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Changes in gene DNA methylation and expression networks accompany caste specialization  
and age-related physiological changes in a social insect

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**Abstract**

Social insects provide systems for studying epigenetic regulation of phenotypes, particularly with respect to differentiation of reproductive and worker castes, which typically arise from a common genetic background. The role of gene expression in caste specialization has been extensively studied, but the role of DNA methylation remains controversial. Here, we perform well-replicated, integrated analyses of DNA methylation and gene expression in brains of an ant (*Formica exsecta*) with distinct female castes using traditional approaches (tests of differential methylation) combined with a novel approach (analysis of co-expression and co-methylation networks). We found differences in expression and methylation profiles between workers and queens at different life stages, as well as some overlap between DNA methylation and expression at the functional level. Large portions of the transcriptome and methylome are organized into ‘modules’ of genes, some significantly associated with phenotypic traits of castes and developmental stages. Several gene co-expression modules are preserved in co-methylation

networks, consistent with possible regulation of caste-specific gene expression by DNA methylation. Surprisingly, brain co-expression modules were highly preserved when compared with a previous study that examined whole-body co-expression patterns in 16 ant species (Morandin et al. 2016), suggesting that these modules are evolutionarily conserved and for specific functions in various tissues. Altogether, these results suggest that DNA methylation participates in regulation of caste specialization and age-related physiological changes in social insects.

### **Keywords**

Co-methylation network, co-expression network, ageing, phenotypic plasticity, caste

### **Introduction**

DNA methylation is the most studied epigenetic mechanism and has been linked to variation in gene regulation in mammals (Maunakea et al., 2010; Shukla et al., 2011), plants (Ecker & Davis, 1986; Zemach, Mcdaniel, Silva, & Zilberman, 2010; Zilberman, Coleman-Derr, Ballinger, & Henikoff, 2008), and insects (Bewick, Vogel, Moore, & Schmitz, 2017; Kucharski, Maleszka, Foret, & Maleszka, 2008; Li-Byarlay et al., 2013). DNA methylation regulates a wide range of cellular processes, such as development or disease (Jirtle & Skinner, 2007; Lister et al., 2009; Waterland & Jirtle, 2003; Wolffe & Matzke, 1999) and has been shown to affect processes such as gene expression (Keshet, Yisraeli, & Cedar, 1985; Tate & Bird, 1993), genomic imprinting (E. Li, Beard, & Jaenisch, 1993; Razin & Cedar, 1991), and transcriptional regulation (Eden & Cedar, 1994; Schübeler, 2015).

Biological characteristics, functions, localization, and even presence of DNA methylation vary greatly among taxonomic lineages (Colot & Rossignol, 1999). For example, in mammals, DNA methylation primarily occurs at CpG dinucleotides (Cheng & Blumenthal, 2008), with 60% to 90% of all CpG sites methylated (Bird, 1986; Lister et al., 2009). DNA methylation is accomplished by DNA methyltransferase 3 (DNMT3) and persists due to the activity of the maintenance methyltransferase, DNMT1 (Cheng & Blumenthal, 2008; Goll & Bestor, 2005; Kim, Samaranyake, & Pradhan, 2009). In mammals, DNA methylation typically occurs in gene promoter regions, where it represses gene transcription (Bird & Wolffe, 1999; Suzuki & Bird, 2008; Weber et al., 2007). In insect species with identified DNA methylation machinery, DNA methylation is predominantly found in coding regions and located in gene bodies of actively expressed genes (Bonasio et al., 2012; Feng et al., 2010; Lyko et al., 2010; Suzuki & Bird, 2008; Zemach et al., 2010). Gene body DNA methylation in insects correlates with alternative splicing and may modulate gene activities (Bonasio et al., 2012; Flores et al., 2012; Foret et al., 2012; Libbrecht, Oxley, Keller, Jan, & Kronauer, 2016; Lyko et al., 2010), or even affect gene function through nucleosome stability (Hunt, Glastad, Yi, & Goodisman, 2013a). This suggests that gene body DNA methylation may be involved in a wide range of biological processes, and that it may be involved in evolution of novel traits, through mechanisms such as genomic imprinting (Amarasinghe, Clayton, & Mallon, 2014). Furthermore, invertebrate genomes often display lower levels of DNA methylation than those of mammals or plants, ranging from 0% (the order Diptera) to 14% of all CpG sites (Asian cockroaches, *Blattella asahinai*) (Bewick et al., 2016). In the honey bee (*Apis mellifera*), a model species for social insect genomic analyses, less than 1% of CpG dinucleotides are methylated (Lyko et al., 2010). Furthermore, in some insect species (e.g. *Aedes aegypti*) no evidence of DNA methylation has been found. In these species, the responsible machinery (cytosine-5 DNA methyltransferases) is absent from their genomes (Falckenhayn et al., 2016; Standage, Berens, Glastad, & Severin, 2016). When present, DNA methylation in insects contributes to diverse processes, such as nutritional control of reproductive status (Kucharski et al., 2008), development (Lyko et al., 2010; Shi, Yan, & Huang,

2013; Yang, Guo, Zhao, Sun, & Hong, 2017), embryogenesis (Kay, Skowronski, & Hunt, 2017), alternative splicing (Bonasio et al., 2012; Flores et al., 2012; Foret et al., 2012; Li-Byarlay et al., 2013; Libbrecht et al., 2016), host-parasite evolution (Vilcinskas, 2016), memory processing (Biergans, Jones, Treiber, Galizia, & Szyszka, 2012; Lockett, Helliwell, & Maleszka, 2010), age-related changes in worker behavior (Herb et al., 2012), modulation of context-dependent gene expression (Wedd, Kucharski, & Maleszka, 2016), maternal care (Arsenault, Hunt, & Rehan, 2018), and defense against territorial intrusion (Herb, Shook, Fields, & Robinson, 2018). Perhaps most dramatically, in social insects, DNA methylation has been proposed to control the developmental path taken by a totipotent egg to either a reproductive queen or a non-reproductive worker (Herb et al., 2012; Kucharski et al., 2008; Yan, Bonasio, Simola, & Berger, 2015). Despite a common genetic background, queen and worker castes acquire extensive behavioral, physiological and morphological differences, such as vast differences in lifespan, which can be on the order of decades (Page & Peng, 2001). Because epigenetic regulation has been proposed as a key mechanism in gene and environment interactions (Liu, Li, & Tollefsbol, 2008), social insects provide an ideal model to investigate the function of DNA methylation on the development of alternative phenotypes (Lyko & Maleszka, 2011).

Since its initial discovery in honey bees (Wang et al., 2007), the role of DNA methylation in regulating caste specification has been controversial. On one hand, many studies have reported DNA methylation differences between female castes [(honey bees: Elango, Hunt, Goodisman, & Yi, 2009; Foret et al., 2012; Kucharski et al., 2008; Lyko et al., 2010) (ants: Bonasio et al., 2012) (bumblebees: Amarasinghe et al., 2014) (termites: Glastad, Hunt, & Goodisman, 2012; Glastad, Gokhale, Liebig, & Goodisman, 2016)] and (non-social Jewel wasps, *Nasonia vitripennis*: Beeler et al., 2014), while others found no effect of DNA methylation on caste regulation (Libbrecht et al., 2016; Patalano et al., 2015). Several factors may explain these discrepancies. For instance, studies that failed to detect significant DNA methylation differences compared reproductive and non-reproductive individuals with similar morphology (Libbrecht et al., 2016; Patalano et al.,

2015). Also, most previous studies did not employ appropriate replication, and those that did, failed to find significant differences between castes (Herb et al., 2012; Libbrecht et al., 2016; Patalano et al., 2015). Thus, it is still unclear whether the lack of biological replicates or the lack of distinct morphological castes explains the inconsistency among studies. Furthermore, only two previous studies have used whole-genome sequencing to investigate DNA methylation pattern differences between adult queen and worker brains in honey bees. Here again discrepancies arise. Lyko et al. (2010), with no technical or biological replicates, found around 600 genes differentially methylated between castes, while Herb et al. (2012), with five replicates, found no significant differences in DNA methylation between irreversible workers and queens.

Thus, to explore the role of DNA methylation on social insect caste and on individual traits important in social organization, a study employing a suitable number of biological replicates and a model system with clear caste differences was needed. To address this matter, we used the ant, *Formica exsecta*, to study changes in brain DNA methylation and gene expression associated with the two female castes. *F. exsecta* has morphologically differentiated castes, with queens living as long as 20 years, while workers have lifespans slightly over 1 year, including a winter hibernation (Pamilo, 1991). Thus, *F. exsecta* provides an extreme contrast in caste physiology and lifespan, especially during the adult stage. In a previous study of the *F. exsecta* transcriptome (Morandin et al., 2015), we found differential expression of DNA methyltransferase 3 (DNMT3, up-regulated in adult workers compared to queens), an enzyme responsible for establishing *de novo* DNA methylation patterns in mammalian genomes (Hata, Okano, Lei, & Li, 2002; Kato et al., 2007; Okano, Bell, Haber, & Li, 1999; Okano, Xie, & Li, 1998), which also affects caste development in honey bees (Kucharski et al., 2008). We hypothesized that DNA methylation states may differ between ant castes, either as a result of differential DNA methylation during larval development, or because of re-programming as adults. To test this

hypothesis, we sampled queens and workers at two adult developmental stages, soon after emergence from the cocoon and well after establishment in specific roles (foraging and social behavior vs. reproduction). We then tested predictions that (a) both DNA methylation and gene expression should differ between these stages; (b) if differences exist, expression and DNA methylation signals would show some level of correspondence; and (c) adults within a caste show persistent differences throughout life, suggesting an action of DNA methylation beyond larval development. While our experimental design cannot prove causality between DNA methylation and caste differentiation, it can show that caste-specific DNA methylation patterns have the potential to underpin differences in caste and adult development, hopefully spurring further functional investigation.

