcriptomic patterns suggest that selection has acted on genes affecting total brain size in a strongly body-size-linked manner as well as loci affecting differential investment in brain compartments resulting in mosaic compartmental allometries. Identifying whether brain gene expression differences regulate task specialization and neuroanatomy in socially complex ants or result from differential task experience and/or differential doses of transcripts guiding neuropil allometries will further characterize functional correlates of division of labor.

AUTHOR CONTRIBUTIONS

I. B. M., S. P. M., and J. F. A. T. designed the study. I. B. M. prepared and quality assessed samples for RNAseq. I. B. M. analyzed RNA sequences. I. B. M. drafted the manuscript. I. B. M., S. P. M., and J. F. A. T. edited the manuscript. J. F. A. T. and S. P. M. secured funding.

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CONFLICT OF INTEREST STATEMENT

We declare that all authors have no competing interests relevant to this study.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in the following repositories: Merged FASTA sequence data from all samples used for *kallisto* pseudoalignment, raw cDNA counts, expression data for all genes in each pairwise comparison (Table S2), DEGs for each pairwise comparison with redundant genes removed (Table

S3), DEGs for each pairwise comparison including genes significant in more than one comparison (Table S4), and colony DEGs available via Dryad: https://doi.org/10.5061/dryad.05qfttf3c. Code used in analysis is available via: https://github.com/lmuratore-bio/ant-transcriptomics

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PEER REVIEW

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ETHICS STATEMENT

We collected incipient colonies of *A. cephalotes*, a nonendangered species, from Trinidad in compliance with the laws of Trinidad and Tobago. We imported and cultured colonies in compliance with USA laws under USDA APHIS Permit P526P-12-04067. We reared all ant colonies under constant photoperiod in environmental chambers under conditions that promote growth and health by maintaining high humidity and temperature to simulate a tropical environment. We housed individual colonies in multiple connected plastic boxes to serve as fungal growth chambers and provided plant material in a foraging arena. We regularly supplied colonies with leaves of diverse plant species to provide substrate for the fungal comb and nutritional variety. We avoided disturbing colonies apart from provisioning times and during the collection of workers for study. Environmental chamber doors were protected with seals and colonies were contained using Fluon® coating on nest boxes and oil traps to prevent escape. Facilities were routinely inspected by the USDA. We balanced sampling ($n = 9$–30 individuals per worker group; all sterile females) while minimizing impact on colony populations to ensure adequate RNA yield. To measure whole-brain gene expression, we cold-anaesthetized and humanely sacrificed workers.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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