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Title: Changes of gene expression but not cytosine methylation are associated with male parental care reflecting behavioural state, social context, and individual flexibility

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Short Running Title: Behaviour and methylation during parental care

Summary Statement

Behavioural state has more influence on gene expression than social context or individual flexibility during a transition to parental care, while cytosine methylation is not associated with differential gene expression.

Abstract

Behaviour is often on the front line response to changing environments. Recent studies show behavioural changes are associated with changes of gene expression; however, these studies have primarily focused on discrete behavioural states. We build on these studies by addressing additional contexts that produce qualitatively similar behavioural changes. We measured levels of gene expression and cytosine methylation, which is hypothesized to regulate the transcriptional architecture of behavioural transitions, within the brain during male parental care of the burying beetle *Nicrophorus vespilloides* in a factorial design. Male parenting is a suitably plastic behaviour because while male *N. vespilloides* typically do not provide direct care (i.e., feed offspring) when females are present, levels of feeding by a male equivalent to the female can be induced by removing the female. We examined three different factors: behavioural state (caring vs non-caring), different social contexts (with or without a female mate), and individual flexibility (if a male switched to direct care after his mate was removed). The greatest number of differentially expressed genes were associated with behavioural state, followed by social contexts, and lastly by individual flexibility. Cytosine methylation was not associated with changes of gene expression in any of the conditions. Our results suggest a hierarchical association between gene expression and the different factors, but that this process is not controlled by cytosine methylation. Our results further suggest that the extent a behaviour is transient plays an underappreciated role in determining its underpinning molecular mechanisms.

Keywords: DNA Methylation, Epigenetics, *Nicrophorus vespilloides*, Social Behaviour, Social Neuroscience

Introduction

Behaviour, like all phenotypes, is traceable to how and when genes are expressed. Transcriptional profiling has revealed distinct transcriptional architectures associated with distinct behavioural states (Zayed and Robinson, 2012; Cardoso et al., 2015; Parker et al., 2015; Palmer et al., 2016; Jacobs et al., 2016), which is further reflected in protein abundance (Cunningham et al., 2017). However, gene expression differences within and across behavioural states are not only a result of the genes that underpin the behaviour itself, but can also reflect other factors, such as social context and simple individual variation. An outstanding question is how much gene expression is associated with each source of variation when more than one factor is manipulated. Assessing the influence of multiple factors requires examining those factors with experimental designs that minimize other differences that can exist when examining highly distinct behavioural states (Benowitz et al., 2017; Kronauer and Libbrecht, 2018) and that allow a partitioning of effects. Furthermore, the mechanisms that regulate the variation of gene expression differences are not fully characterised but need to be to fully understand of the evolution and mechanistic basis of behaviour.

In this study, we sought to estimate the association of gene expression with three different factors: (1) differences of behavioural state, (2) differences social contexts, and (3) differences of individual flexibility. We examined all three of these factors influencing male parental care behaviour of the subsocial beetle *Nicrophorus vespilloides*. This social behaviour displays considerable plasticity, making it accessible for the investigation of transcriptional architecture of flexible social behaviours under different conditions. In this species, males but not female parental care is naturally plastic (Smiseth et al., 2005). With a mate, males can but do not always participate in the feeding of the offspring but instead provide indirect forms of care, such as excretion of anti-microbial compounds to cover the carcass (Smiseth et al., 2005; Capodeanu-Nägler et al., 2018). With the removal of his female mate, some males begin feeding offspring (Smiseth et al., 2005). Because we can manipulate male parental care *via* changes of social context, we can generate factorial crosses of males with or without mates that do or do not feed offspring. This helps us directly isolate associations of gene expression with behavioural state, social context, and individual flexibility displayed for those behaviours (Table 1). This is important because social behaviour is multifaceted and moving to factorial designs helps us to begin disentangling single variables, rather than comparing gene expression across behavioural states that necessarily differ by many variables (Lockett et al., 2012; Benowitz et al., 2017).

Within this experimental design we also tested if cytosine methylation regulated any rapid changes of gene expression during socially responsive parental care. Cytosine methylation is a core mechanisms regulating gene expression (Cardoso et al., 2015). It is stable (Turecki, 2014; Yan et., 2015), reversible (Yan et al., 2015), and can have relatively short-term turnover in animals (Levenson et al., 2006; Guo et al., 2011; Herb et al., 2012; Mizuno et al., 2012; Baker-Andresen et al., 2013; *contrario sensu* Cardoso et al., 2015). Therefore, cytosine methylation has excellent characteristics to regulate the gene expression underlying behaviours (Cardoso et al., 2015; Yan et al., 2015). Cytosine methylation is associated with different behaviours of a range of insects, including different hymenopterans (Kucharski et al., 2008; Lyko et al., 2010; Bonasio et al., 2012; Foret et al., 2012; Herb et al., 2012; Lockett et al., 2012; Amarasinghe et al., 2014; Kucharski et al., 2016; Herb et al.., 2018) and an orthopteran (Wang et al., 2014). However, its association with behaviour is not ubiquitous, as cytosine methylation is not