Here, for the first time in social insects, we examined the relationships between networks of co-expressed and co-methylated genes. Co-methylation networks were first used to describe relationships among methylation profiles (Busch et al., 2016; Eijk et al., 2012; Horvath et al., 2012, 2016; Rickabaugh et al., 2015). Co-methylation analysis relies on the fact that adjacent CpG sites can be co-methylated due to locally coordinated activities of methyltransferases or demethylases. Weighted network methods (such as WGCNA) can be used on any high-throughput, continuous, or semi-continuous datasets and preserves the continuous nature of co-methylation information (Langfelder & Horvath, 2008; B. Zhang & Horvath, 2005) by grouping highly correlated DNA methylation profiles into modules of genes. The overall DNA methylation level of genes clustered in a module can be represented by the module eigengene (Langfelder & Horvath, 2007; Langfelder, Mischel, & Horvath, 2013), which can later be correlated with several phenotypic traits. In addition, robust preservation statistics are also implemented in WGCNA and can be used to detect connections between modules of co-expressed and co-methylated genes (Langfelder, Luo, Oldham, & Horvath, 2011).

Using DNA methylomes and expression profiling of individual queen and worker brains, we find a number of differentially methylated genes and CpG sites associated with either caste or developmental stages (newly emerged vs. old). In parallel, we find that the transcriptome and methylome can be partitioned into conserved modules of co-expressed and co-methylated genes, which are associated with caste and age-related physiological changes. Furthermore, some methylation modules are preserved in the gene expression data, consistent with a possible regulatory role of DNA methylation.

## **Materials and Methods**

### **Sample collection and brain extractions**

All samples of *F. exsecta* were collected from colonies around the Tvärminne Zoological Station in the Hanko Peninsula, southwestern Finland, in the spring of 2013. Old adult queens and old adult workers were collected in April, when ants come to the colony surface for warmth, providing the only opportunity to easily collect egg-laying queens in the wild. The age of overwintered queens could not be controlled; however, they were all found in large mature colonies and were physogastric at the time of sampling (i.e., with greatly enlarged gasters due to egg production). At this time of year, all workers have overwintered once and are reaching the ends of their lives. Emerging queens were collected in June and emerging workers in July, right after they emerged from their cocoons. Samples were collected randomly from 19 colonies in close proximity, without bias toward specific ages or castes. After collection, samples were frozen immediately at -80 °C. Brains were dissected on ice and stored in 180 µL buffer ATL and 20 µL proteinase K overnight (DNA, QIAamp DNA Micro Kit, Qiagen) or 350 µL buffer RLT (RNA, RNeasy Micro Kit, Qiagen) for further extractions.



### ***Formica exsecta* genome data**

*Formica exsecta* genome sequences and annotation (NCBI BioProject ID PRJNA393850 and BioSample: SAMN07344805) were obtained pre-publication from the authors (Dhaygude, Nair, Johansson, Wurm, & Sundström, 2018). In brief, the genome assembly consists of 14,617 contigs and scaffolds comprising a total of 278 Mb with an overall GC content of 36%. Annotation of the genome reported 13,637 protein-coding genes, labeled FEX0000001 to FEX0013637. Functional annotation was not provided, and classification of gene models for this study was accomplished using BLAST ([blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)) (Altschul, Gish, Miller, Myers, & Lipman, 1990) and Blast2Go ([www.blast2go.com/](http://www.blast2go.com/)) (Conesa et al., 2005) searches, as described below.

### **DNA methylation analysis**

**DNA extraction, reduced representation bisulfite sequencing (RRBS) library preparation, and sequencing.** RRBS was performed on 24 libraries from single ant brains (6 emerging queens, 5 old queens, 6 emerging workers, 7 old workers), representing biological replicates of each caste and developmental stage. Total DNA from each brain was extracted using a DNA micro kit (QIAGEN) and diluted in 20 µL of buffer AE (QIAGEN). Concentration and quality of extracted DNA was examined with an Agilent 2100 bioanalyzer (Agilent Technologies).

*Restriction enzyme digestion.* DNA was digested with two enzymes (MspI and TaqI) prior to bisulfite conversion (Gu et al., 2011). The following procedure was carried out for each enzyme separately before combining samples at a later stage. First, 50 ng of DNA were mixed with 2 µL of 10 x T4 DNA ligase buffer, 1 µL unmethylated lambda DNA (0.45 ng/µL), 0.5 µL of the enzyme (20 U/µL), and distilled H<sub>2</sub>O up to 20 µL. The mixture was then incubated at 37 °C overnight.

*Adaptor ligation.* 1.25  $\mu\text{L}$  of methylated adaptor (15  $\mu\text{M}$ ), 0.5  $\mu\text{L}$  of 10 x T4 DNA ligase buffer, 1  $\mu\text{L}$  of T4 DNA ligase (2,000 U/ $\mu\text{L}$ ), and 2.25  $\mu\text{L}$  of distilled  $\text{H}_2\text{O}$  were added to each mixture.

Sequences of the methylated adaptors can be found in Table S1. The mixture was incubated at 4  $^\circ\text{C}$  overnight.

*Size selection.* Complete details of the size selection step can be found in Tan & Mikheyev (2016). In brief, in the first selection step, 100  $\mu\text{L}$  of 13% PEG-6000/NaCl/Tris and 10  $\mu\text{L}$  prepared Dynabeads were added to the mixture and resuspended. The mixture was incubated for 5 minutes and placed on a magnetic stand for 5 minutes. The supernatant (150  $\mu\text{L}$ ) was transferred to a new tube, and the beads were discarded. In the second selection step to select fragments between 200 and 400bp, 100  $\mu\text{L}$  of 13.5 % PEG-6000/NaCl/Tris and 10  $\mu\text{L}$  prepared Dynabeads were added to the supernatant and mixed. The mixture was incubated for 5 minutes followed by bead separation on a magnetic stand. This time, the supernatant was discarded, and the beads were collected. The beads were washed twice with 70% ethanol (with 10 mM Tris, pH 6) and dried for 5 minutes. The tubes were then taken off the magnetic stand, and DNA was eluted from the beads by resuspending them in 15  $\mu\text{L}$  EB.

*Bisulfite conversion.* Unmethylated cytosines were converted to uracils using the Qiagen EpiTect Bisulfite Kit. The kit was used twice on each sample following the manufacturer's instructions.

*Library amplification.* Bisulfite libraries were synthesized with a limited number of PCR cycles (20). The 50  $\mu\text{L}$  PCR reaction consisted of 5  $\mu\text{L}$  of the bisulfite-converted genomic DNA from the previous step combined with 5  $\mu\text{L}$  of 10 x Advantage 2 PCR buffer (Clontech), 1.25  $\mu\text{L}$  of 10 mM dNTP mix, 2.5  $\mu\text{L}$  of 5  $\mu\text{M}$  Illumina primer, 1  $\mu\text{L}$  of 50x Advantage 2 DNA Polymerase (Clontech) and 35.25  $\mu\text{L}$  of distilled  $\text{H}_2\text{O}$ . 50- $\mu\text{L}$  PCR reactions were set up for each bisulfite-converted DNA sample. PCR reactions were carried out under the following conditions: initial denaturation at

95 °C for 1 minute, with 20 cycles of denaturation at 98 °C for 10 seconds, 65 °C for 1 min, followed by final extension at 72 °C for 5 minutes. PCR products were purified by solid phase reversible immobilization using Dynabeads MyOne Carboxylic Acid (Invitrogen). We followed the above protocol using 14.5% PEG for purification. Prior to pooling, libraries were analyzed with a Bioanalyzer High-Sensitivity DNA Kit (Agilent Technologies). The quantity of the library was estimated using a Quant-iT PicoGreen dsDNA Assay Kit and libraries were pooled. Quantitative PCR (KAPA Biosystems) was used to estimate library concentrations. Pooled libraries were sequenced single-end for 50 cycles (1 x 50bp) on an Illumina Hiseq 2000 system at the Okinawa Institute of Science and Technology. Adaptors were removed from the raw reads. Subsequently the reads were parsed through quality filtration (Trimmomatic (Bolger, Lohse, & Usadel, 2014), options: MAXINFO:40:0.8 MINLEN:10). Read quality was inspected with FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Raw sequences were submitted to the DDBJ database (see Table S2 for the accession numbers), ranging from 198 Mb to 802 Mb of total sequence data per sample. Median trimmed read length for the samples ranged between 33 and 46 nucleotides. Mapping efficiencies (next section) ranged between 64.3% and 73.3%, with median 69.5% (Table S2). To estimate the number of genomic cytosines accessible to mapping after the RRBS protocol of fragmentation and size selection, we compared whole genome mapping efficiencies with mapping efficiencies obtained against in silico fragmented genomes. Results showed that mapping against fragments in the 50-440-bp range gave almost the same mapping efficiencies as for mapping against the whole genome. Based on this result, we estimate that about 11 million of the 24.5 million genomic CpGs were accessible with our RRBS protocol.

**Computational processing of BS-seq data.** Individual read samples were mapped to the *Formica exsecta* genome and DNA methylation calls were tallied using Bismark (Krueger & Andrews, 2011) with the BWASP workflow (<https://github.com/brendelgroup/BWASP>), modified for RRBS data. In brief, BWASP is a workflow-enabling wrapper for Bismark, a BS-seq analysis tools that executes read and mapping quality control and produces sets of highly-supported DNA methylation (hsm) sites. hsm sites are a subset of sufficiently covered sites (scd), i.e. genomic cytosines covered by enough reads to assess statistically significant DNA methylation at that site. Requisite calculations are done in the BWASP Creport2Cxreport.py and Cxreport2hsm.py scripts. Based on an assumed bisulfite conversion accuracy of 99.5%, a binomial test is performed to determine whether the observed DNA methylation frequency at a given site can be rejected as a chance event (1% significance level, Bonferroni adjusted). scd sites are sites with sufficient coverage (here: 4 reads; Table S3) to allow detection of significant DNA methylation, and hsm positions are where significant DNA methylation occurs (e.g., 4/4 DNA methylation calls). Overall levels of DNA methylation in CpG, CHG, and CHH sequence contexts were estimated from mapped reads with BWASPR, as well as mean CpG methylation levels of introns, exons, 5' UTRs, 3' UTRs and intergenic regions. We also calculated and reported per-gene CpG methylation levels for queens, workers, emerging, and old samples. Samples were compared only on the basis of sites that were covered in both samples when replicates were averaged. Analysis of these sets of sites was done with a set of R functions that are available in the BWASPR package (<https://github.com/brendelgroup/BWASPR>). A typical BWASPR workflow reads the specified mcalls files (produced by Bismark) and generates various output tables and plots, including differential DNA methylation analysis, as described briefly below. Entire workflows are reproducible on any Linux system, following installation of the packages. For convenience, we made all workflow documentation and scripts available on the bgRAMOSE virtual machine (VM) image on the Jetstream scientific cloud computing platform (<https://jetstream-cloud.org>). Users can deploy an example of this VM image and can follow instructions from `/usr/local/share/bgRAMOSE/MBSHM2018/0README` to recap all of

our DNA methylation analysis workflows with a few keystrokes. Bismark's sam file output (mcall) was used as input to methylKit (Akalin et al., 2012) and data were imported using the function read.bismark. Differentially methylated CpG sites were determined with BWASPR using logistic regression implemented in MethylKit (Akalin et al., 2012) from calls using the functions methylKit:calculateDiffMeth() and getMethylDiff. For site-level analysis, we discarded CpG sites covered by fewer than 10 reads, and we considered sites differentially methylated if they showed 25% methylation differences and a qvalue of less than 0.01. pvalues were adjusted to qvalues to account for multiple testing using the SLIM method. At the gene level, differentially methylated gene lists for all four comparisons (OQ vs. OW; EQ vs. EW; OQ vs. EQ; OW vs. EW) were compared among samples with the Wilcoxon paired ranked sign test, applied only to genes of at most 20 kb and with at least two differentially methylated sites (restrictions applied to focus on the genes with highest concentration of sites).