associated with different behaviours of several hymenopterans (Patalano et al., 2015; Libbrecht et al., 2016; Gladstad et al., 2017; Toth and Rehan, 2017), nor with the evolution of social behaviour of insects in general (Bewick et al., 2017). We have a sequenced and annotated genome for *N. vespilloides*, and there is gene body cytosine methylation allowing us to directly assess the association of cytosine methylation with different factors (Cunningham et al., 2015). The role of cytosine methylation underlying gene expression differences of transient behaviours has not been assessed. More generally, it is still unknown the mechanisms underlying rapid, transient, and flexible transitions of behaviour are the same as those that are associated with longer-term behavioural transitions.

Our first goal was to identify gene expression associated with three different factors; differences between behavioural state (directly caring vs non-caring), differences of social context (with or without a mate), and differences of individual flexibility (whether an individual did or did not change their behaviour during the study; Table 1). We predicted that differences of behaviour would have a large influence on gene expression (Parker et al., 2015), followed by difference of social context (Parker et al., 2015), and the influence of individual flexibility of behaviour is largely unknown. We also predicted the possible change of expression of several pathways, including neuropeptides (Cunningham et al., 2016; 2017; Bukhari et al., 2017), neural remodelling factors (e.g, *bdnf*; Cunha et al., 2010), and genes associated with transcriptional regulation in general (Cardoso et al., 2015). Our second goal was to assess if cytosine methylation underpinned the rapid changes of gene expression seen during rapid changes of behaviour using whole genome bisulfite sequencing (WGBS) of the same males used for the gene expression experiments. Assuming cytosine methylation underpins behavioural changes, we expected to see cytosine methylation levels would change for behaviourally-responsive genes (Cardoso et al., 2015; Yan et al., 2015). We found many differences of gene expression between caring and non-caring behavioural states, fewer expression differences due to social context or individual flexibility. Very few cytosine methylation changes were associated with any of the factors we manipulated. Thus, differential expression of genes accompanies rapidly changing behaviour with a hierarchy from behavioural state, social context, and individual flexibility; however, cytosine methylation does not appear to underpin any of these rapid changes and the epigenetic mechanisms that influence this process remain to be identified.

Materials and Methods

Parental Care of *N. vespilloides*

The parental care behaviour of *N. vespilloides* is multifaceted, easily observed, and reliably scored (Smiseth et al., 2004, 2005; Walling et al., 2008). Parental care in all burying beetle species is extensive and elaborate, including direct provisioning of regurgitated food to begging offspring (Eggert and Müller, 1997; Scott, 1998). Parental care can be uniparental or biparental, often within a species. Adults search for and bury a small vertebrate carcass on which they feed and rear offspring. Parents provide both indirect and direct care. Before offspring are born there is indirect care involving stripping the fur (or feathers or scales) from the carcass, forming it into a nest, and preventing microbial growth on the carcass through excretions (e.g., Palmer et al., 2016; Duarte et al., 2018; Shukla et al., 2018; Wang and Rozen, 2018). The latter form of indirect care also occurs after offspring are present, along with resource defence (Walling et al., 2008). Eggs are deposited away from the carcass while it is being manipulated into a suitable larval food resource. When eggs hatch, the larvae crawl to the carcass and reside in a small cavity

excised by the parents in the carcass. Parents provide direct care by regurgitating pre-digested carrion directly to their dependent, begging offspring and by depositing oral secretions into the larval cavity to prepare it for larval occupation and consumption (Scott, 1998). In *N. vespilloides*, the species studied here, parental care can be provided under multiple social contexts; by both parents or either individually without influencing the survival or vigour of larvae (Parker et al., 2015). When both parents are present, females bias their time toward direct care of offspring (i.e., feeding them) while males spend more time on indirect care (Smiseth et al., 2005). This system is amenable to our experimental manipulation (Table 1) as removing females while larvae are still young results in some males initiating direct care (Smiseth et al., 2005), while other males will maintain their level of direct care observed when a female is present (generally, high level or none). This allowed us to create a factorial design to separate the influence of behavioural state, social context, and individual flexibility on gene expression.