**Weighted correlation network analysis of DNA methylation.** We employed weighted co-methylation networks analysis using the R package WGCNA (version 1.61.86, (Langfelder & Horvath, 2008)) to find weighted signed co-methylated sets of genes (modules) associated with caste and/or developmental stages, an approach analogous to that employed for gene expression data. The goal of our network analysis was to 1) identify sets of co-methylated genes (modules), 2) calculate module eigengenes (i.e., representative values for each module), and 3) correlate module eigengenes with phenotypes of interest (caste and stage). WGCNA identifies modules of co-methylated genes starting at the level of DNA methylation and correlates these modules to phenotypic traits. The network is created purely by gene DNA methylation levels and does not require genes to be classified into binary categories (i.e., whether a gene is methylated or not), as is typical for gene-level differential DNA methylation tests. Thus, it overcomes the problem of multiple comparisons. The input dataset (Table S4) consisted of results from DNA methylation calls of 12,112 genes measured as the average percentage of CpG methylation per site per gene, restricted to sites with highly-supported methylation data (high

coverage, at least 10 reads). These proportional levels control for the number of restriction enzyme sites present on each gene, gene length, and quality of mapping to avoid any biases. WGCNA can be used on any high-throughput continuous or semi-continuous data, and can calculate correlations from proportional DNA methylation data without requiring normalization for gene length. Our dataset was first filtered to remove genes with too many missing values, following WGCNA cutoff threshold recommendations using the function `goodSamplesGenes` (Langfelder & Horvath, 2008). After considering a range of soft thresholding power (10 to 30), a power of 20 was chosen based on the criterion of approximate scale-free topology and  $R^2$ . After calculating topological overlap values for all pairs of genes, a hierarchical clustering algorithm identifies modules of highly interconnected genes. To define modules of co-methylated genes, we used average linkage hierarchical clustering with the topological overlap-based dissimilarity measure. Subsequently, modules of highly co-methylated genes were merged using a cut-off value of 0.45. The minimum module size was set to 30 (Langfelder & Horvath, 2008). As detailed in Morandin et al. (2016), we next calculated average signed, normalized gene DNA methylation values (called an 'eigengene') to determine the relationship between modules and phenotypic traits (e.g., caste and developmental stage). The eigengene is defined as the first principal component of a module and represents the gene DNA methylation profile. For each module, the eigengene can be used to define a measure of module membership, which indicates how close a DNA methylation profile is to the module. A general linear model was then used to find the association between external phenotypic traits (caste and developmental stage, and their interaction) and modules' eigengenes. The general linear model approach provides a convenient means of testing the correlation of multiple traits with module eigengenes using a single model-relating eigengene of expression or DNA methylation modules to caste phenotype and stage. We used the `glm` function in R with 1000 bootstrap pseudoreplicates, with caste and stage as the explanatory variables and their interactions. p-values were FDR-corrected to account for multiple testing.

## Gene expression analysis

**RNA extraction, cDNA synthesis, and library preparation.** Four independent replicates for each caste (queen, worker) and developmental stage (emerging, old), using single brains, were used in this study. Total RNA from each brain was extracted using an RNeasy® micro kit (QIAGEN) and diluted in 14 µL of RNase-free water. Concentrations and qualities of extracted RNA were examined with an Agilent 2100 bioanalyzer (Agilent Technologies). Total input RNA was standardized to 100 ng prior to cDNA synthesis. cDNA synthesis and library preparation were done following an in-house protocol (Aird et al., 2013). Libraries were analyzed with a Bioanalyzer High-Sensitivity DNA Kit (Agilent Technologies). Library quantities were estimated with a Quant-iT PicoGreen dsDNA Assay Kit and equimolar concentrations of libraries were pooled. Quantitative PCR (KAPA Biosystems) was used to estimate the concentration of the libraries. Pooled libraries were sequenced paired-end with an Illumina NextSeq High Output 2 x 150 bp (400M PE reads) at the FuGU lab in Helsinki (Finland).

**Read mapping and differential expression analysis.** Raw read quality was assessed with FastQC tools (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc>), and adaptor sequences were removed using cutadapt (Martin, 2011). Raw reads were subsequently parsed through quality filtration (Trimmomatic (Bolger et al., 2014), options: MAXINFO:40:0.8 MINLEN:30), and aligned to the reference *F. exsecta* genome (BioProject ID PRJNA393850, Dhaygude et al., 2018) using Tophat2 (Kim et al., 2013) and Cufflinks (Trapnell et al., 2012). The genome alignment output file was then used to reconstruct known transcripts using Cufflinks (Trapnell et al., 2012). The transcriptome alignment output file of cuffmerge was subsequently used to quantify expression levels of genes and transcripts using RSEM (Li & Dewey, 2011). The resulting expected counts were used in the differential gene expression analysis with the R Bioconductor package, EdgeR (Robinson, McCarthy, & Smyth, 2010). Reads generated from the 24 samples were used as replicates, and comparisons were made across castes and

developmental stages (OQ vs. OW; EQ vs. EW; EQ vs. OQ; EW vs. OW). For all comparisons, we first filtered out transcripts with very low read counts by removing loci lower than 1 per kilobase of exons per million fragments mapped in at least half of the sequenced libraries, as recommended by EdgeR. TMM normalization was applied to account for compositional differences between libraries, and expression differences were considered significant at a false discovery rate of FDR < 0.05.

**Weighted correlation network analysis of expression.** Weighted gene co-expression network analysis was conducted using the R package WGCNA, as for the co-methylation network detailed above. The input dataset consisted of a matrix with 16 libraries from either queens or workers from both developmental stages, and 13,041 gene expression levels (Table S5). Log-transformed FPKM values were used as input to avoid gene length biases, and as recommended by Langfelder & Horvath (2008), the same procedure as in the co-methylation network analysis was used to construct the co-expression network, with two exceptions. After considering a range of soft thresholding power (10 to 30), a power of 20 was chosen, based on the criterion of approximate scale-free topology and  $R^2$ , and modules of highly co-expressed genes were merged using a cut-off value of 0.2 (Langfelder & Horvath, 2008).

#### **Functional analysis and overlap between differentially methylated and differentially expressed genes**

The software Blast2GO ([www.blast2go.com](http://www.blast2go.com)) was used to infer functional annotation of the *F. exsecta* gene set using structural similarity (BLASTx with an e-value cut-off  $\leq 10^{-3}$ ). The GOstat package for R (Beissbarth & Speed, 2004) was used to conduct GO term enrichment analysis on differentially expressed and differentially methylated gene sets, using all genes having GO terms as the universe. A similar procedure was used to conduct GO-term enrichment analysis on co-expressed and co-methylated gene sets retrieved from WGCNA. Overlaps between differentially



expressed and differentially methylated gene lists were visualized using a Venn diagram (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) and statistical analysis of the significance of overlaps between the two gene sets was calculated using the GeneOverlap BioConductor package (Shen & Sinai, 2016).

### **Association between DNA methylation and expression at the network level**

To assess preservation of modules between the expression and DNA methylation networks, we used the network module preservation statistics Zsummary methods implemented in the R function modulePreservation in the WGCNA R package (Langfelder & Horvath, 2008; Langfelder et al., 2011). Network module preservation statistics assess whether the density (strength of interactions among genes in a module) and connectivity patterns of modules (for example hub/central genes) are preserved between two independent datasets. This method does not require that modules and genes be identified in the target network (only in the reference data set); therefore, it is independent of ambiguities associated with module identification (Langfelder et al., 2011). The Zsummary statistic result summarizes the evidence that network connections of the modules are significantly more preserved than connections of random sets of genes of equal size (Langfelder et al., 2011). We used the 'modulePreservation' function in the WGCNA R package (Langfelder & Horvath, 2008) that enables rigorous testing of module preservation with 100 permutations. This reproducibility method was used to estimate the relationship between modules obtained from our co-expression and co-methylation network. In addition, to understand the biological relevance and reproducibility of our analysis, we conducted the same module preservation statistics (Langfelder et al., 2011) between our co-expression network modules and modules retrieved from a recent study of caste specialization across 16 ant species (Morandin et al., 2016).

Detailed scripts can be found in <https://github.com/oist/formica-methylation> under a MIT license.

## Results

### DNA methylation profiles

*Overall patterns:* DNA methylation frequency was assessed in two ways. The overall level of DNA methylation in the mapped reads (e.g. as reported by Bismark (Krueger & Andrews, 2011) and referring to all DNA methylation calls made on all mapped reads) was found to be about 1% in all samples. The proportions of highly supported DNA methylated (hsm) CpGs in the respective sets of detectable (scd) sites were 2.93% for emerging queens, 1.63% for emerging workers, 1.71% for old queens and 1.95% for old workers (Table S6 and S7; Figures S1, S2). Hsm statistics/proportions allow us to establish whether DNA methylation is localized. These low DNA methylation levels are similar to previous observations for honeybees (0.1%, Lyko et al., 2010), the ants, *Camponotus floridanus* (0.3%, Bonasio et al., 2012), *Harpegnathos saltator* (0.2%, Bonasio et al. 2012), and *Cerapachys biroi* (2.1%, Libbrecht et al. 2016). Consistent with observations on other hymenopterans, cytosine DNA methylation was found almost exclusively in a CpG context (Table S8). Highly supported methylated DNA CpGs sites occur in all annotated genomic regions, but are observed at about 2.5-fold higher numbers in coding regions than expected by random distribution over all scd sites (Tables S9 and S10). DNA methylation was found mostly within genes (90.8% +/- 3.5%), and predominantly in exons (64.5% +/- 8%) (Figure 1, Tables S9 and S10). There was a positive correlation between expression levels and the mean % DNA methylation per site, implying that highly expressed genes are more likely to be highly methylated (cor = 0.28,  $p < 0.001$ ; Spearman's product-moment correlation, Figure 2). Results of the Wilcoxon rank-sum test showed that overall DNA methylation levels were significantly higher in queens compared to workers ( $p < 0.001$ ), at both emerging and old stages. Emerging ants had higher overall DNA methylation than old ants among both queens and workers ( $p < 0.001$  each; Wilcoxon signed rank test).

As a control for our experiments, lambda DNAs were included to rule out any issues with conversion efficiency differences between samples. Conversion rates were verified by mapping the BS-reads to the lambda genome. None of the samples show any conversion problems. For instance, for the 6 emerging worker samples, the % of unmethylated C calls on lambda DNA by Bismark were between 99.38% and 99.71%. Our sequencing depth was not sufficient to meaningfully assess and compare the DNA methylation status of replicates individually. Rather, the statistical analysis relies on comparisons made between the replicated aggregate groups with safeguards against accidental reliance on a non-representative individual.

*Differentially methylated sites:* Across castes, and after correcting for multiple testing, we found 1,528 sites differentially methylated between old queens and old workers (869 up-methylated in old workers and 659 in old queens), and 1,620 differentially methylated sites between emerging queens and emerging workers (886 sites up-methylated in workers, and 734 in queens). Similarly, between developmental stages, we found 1,344 sites differentially methylated between emerging and old queens (692 sites up-methylated in emerging queens and 652 sites up-methylated in old queens). Likewise there were 1,894 sites differentially methylated between emerging and old workers (949 sites up-methylated in emerging workers and 945 sites in old workers) (Table S11).

*Differentially methylated genes:* At the gene level and across castes, these differentially methylated CpG sites resulted in 226 genes (1.7%) having at least two sites differentially methylated between old queens and old workers (118 genes up-methylated in queens and 108 in workers), with no significant differences in the number of differentially methylated genes ( $p = 0.55$ , Fisher's exact test). Between emerging queens and emerging workers, 264 genes (1.9%) were differentially methylated with 164 genes up-methylated in emerging queens and 100 genes in emerging workers. Emerging queens up-methylated significantly more genes than emerging workers ( $p < 0.001$ , Fisher's exact test, Figure S3). 75 genes were differentially

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methylated between castes in both old and emerging stages, which is more than expected by chance (Figure S4, GeneOverlap R package,  $p < 0.001$ ). Over half of those genes were caste-biased in opposite directions across both developmental stages (41 genes, 55%). These results parallel expression patterns, suggesting that caste-biased genes are more specific to developmental stage than to caste.

Across stages, 198 genes (1.5%) had at least two sites differentially methylated between emerging queens and old queens (105 sites up-methylated in emerging queens and 93 in old queens). No significant differences were found in the number of genes up-methylated in emerging and old queens ( $p = 0.43$ , Fisher's exact test). And 300 genes (2.3%) had at least two sites differentially methylated in emerging and old workers (172 genes up-methylated in emerging and 128 in old workers). A significant difference was found in the number of genes up-methylated between emerging workers and old workers ( $p = 0.01$ , Fisher's exact test, Figure S3). There were 66 genes in common among those differentially methylated between stages for queens and workers; however, this is not more than expected by chance (Figure S4, GeneOverlap R package,  $p = 0.2$ ). However, half of those genes were over-methylated in different direction (35 genes, 53%). Blast annotations of the differentially methylated gene lists for all comparisons can be found in Table S12.

*Differential DNA methylation GO term annotation:* The complete list of enriched GO terms can be found in Table S13, but here we summarize some of the most conspicuous findings. Between old queens and old workers, the queen up-methylated gene list included GO terms such as response to stress and DNA repair, whereas worker up-methylated genes were associated with oxoacid metabolic process and ncRNA metabolic process. Between emerging queens and emerging workers, the queen up-methylated gene list included GO terms similar to the old stage, such as DNA repair and ncRNA metabolic process. Worker gene list included GO terms such as cellular

response to DNA damage stimulus and positive regulation of catabolic processes. Across queens, old ants up-methylated genes that were related to GO terms such as ncRNA metabolic processes and DNA repair and methylation, whereas visual perception and eye morphogenesis were enriched in the emerging ant up-methylated gene set. Across workers, old ants up-methylated genes that were related to GO terms such as oxoacid metabolic processes and ATP metabolic processes. In contrast, RNA processing and DNA replication initiation were enriched for the emerging ant up-methylated gene set. Sets of GO terms associated with caste and/or developmental stage differences are consistent with hypothetical regulatory roles for differential DNA methylation (similar to previous studies (Foret et al., 2012; Kucharski et al., 2008; Libbrecht et al., 2016; Lyko et al., 2010)).

### Expression profiles

*Overall patterns:* We recovered 99 Gb of 100-bp paired-end reads from the 16 libraries. Following quality filtering, we realigned the reads to the *F. exsecta* genome, and on average a mapping rate of ~84% was obtained. A total of 10,874 genes were expressed, with over 1 count-per-million in at least half of the samples (Robinson, McCarthy, & Smyth, 2010).

*Differential expression patterns:* Among old queens and old workers, a total of 1,185 genes (8.7% of the total number of genes present in the genome) were differentially expressed, with queens up-regulating significantly more genes than workers (675 vs. 510,  $p < 0.001$ , Fisher's exact test). Between emerging queens and emerging workers, a total of 416 genes (3.1%) were differentially expressed, with queens over-expressing more genes than workers (291 vs. 125,  $p < 0.001$ , Fisher's exact test) (Figures S3, S5). We compared the list of genes differentially expressed between castes across old and emerging stages and found 164 genes overlapping. These genes are always differentially expressed between queens and workers regardless of the developmental stage (Figure S4, GeneOverlap R package,  $p < 0.001$ ). However, many genes (48

genes, 29.3%) were caste-biased in opposite directions in different developmental stages, meaning that caste-biased genes tend to be specific to developmental stages. A similar pattern was also found in a previous study looking at caste-biased genes over several development stages in the same species (Morandin et al., 2015).

Across stages, within the queen caste, a total of 892 genes (6.5% of all genes in the genome) were found differentially expressed between emerging and old ants, with no significant differences in the number of genes over-expressed (433 vs. 459,  $p = 0.4$ , Fisher's exact test). Within the worker caste, a total of 1,568 genes (11.5%) were differentially expressed between emerging and old workers, with more genes up-expressed by the emerging workers (893 vs. 675,  $p < 0.001$ , Fisher's exact test, Figure S3, S5). We compared the genes differentially expressed between stages across queen and worker castes, and found 292 common genes. These genes are always differentially expressed between stages regardless of caste (Figure S4, GeneOverlap R package,  $p < 0.001$ ). Many genes were consistently over-expressed by the same caste (269 genes, 92.1%) between developmental stages. Blast annotations of the differentially expressed gene lists for all comparisons can be found in Table S12.

*Differential expression GO term annotation:* The complete list of enriched GO terms can be found in Table S13. Between old queens and old workers, GO terms enriched for oxidation-reduction process and hormone transport were associated with queens, and terms such as social behavior and multi-organism behavior were enriched for workers. Between emerging queens and emerging workers, GO terms such as oxidation-reduction process and response to hormone were associated with queens, and cellular response to stimulus for workers. Between old and emerging queens, old ants up-regulated genes for telomere organization and response to stress, while emerging ants up-regulated genes associated with GO terms such as oxidation-reduction process and regulation of TOR signaling. Comparing old and emerging workers, old ants

enhanced expression of gene for DNA recombination and sensory perception of smell, while emerging ants over-expressed genes associated with regulation of hormone levels and response to stimulus.

### **Overlap between differentially expressed and differentially methylated genes**

To examine the hypothesis that expression and DNA methylation signals would show correspondence when looking at caste and developmental stage differences, we investigated whether list of differentially expressed and differentially methylated genes (all comparisons) might overlap at three different levels: genes and GO terms, in addition to network-based analyses described below. When comparing the lists of genes, very few were both differentially expressed and differentially methylated. We found 19 genes differentially expressed and differentially methylated between old queens and old workers, and 6 genes between emerging queens and emerging workers. We also found 8 genes that are both differentially expressed and differentially methylated between emerging and old queens, and 37 genes between emerging and old workers (Figure 3). The lists of genes differentially expressed and differentially methylated genes across the four comparisons did not overlap significantly for any comparisons (GeneOverlap R package, Old queens vs. workers (1185 vs. 226,  $p = 0.6$ ); Emerging queens vs. workers (416 vs. 264,  $p = 0.82$ ); Emerging queens vs. old queens (892 vs. 198,  $p = 0.95$ ); Emerging workers vs. old (1568 vs. 300,  $p = 0.35$ ); Figure 3). We also examined whether the direction of up/down expression and DNA methylation (e.g. whether a gene that is more expressed in one caste is also more methylated in the same caste). Surprisingly the direction of overexpression/DNA methylation only matched in around half of the genes (Figure S6; OQ vs. OW: 9 genes out of 19; EQ vs. OQ: 4 genes out of 8; EW vs. OW: 15 out of 37), except in the case of emerging queens vs. emerging workers, where all genes (8), both differentially expressed and differentially methylated, were upregulated by the emerging queens.

Next, we investigated whether we could find a correspondence between expression and DNA methylation and gene function, looking at the overlap between lists of GO terms. Surprisingly, given the small number of genes, we found a significant overlap between lists of GO terms associated with genes differentially expressed and differentially methylated genes across the four comparisons (GeneOverlap R package, Old queens vs. workers (158 vs. 162,  $p < 0.001$ ); Emerging queens vs. workers (51 vs. 243,  $p < 0.001$ ); Emerging queens vs. old queens (68 vs. 137,  $p < 0.001$ ); Emerging workers vs. old (136 vs. 264,  $p < 0.001$ ); Figure 4 and Table S13). Despite possible limitations with GO terms analyses, which rely on orthology with distantly related references, they provide insights into biological processes possibly involved, beyond what can be gleaned from gene lists alone.

### **DNA methylation and expression modules correspond to castes and developmental stages**

We separately constructed co-expression and co-methylation networks from the expression and DNA methylation datasets using the Weighted Correlation Network Analysis approach (WGCNA (Langfelder & Horvath, 2008)). In the methylation dataset, 8,200 genes were retained for further analyses following the cleaning step. Due to low coverage, one old queen sample and three old worker samples were removed from the co-methylation network input dataset. A total of 6 emerging queens, 6 emerging workers, 4 old queens and 4 old workers were used for this analysis. A total of 348 genes were not co-methylated and were excluded from further analysis. A total of 20 co-methylated modules (labelled M1-M20) were identified, ranging in size from 56 (M1) to 3,230 (M18) genes, with 393 genes per module on average. In the expression dataset, 10,700 genes were retained after removing genes with too many missing values, and 6,570 genes were subsequently assigned to one of the co-expression modules. A total of 4130 genes were not co-expressed and were consequently removed from further analysis. We identified 14 co-expression modules (labeled E1-E14) with sizes ranging from 46 (E11) to 2,454 (E9) genes,



with an average of 469 genes per module. For both datasets, we calculated the module eigengenes which are defined as the first principal component of a module and are representative of gene expression or gene DNA methylation profiles in a module (Langfelder & Horvath, 2008). Afterward, we correlated these eigengenes with two phenotypic traits, *i.e.*, caste and stage, using a glm approach. When the eigengene of a module is correlated with a trait of interest, it means that most/all genes in the module exhibit a significant correlation/association with the trait, and we can define which genes/modules are likely to underlie the phenotype via gene expression. For the methylation dataset, one of the modules was significantly correlated with one of the caste phenotypes (M2; Queen phenotype), and 11 of 20 modules were significantly correlated with stage (Emerging phenotype). For the expression dataset, 7 out of 14 modules were correlated with one of the two female castes (4 associated with the worker phenotype and 3 with the queen) and 9 modules were correlated with stage (5 with the old phenotype and 4 with the emerging). In addition, 3 methylation modules and 5 expression modules were significantly associated with the interaction of caste and stage phenotypes (Figure 5, Table 1).

To gain insight into the biological relevance and functional significance of modules, we performed GO term enrichment analysis on the genes in each module (Tables S14 and S15).

Here we summarize some of the main findings. In the methylation dataset, the module associated with the queen phenotype (M2) was correlated with gene expression and RNA metabolic processes. Modules associated with the emerging phenotype were correlated with gene expression (M3), response to pheromone (M7), ATP biosynthetic process (M8), RNA metabolic process (M11), mRNA transport (M12), oxidative phosphorylation (M14), nerve development (M15), regulation of RNA biosynthetic process (M16), social behavior (M17), developmental process (M18) and DNA repair (M19) (Table S15). In the expression dataset, queen-associated modules were related to detection of chemical stimulus involved in sensory perception of smell (E7), oxoacid metabolic process (E13) and oxidation-reduction process

(E14). Worker-associated modules were linked to social behavior (E1), TOR signaling (E2), response to stress (E3) and sensory perception (E5). Modules associated with stage in the expression dataset, were correlated with DNA recombination (E4), detection of chemical stimulus (E5), growth (E6), muscle contraction (E7) and development of the central nervous system in old adults (E8), while modules associated with the emerging phenotype were associated with biological functions such as response to growth factor (E11), regulation of cell death (E12), cell cycle (E13) and response to hormone (E14) (Table S15). As a precaution, it is worthy to note that GO terms associated with eye pigmentation (M14) were also enriched and could potentially be a sign of contamination.

### **Preservation of co-expression sets of genes in DNA methylation data**

Next, we looked for evidence of preservation between the co-expression and co-methylation networks using the WGCNA R package. Values of “*Zsummary* below 2” indicate no evidence of preservation. Values between 2 and 5 indicate moderate evidence for preservation, while values over 5 indicate strong evidence of preservation. Although conservation of modules between co-expression and co-methylation datasets was weak, four expression modules were conserved in the co-methylation data. Based on the preservation statistic *Zsummary*, we found that four co-expression sets of genes (E1, E6, E9, and E10) exhibited moderate preservation within the co-methylation network (Figure 6). The expression module E6 is correlated with the old phenotype, module E9 with the association of both phenotypes, and module E1 with the worker phenotype (Table S16). An online resource has been created to simplify visualization of module organization and the association between expression and DNA methylation networks (the website is available at <https://mikheyev.github.io/formica-brain-expression-methylation/>). The online tool allows users to visualize caste and stage association for each module (Figure 5). Many of the modules belonging to the co-expression or co-methylation networks were correlated with either caste (inner band), or stage (outer bands).

## **Preservation of co-expression sets of genes with a previous study**

Similarly, we looked for preservation between our brain co-expression network and our multiple species co-expression network published in an earlier study (Morandin et al., 2016). Based on the preservation statistic Zsummary, we found that 3 out of 36 of our 2016 co-expression modules exhibited moderate preservation with our brain co-expression network. Additionally, 9 out of 14 brain co-expression modules in return exhibited moderate to strong preservation with the 2016 co-expression network (e.g. modules E1, E2, E3, E6, E8, E9, E10, E12 and E13) (Figure S7, Table S17). This module preservation analysis confirmed that our modules are found in an independent data set.

## **Discussion**

The goal of this study was to examine whether socially important phenotypic traits correlate with divergence in DNA methylation and expression. We found distinct transcriptional and methylation differences between castes within a developmental stage and different developmental stages within a caste. Furthermore, there was some evidence of overlap between methylation and gene expression states, but only at the functional level, that of biological processes gene ontology terms. More specifically, some modules in the co-expression data are preserved in co-methylation modules (retain similar network structure (genes) and network properties), though not *vice versa*. This is consistent with a role of DNA methylation as a proximate mechanism regulating gene expression, which is already known to affect caste specialization, as well as task specialization and ageing, in social insects (Kozieretska, Serga, Koliada, & Vaiserman, 2017; Yan et al., 2015), though further investigations are necessary to confirm this link.

Here we used individual brains to conduct the first reduced representation bisulfite sequencing study on social insects. RRBS brings down the scale and cost of whole genome bisulfite sequencing by only analyzing a representative portion of the genome. In vertebrates, this approach has been shown to capture around 85% of the CpG islands and 60% of promoters while requiring very little input material (Gu et al., 2011), and it permits more replicates per experiment while providing an efficient way to generate overall quantification of DNA methylation, and to apply powerful network-based analysis methods with individual-relevant replication. Nonetheless, it is important to note the limitations of RRBS. Because restriction enzymes digest DNA sequences at restriction sites randomly across the genome, many relevant methylated fragments in each sample are missed. Thus, these results encompass only a fraction of all methylated sites. As a result, global patterns are more easily captured compared to specific mechanisms. Furthermore, the size selection step associated with RRBS results in stochastically uneven coverage across samples, and large amounts of missing data. This must be carefully accounted for in the analysis, and it also makes per-site comparisons, and comparisons across more than two conditions problematic. In this analysis, after rigorously filtering sites based on coverage and quality, we conducted analyses aggregated at the gene level. Despite these limitations, RRBS produces data that are comparable across different experimental conditions, and can provide biological insights, given the right statistical approach. In particular, co-methylation analysis overcomes some of the limitations introduced by data sparseness, as DNA methylation levels at nearby CpG-sites tend to be highly correlated. For instance, variation in DNA methylation across treatments has been showed to occur more frequently in aggregated CpGs (Jaffe, Feinberg, Irizarry, & Leek, 2012). Because co-methylation analysis leverages information from the entire data set to construct the network, sporadic missing sites do not significantly alter the overall data structure. Thus, this type of analysis is more powerful than those focusing on detecting single-site differences, and allows more sophisticated types of analyses, such as full-factorial designs with main effects and interaction terms (Table 1).

Previous work proposed that social insect castes differ in DNA methylation states, which are established during caste differentiation that occurs during larval development (Bonasio et al., 2012; Elango et al., 2009; Foret et al., 2012; Glastad et al., 2016; Kucharski et al., 2008; Lyko et al., 2010), though that view has been challenged (Herb et al., 2012; Libbrecht et al., 2016; Patalano et al., 2015). We tested this hypothesis, also expanding it to include changes in DNA methylation states that take place in the course of adult development and ageing. To do this we sampled queens and workers at different ages, and made comparisons both within castes across developmental categories, as well as between castes within the same age category, without maintaining that “old” and “emerging” are necessarily equivalent states for the two castes. Indeed, emerging and old ants were sampled a few weeks apart, and emerging queens are also unmated compared to old queens. Keeping in mind that age categories are difficult to standardize between castes, we found significant changes in DNA methylation and gene expression between these phenotypic endpoints, showing that DNA methylation differs between ant castes, as well as across the lifetime of the adult. The latter finding is particularly interesting since it suggests that caste differences due to DNA methylation may be dynamic, and not necessarily fully fixed during the developmental program when the castes differentiate. The same pattern holds true when comparing DNA methylation between castes over the two developmental stages, suggesting that age-related changes in DNA methylation differ between queens and workers at least at the adult stage. Developmental specificity of DNA methylation bias in general is certainly a promising direction for future research. Furthermore, we found some significant interaction terms in co-expression and co-methylation analysis, suggesting that gene expression and DNA methylation levels do not necessarily change in the same direction as a function of caste and adult developmental stage (Table 1). This suggests that ants have a dynamic DNA methylation system that is active throughout their lives. Indeed, the overall positive relationship between gene body DNA methylation and expression (Figure 2), which is a hallmark of other insects, suggests that DNA methylation in ants largely functions in the same way as in other species. From this perspective, finding differences between castes and

developmental stages, as we did in this study, is not surprising given the many ancient developmental and regulatory mechanisms coopted into caste differentiation (Robinson, Grozinger, & Whitfield, 2005).

**Patterns of DNA methylation.** We found more overall DNA methylation in queens compared to workers, at both developmental stages, in contrast to previous findings that found a lower level of DNA methylation in queens during the larval stage (Shi et al., 2013), or even no differences in the adult stage (Lyko et al., 2010) in the honey bee. However, similar results were observed for the ant, *Pogonomyrmex barbatus*, in which virgin queens exhibited higher levels of DNA methylation compared to workers (Smith et al., 2012). Unfortunately, studies are still too few to draw any general patterns. Also, considering that *Formica exsecta* queens live ~20 years and the workers just over a year (Pamilo, 1991), if DNA methylation accumulated over time/ageing, we would expect to find large differences in overall DNA methylation between old queens and emerging queens, and more subtle differences between emerging and old workers. However, contrary to this hypothesis, we found the opposite pattern, with emerging ants showing more DNA methylation overall than old ants. The important role of DNA demethylation in diverse biological processes by regulating gene expression has been well documented in mammals (Richardson, 2003), but its exact role with gene body DNA methylation remains unclear. Further experimental studies of DNA demethylation in social insects (especially considering caste longevity and behavior differences) are needed to further understand how DNA demethylation is transduced into physiological changes over time in the two castes.

Previous work has demonstrated the usefulness of network-based approaches for detecting links between expression and DNA methylation that could be missed by more commonly used approaches focused on comparing lists of differentially expressed and differentially methylated genes (Davies et al., 2012; Eijk et al., 2012; Horvath et al., 2012; J. Zhang & Huang, 2017). Our

results reveal that both the brain transcriptome and methylome can be organized into modules. All co-methylation and co-expression modules are significantly enriched with gene ontology categories (Table S14), thus providing additional evidence that these modules are biologically meaningful. For instance, co-expressed modules associated with castes were enriched for gene functions such as social behavior, TOR pathways and pheromone synthesis, while co-methylation modules were enriched for core biological functions such as DNA repair. Interestingly, co-expression modules identified in this study in brain tissue were also conserved in whole-body transcriptional data from an earlier study (Figure S7), suggesting that similar gene regulatory processes act at both tissue-specific and whole-body levels.

**Conclusion.** We propose that in addition to action on single genes or their isoforms, gene DNA methylation may be thought of in a network context, with co-methylation modules associated with specific phenotypes, *e.g.*, caste and stage (Table 1). We hope that future work will focus on reconstructing ever more accurate co-methylation networks, which will require large numbers of replicates across different phenotypic states to fully understand the role of DNA methylation and how it interacts with gene co-expression to generate phenotypic novelty, as it has been done recently for gene co-expression networks (Morandin et al., 2016). Investigations of diverse taxa using similar methodologies would be particularly useful for identifying the extent to which DNA methylation is associated with caste or other phenotypic traits among social insects, and how it evolves.

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### **Data Accessibility Statement**

The raw reads are publicly available in the DNA Data Bank of Japan under bioproject ID PRJDB6378. The raw reads of the transcriptome are publicly available under sample accession numbers ID SAMD00094824 - SAMD00094838. The raw reads of the methylome are publicly available under sample accession numbers ID SAMD00094839 - SAMD00094858.

### **Authors contribution**

Conceptualization, CM; Methodology, CM and ASM; Fieldwork: HH and LS; Investigation, CM, ASM and VPB; Writing, CM and ASM; Review & Editing, All authors; Funding Acquisition, CM, HH, LS, ASM. All authors read and approved the final manuscript.

### **Competing interests**

The authors declare that they have no competing interests.

## Figure legends

### Figure 1

**Mapping of methylation sites on the genome annotation.** Output was generated by BWASPR::map\_methylome() and accounts for where CpGhsm and CpGscd (control) sites reside relative to the genome annotation, in every sample. a) All regions b) Exon regions. Cytosine DNA methylation was found almost exclusively in a CpG context. Highly supported DNA methylated CpGs sites are observed at ~2.5-fold higher numbers in coding regions than expected by random distribution over every scd site.

### Figure 2

**Scatter plot showing the correlation between expression level (FPKM) and average percentage of DNA methylation per site.** A positive correlation between expression and DNA methylation was found, implying that highly expressed genes are more likely to be highly methylated ( $\text{cor} = 0.28$ ,  $p < 0.001$ ; Spearman's product-moment correlation). This is the typical pattern found in insect genomes that rely on DNA methylation (Hunt, Glastad, Yi, & Goodisman, 2013b; Xiang et al., 2010), providing a level of validation for these findings.

### Figure 3

**Venn diagram summarizing overlap between differentially expressed and differentially methylated genes between all comparisons at the gene level.** No significant overlap was found at the gene level for any of the four comparisons. Statistical analysis of the significance of the overlap between the two gene sets was calculated using the GeneOverlap BioConductor package.

#### Figure 4

**Venn diagram summarizing overlap between GO terms of differentially expressed and differentially methylated genes between comparisons.** Significant overlap was found at the GO term level for all comparisons. Statistical analysis of the significance of the overlap between the two gene sets was calculated using the GeneOverlap BioConductor package.

#### Figure 5

**Visual representation of the link between co-expression and co-methylation modules.**

Many modules belonging to the co-expression and co-methylation networks were correlated with either caste (inner band), or stage (outer bands). **Significant correlations with the queen caste and developmental maturity are highlighted in red, while worker and newly eclosed developmental stage correlations are highlighted in blue. Significant caste by developmental stage interactions are highlighted in purple.** Although conservation of modules between co-expression and co-methylation datasets was generally weak, four expression modules (E1, E6, E9 and E10) were conserved in the co-methylation data. Their connections are highlighted in orange.

#### Figure 6

**Preservation and association of co-expression and co-methylation modules using the Module preservation statistic, *Zsummary*, of WGCNA.** a) preservation of expression modules in the methylation dataset b) preservation of methylation modules in the expression dataset. Values of *Zsummary* below 2 indicate no evidence of preservation; values between 2 and 5 indicate moderate evidence for preservation; values above 5 strong evidence for preservation. Four co-expression modules (E1, E6, E9 and E10) were conserved in the co-methylation data.

## Table legends

**Table 1**

**Correlation between module eigengenes and biological traits (caste and stage, and caste/stage interactions).** Expression and DNA methylation patterns of most modules are strongly associated with developmental stage phenotype. In addition, expression of several of these modules was also associated for both phenotypes and phenotype interactions. This shows that modules likely play multiple roles, and that their constituent genes have many functions. Caste/black: module associated with queen phenotype. Caste/grey: module associated with worker phenotype. Stage/black: module associated with old phenotype. Stage/grey: module associated with emerging phenotype. Caste x Stage/grey/black: module associated with both caste and stage.

## Supplementary figures

**Figure S1**

**Histogram of CpG read coverage per cytosine for all aggregate samples of emerging queen, old queen, emerging worker, old worker.** The range of coverage was restricted to the range [10-1000] for easy comparison. Plots were generated with `methyKit:getCoverageStats()` as implemented in the BWASPR workflow.

**Figure S2**

**Histogram of % DNA methylation per CpG site for all aggregate samples of emerging queens, old queens, emerging workers, old workers.** The range of coverage was restricted to the range [10-1000] for easy comparison. Plots were generated with `methyKit:getMethylationStats()` as implemented in the BWASPR workflow.

### Figure S3

**Barplot showing the number of genes differentially expressed and differentially methylated** across castes (Old queens (OQ) vs. old Workers (OW); Emerging queens (EQ) vs. Emerging Workers (EW)) and across stages (Emerging Queens (EQ) vs. Old Queens (OQ); Emerging Workers (EW) vs. Old Workers (OW)). A larger number of genes were found differentially expressed than differentially methylated. \* $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

### Figure S4

**Venn diagram summarizing numbers of genes that are found always differentially expressed/methylated between castes regardless of the developmental stage (OQW vs. EQW) and genes that are always differentially expressed/methylated between developmental stages regardless of the caste (Queen vs. Worker).** Three of the four comparisons presented a significant overlap, implying that a larger number of genes is consistently differentially expressed by caste or developmental stage than by chance. For methylation, a larger number of genes was consistently caste differentially methylated across stages than expected by chance. Statistical analysis of the significance of the overlaps between the two gene sets was calculated using the GeneOverlap BioConductor package.

### Figure S5

**Differentially expressed and differentially methylated genes across comparisons (Old queens vs. Old workers; Emerging queens vs. Emerging workers) visualized as 'MA' plots** (log ratio versus abundance).

## Figure S6

**Caste- and developmental stage- bias direction of the genes that were both differentially expressed and differentially methylated across all treatments (OQ vs. OW; EQ vs. EW; EQ vs. OQ; EW vs. OW).** Only half of the genes that were over-expressed in one direction tended to be also over-expressed in the same direction, apart from the emerging queens vs. emerging workers comparison, where all eight genes were over-expressed and over-methylated in emerging queens.

## Figure S7

**Preservation and association of *F. exsecta* brain co-expression and 16 ant species co-expression network published in an earlier study (Morandin et al. 2016) using the Module preservation statistic *Zsummary* of WGCNA.** a) preservation of expression modules in the 2016 expression data b) preservation of expression modules in the 2016 expression data. Values of “*Zsummary* below 2” indicate no evidence of preservation. Values between 2 and 5 indicate moderate evidence for preservation. Values above 5 demonstrate strong evidence for preservation. Three 2016 expression modules were preserved in the present study expression modules, while 9 expression modules from the present study were preserved in the 2016 expression modules.

## Supplementary tables

### Table S1

**RRBS primer sequences.** Sequences of RRBS primer used for the RRBS library preparation (see methods for more details).

## Table S2

**Statistics of deposited RRBS and RNAseq samples.** All methylation data were derived with the BWASP/BWASPR workflows. Median length was determined with FastQC. Mapping efficiency was determined with Bismark software, as implemented in BWASPR. All expression data were derived with the Tophat2, cufflinks and RSEM pipeline.

## Table S3

**Details of the analysis performed to find minimal coverage of sites required to potentially observe significant DNA methylation.** Following this analysis, a cut-off of 4 reads was set as the threshold to detect sites with enough coverage (scd sites) to perform detection of significant DNA methylation.

## Table S4

**Dataframe used as input for the co-methylation network analysis with WGCNA.** Each column represents one sample analyzed and each row represents the DNA methylation level of one gene across all samples. The input dataset consists of results from DNA methylation calls of the 12,112 genes measured, as the average percentage of DNA methylation per site per gene, restricted to sites with highly-supported DNA methylation data (high coverage, at least 10 reads). These proportional levels control for the number of restriction enzyme sites present on each gene, gene length, and quality of mapping to avoid any biases.

#### Table S5

**Dataframe used as input for the co-expression network analysis with WGCNA.** Each column represents one sample analyzed and each row represents the expression level of one gene across all samples. The input dataset consists of the expression level (raw counts) of the 13,041 genes evaluated. Raw counts were log-transformed as recommended by Langfelder & Horvath (2008).

#### Table S6

**Overall level of DNA methylation** (percent reads reporting conversion or non-conversion) on all C, CpG sites, CHG sites and CpG sites for all biological samples (old queens, emerging queens, old workers, emerging workers).

#### Table S7

**Number of CpG sites, CHG sites, CHH sites, for each biological sample (old queens, emerging queens, old workers, emerging workers).**

#### Table S8

**Per aggregate sample DNA methylation statistics.** scd, sufficiently covered detectable sites, and hsm, highly supported methylated sites, were reported by the BWASPR workflow. The scd percentage refers to the proportion of genomic CpG that are scd. The hsm percentage refers to the proportion of scd that are hsm. The overall DNA methylation level refers to the proportion of Cs in all mapped reads that are called methylated, as determined by Bismark software as implemented in the BWASPR workflow.



#### Table S9

**Distribution of CpG (hsm) sites in genome feature regions** for all biological samples (old queens, emerging queens, old workers, emerging workers). Cytosine DNA methylation was found almost exclusively in CpG contexts, as expected for insects.

#### Table S10

**Distribution of CpG (hsm) sites in exon feature regions** for all biological samples (old queens, emerging queens, old workers, emerging workers). Highly supported DNA methylated CpGs sites are observed at about 2.5-fold higher frequencies in coding regions than expected by random distribution over every scd site.

#### Table S11

**List of differentially methylated sites** as determined by methylKit::getMethylDiff with qvalue < 0.01. The list of differentially methylated CpG regions was based on q-values (0.01) and percent DNA methylation difference cutoffs (25%) for sites with coverage of at least 10 reads, for all four comparisons using for multiple testing correction.

#### Table S12

**List of differentially expressed and differentially methylated genes for all four comparisons (OQ vs. OW; EQ vs. EW, OQ vs. EQ; OW vs. EW). Blast annotations for the genes are provided, as well as differential analysis results (EdgeR results for DEG and BWASP outputs for DMG).** The software Blast2GO ([www.blast2go.com](http://www.blast2go.com)) was used to infer

functional annotation of the *F. exsecta* gene set using structural similarity (BLASTx with an e-value cut-off  $\leq 10^{-3}$ ).

#### Table S13

**List of enriched GO terms for differentially expressed and differentially methylated genes found in all four comparisons.** Gene ontology (GO) terms for all genes of the *F. exsecta* gene set were determined using BLAST2GO (using BLASTp with an e-value cut-off  $\leq 10^{-3}$ ). We used the Gostats package in R to conduct GO term enrichment analysis on the list of differentially expressed/methylated genes presented in Table S12, using the set of all genes for which GO terms were available, as the universe.

#### Table S14

**List of enriched GO term for each module found in the co-methylation and co-expression network.** We used the Gostats package for R to conduct GO term enrichment analysis on gene sets included in the co-expression and co-methylation modules, using the set of all genes for which GO terms were available as the universe. Modules were enriched with gene ontology categories, which provides indirect evidence that these sets of co-expressed genes are biologically meaningful.

#### Table S15

**List of blast annotations for all genes belonging to a co-methylation or a co-expression module.** The software Blast2GO ([www.blast2go.com](http://www.blast2go.com)) was used to infer functional annotation of the *F. exsecta* gene set using structural similarity (BLASTx with an e-value cut-off  $\leq 10^{-3}$ ).

## Table S16

**Preservation between co-expression and co-methylation networks.** Module preservation statistic *Zsummary* that summarizes evidence of preservation of expression modules in DNA methylation data, and preservation of DNA methylation modules in expression data. Values of *Zsummary* below 2 indicate no evidence of preservation; values between 2 and 5 indicate moderate evidence for preservation; values above 5 strong evidence for preservation. Four co-expression modules (E1, E6, E9, and E10) were conserved in the co-methylation data.

## Table S17

**Preservation between the brain co-expression network and the co-expression network from Morandin et al. (2016).** The module preservation statistic, *Zsummary*, that summarizes evidence of preservation of expression modules in our 2016 study (Morandin et al. 2016), and vice versa. Values of *Zsummary* below 2 indicate no evidence of preservation. Values between 2 and 5 indicate moderate evidence for preservation. Values above 5 strong evidence for preservation. Three 2016 expression modules were preserved in the present study expression modules, while 9 expression modules from the present study were preserved in the 2016 expression modules.

## Table S18

**List of genes defined as hub genes** for co-expression and co-methylation networks (High connectivity ((cor.geneModuleMembership > 0.8) and high gene significance (cor.geneTraitSignificance > 0.5)). Hub genes are genes that are highly connected within a module, and that participate in biological processes associated with the modules (He & Zhang, 2006; Langfelder & Horvath, 2008; Langfelder et al., 2013).

# Accepted Article

## Table S19

Expression and DNA methylation profiles of the ten hub genes belonging to module E6 (correlated with stage, the interaction of caste x stage, and is preserved across the co-methylation modules). Two of the ten hub genes were also found differentially expressed across caste (OQ vs. OW) and across stages (OQ vs. EQ).

a) Methylation  
network

	Caste			
	CI	pvalue	FDR	pvalue
M1	- 1.04	- 0.0011	0.041	0.112
M2	- 1.037	- 0.0119	0.011	0.044
M3	- 0.009	1.053	0.148	0.278
M4	- 0.051	0.047	0.937	0.937
M5	- 0.024	0.093	0.696	0.743
M6	- 0.031	0.02	0.696	0.743
M7	- 0.026	1.048	0.124	0.257
M8	- 0.015	0.043	0.242	0.392
M9	- 0.991	0.038	0.356	0.521
M10	- 1.038	0.039	0.437	0.6
M11	- 0.051	0.074	0.489	0.667
M12	0.011	0.997	0.029	0.087
M13	- 0.079	0.044	0.882	0.897
M14	- 0.099	0.0522	0.193	0.333
M15	- 0.091	0.033	0.685	0.743
M16	- 0.052	0.048	0.387	0.553
M17	- 0.034	0.03	0.606	0.699
M18	- 0.059	0.09	0.261	0.412
M19	0.008	0.977	0.027	0.085
M20	- 0.0124	1.051	0.194	0.333

Stage			
CI	pvalue	FDR	pvalue
- 1.03	0.016	0.175	0.318
- 1.02	0.02	0.216	0.36
0.017	0.104	0.009	0.042
- 0.016	0.078	0.146	0.278
0.007	0.194	0.018	0.068
0.002	0.788	0.039	0.111
0.029	0.218	0.005	0.025
0.017	0.121	0	< 0.001
- 0.973	0.137	0.572	0.68
- 0.992	0.113	0.531	0.678
0.025	0.712	0	< 0.001
0.031	0.361	0.002	0.011
0.001	0.408	0.046	0.12
- 0.03	0.919	0	< 0.001
0.018	0.404	0	< 0.001
0.051	0.793	0	< 0.001
0.029	0.403	0	< 0.001
0.032	1.066	0	< 0.001
0.016	1.943	0.002	0.011
- 0.038	0.089	0.718	0.743

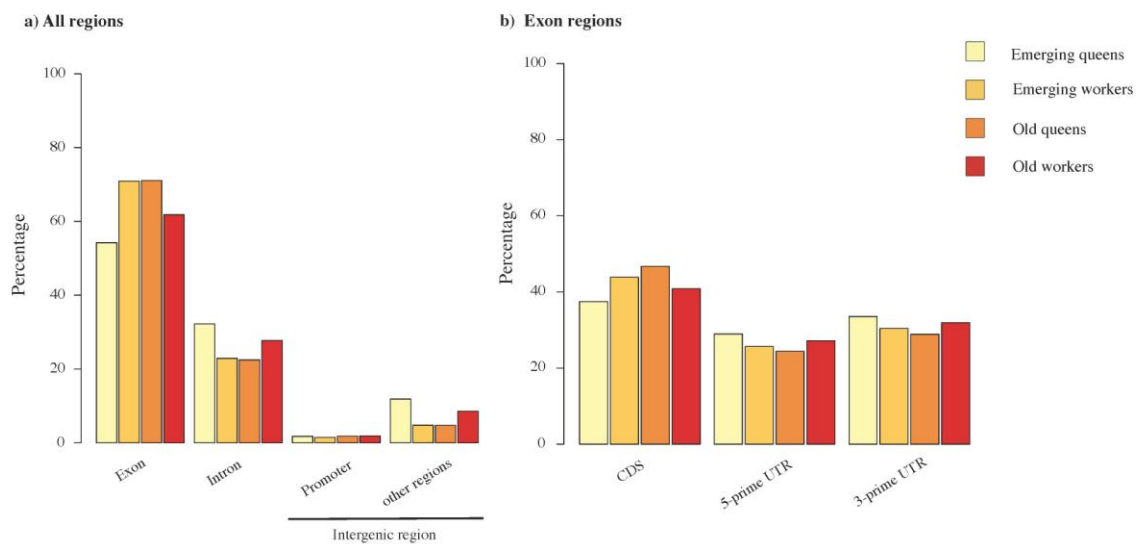
Caste * Stage			
CI	pvalue	FDR	pvalue
0.004	1.069	0.061	0.153
- 0.031	1.015	0.284	0.437
- 1.066	0.01	0.109	0.234
- 0.031	0.97	0.302	0.453
- 0.064	0.742	0.513	0.669
- 0.775	0.009	0.099	0.227
- 1.075	0.0073	0.067	0.161
- 0.062	0.659	0.574	0.68
- 0.132	0.98	0.548	0.68
- 0.084	1.024	0.5	0.667
- 0.673	- 0.008	0.026	0.085
- 1.01	- 0.032	0.011	0.044
- 0.178	0.506	0.707	0.743
- 0.845	- 0.007	0.026	0.085
- 0.159	0.56	0.578	0.68
- 0.768	- 0.03	0.002	0.011
- 0.193	0.542	0.709	0.743
- 1.032	0.011	0.102	0.227
- 0.984	- 0.039	0	< 0.001
- 1.059	0.013	0.141	0.278

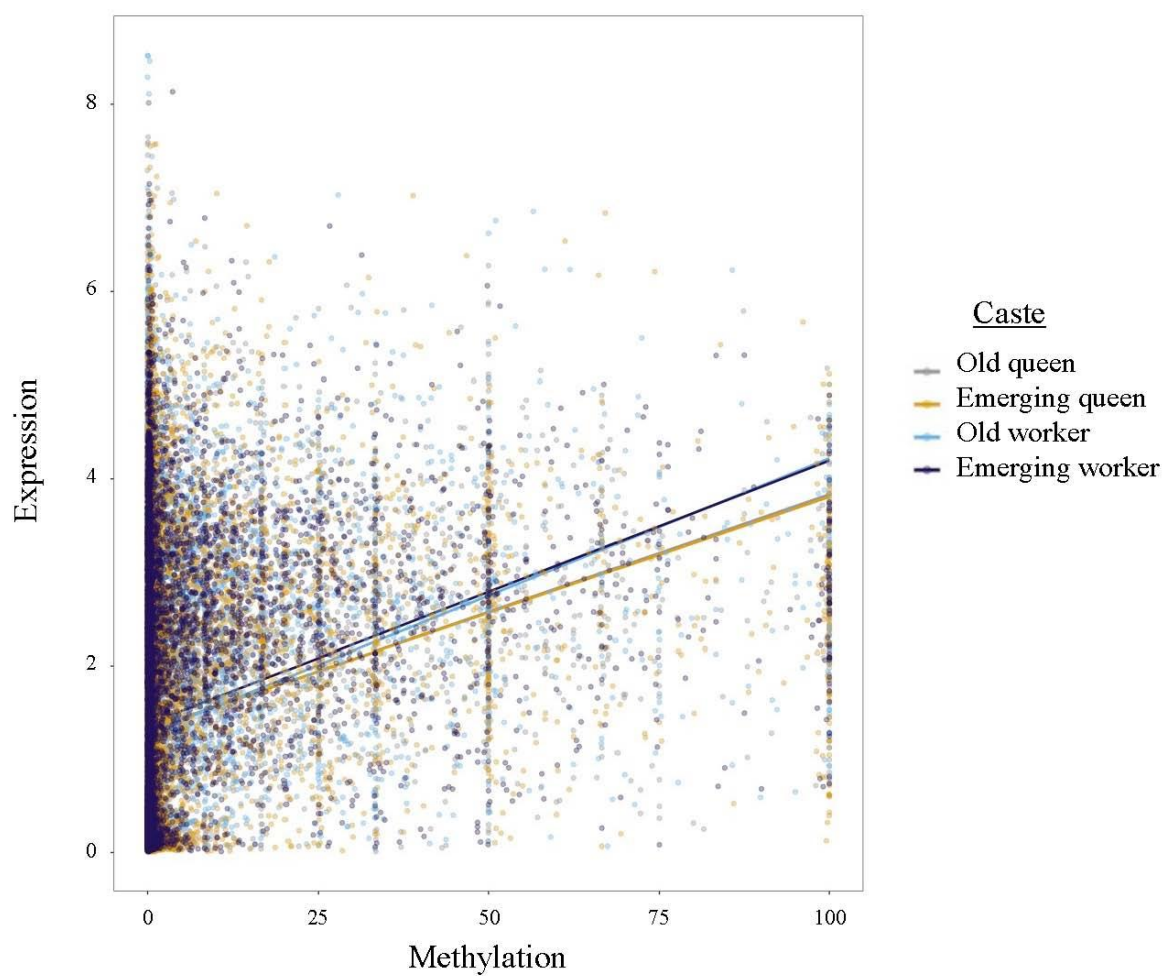
b) Expression  
network

Caste				
CI		pvalue	FDR	pvalue
E1	0.06	1.029	0	< 0.001
E2	0.057	0.981	0	< 0.001
E3	0.305	0.585	0	< 0.001
E4	- 0.159	0.513	0.15	0.21
E5	0.007	0.932	0	< 0.001
E6	- 0.949	0.053	0.13	0.188
E7	- 0.827	- 0.151	0	< 0.001
E8	- 0.885	0.01	0.33	0.42
E9	- 0.463	0.058	0.13	0.188
E10	- 0.011	0.49	0.06	0.1
E11	0.002	0.244	0.03	0.055
E12	- 0.069	0.242	0.51	0.591
E13	- 0.658	- 0.183	0	< 0.001
E14	- 0.558	- 0.122	0	< 0.001

Stage			
CI		pvalue	FDR pvalue
- 0.095	- 0.003	0.035	0.061
- 0.11	- 0.003	0.04	0.067
- 0.135	0.123	0.57	0.614
- 0.445	- 0.078	0	< 0.001
- 0.294	- 0.097	0	< 0.001
- 1.047	- 0.065	0	< 0.001
- 0.998	- 0.384	0	< 0.001
- 1.041	- 0.138	0	< 0.001
- 0.28	0.24	1	1
- 0.223	0.228	0.57	0.614
0.327	0.701	0	< 0.001
0.088	1.038	0	< 0.001
0.067	0.459	0	< 0.001
0.026	0.401	0	< 0.001

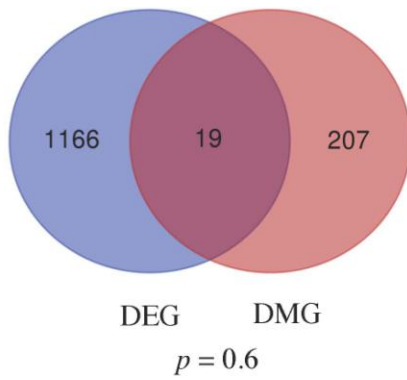
Caste * Stage			
CI		pvalue	FDR pvalue
- 0.964	0.055	0.252	0.33
- 0.863	0.128	0.37	0.457
- 0.86	- 0.114	0	< 0.001
- 0.502	0.25	0.24	0.325
- 0.809	0.135	0.45	0.54
0.032	1.017	0.01	0.02
0.255	0.927	0	< 0.001
- 0.04	0.943	0.1	0.156
0.11	0.948	0	< 0.001
0.024	0.646	0.03	0.055
- 0.311	0.148	0.71	0.727
- 0.903	0.179	0.521	0.591
0.074	0.583	0	< 0.001
- 0.356	0.218	0.7	0.727



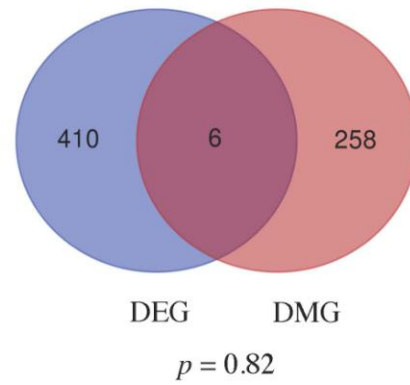




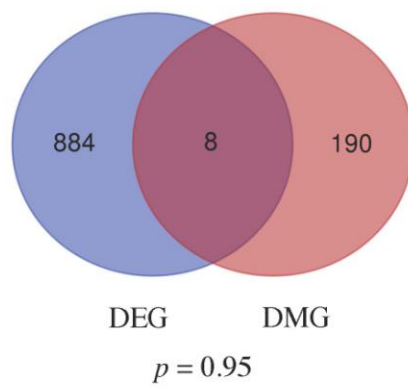
a) Old queens vs. Old workers



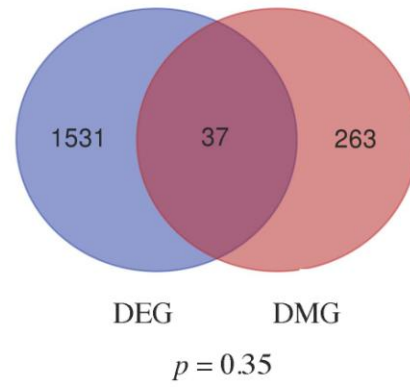
b) Emerging queens vs. Emerging workers



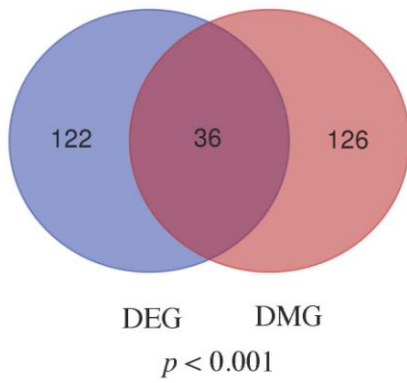
c) Emerging queens vs. Old queens



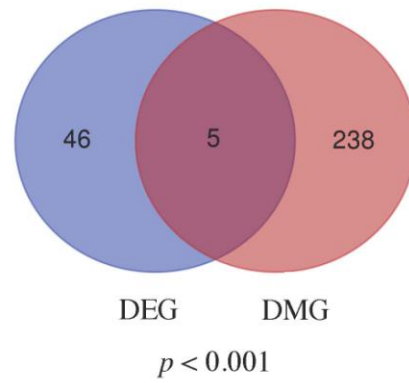
d) Emerging workers vs. Old workers



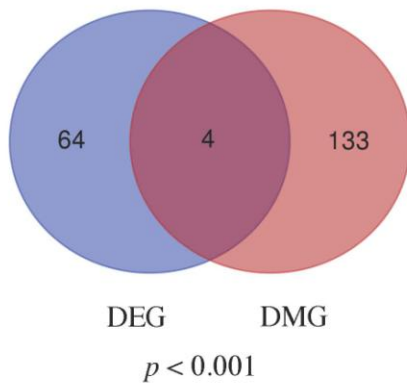
a) Old queens vs. Old workers



b) Emerging queens vs. Emerging workers



c) Emerging queens vs. Old queens



d) Emerging workers vs. Old workers