Experimental Design and Behavioural Observations

We obtained beetles from an outbred colony of *N. vespilloides*, originating from Cornwall, UK, and maintained at the University of Georgia (Cunningham et al., 2014, 2017). This colony is augmented with new families yearly from the same origin population. We followed the protocol of Smiseth et al. (2005) to generate flexibly caring males. Unrelated female and male pairs (age 14 – 29 days) were placed into a mating box with a mouse carcass (19-21g) and 2.54 cm of moist soil. The boxes were observed every morning (approximately 9:00 am) and evening (approximately 17:00) starting at 60 h post-pairing until larvae arrived at the carcass. 21 h after larval arrival, each pair was observed using 1 minute scans for 10 minutes an hour for four observation periods. We then repeated the observation protocol 24 h later. There were two treatments on Day2: we removed half the female from pairs where the males showed no direct care on Day1 and left the pairs intact for the other half. If males were observed caring on Day 1, we left the pair intact. All pairs were observed both days regardless of treatment.

Because we were first interested in separating the influence of three factors on gene expression, we designed an experiment that manipulated males into one of four different experiences that allowed us to assess three a priori contrasts (Table 1a): The first sample, phenotypically “Flexible care”, contained males that initiated direct care (directly feeding offspring) when the female was removed. The second, “Non-flexible no-care”, contained males that were not observed to directly care for offspring even if the female was removed. The third sample, “Biparental no-care”, contained males that never directly care for offspring with the female present both days. The fourth sample, “Biparental care”, contained males that that always directly cared for offspring with the female present both days. No-care samples might have dispersed from the carcass, which is why they might not have been observed to care. It is hard to tell with our protocol as the boxes are so small that males are often found near but not on the carcass. Near but not on can signify rest or dispersal, which can only be distinguished with multiple widely spaced observations that directly opposes our ability to gather samples directly after the behavioural observations. Other samples are not available as males do not provide direct care on Day 2 if they do not provide direct care on Day 1 in the presence of females, and males that provide direct care on Day 1 rarely change to no direct care on Day 2 regardless of the presence or absence of the female. To maximize power, we only selected males for analysis that showed “pure” phenotypes; that is, consistently high care or absolutely no direct care throughout all observation periods. We observed 104 pairs in total (Table S1).

mRNA-sequencing (RNA-seq) Preparation, Sequencing, and Quality Control

We dissected brains from individual males as in Cunningham et al. (2014), with the exception that samples were snap frozen in liquid nitrogen after dissection and stored at -80 ˚C. From these samples we extracted RNA and genomic DNA (gDNA) simultaneously using Qiagen's AllPrep DNA/RNA Mini Kit (cat. # 80284; Hilden, Germany) following the manufacturer's protocol after homogenization with a Kontes handheld pestle (Kimble Chase, Rockwood, TN, USA) to allow us to quantify both gene expression and methylation level from the same individual. We quantified RNA and gDNA with a Qubit 2.0 Flourometer (Invitrogen, Carlsbad, CA, USA) using the RNA Broad Range and dsDNA High Sensitivity protocols, respectively, following the manufacturer's instructions.

We prepared libraries for RNA-seq with a modified Smart-seq2 protocol (Picelli et al., 2014) using a target of 80 ng of total RNA per library and barcoded with Illumina TruSeq indexes. Libraries were SPRI'ed to select for fragments between 300-1000 bp and insert size was estimated with a Fragment Analyzer Automated CE System (Advanced Analytical, Ankeny, IA, USA). We sequenced 24 samples (six from each of the four behavioural states), assigned to one of two pools to evenly distribute samples based on experimental factors across the two lanes, with a 75bp single-end (SE) protocol using to Illumina's NextSeq500 with a High-Output flow cell targeting 35 million reads per sample at the University of Georgia's Georgia Genomics Facility (Table S2).

We assessed the quality of the raw sequencing reads using FastQC (v0.11.4; default settings; bioinformatics.babraham.ac.uk/projects/fastqc). We trimmed for the transposase adapter, reads based on quality (Phred > 15 at both ends), trimmed the last two base pairs of the reads due to highly skewed nucleotide frequencies, and reassessed quality of the reads using FastQC (v0.11.4) and Cutadapt (v1.9.dev1; Martin, 2011).

Differential Gene Expression and Gene Ontology (GO) Analysis

We combined data from the four groups of males with different experiences of parental care, social context, and individual flexibility to parse the effect of different influences and refine the potential causal differences (Table 1b). We preformed three contrasts. First, we compared all those individuals that displayed direct care to those that did not, regardless of social context (Behavioural State contrast). This compares males that transition from a no-care state to a direct care state versus those that do not make this transition with or without the female. Next, we compared individuals in the presence of a female both days to those where a female was absent the second day, regardless of their own behaviour (Social Context contrast). This tests for the transcriptional response to difference of the social context. Finally, we tested for the transcriptional response to changes of individual behaviour regardless of social context or starting behaviour (Individual Flexibility contrast). This directly compares flexible to non-flexible individuals. Taken together, then, these three contrasts lead to a description of gene expression unique to each source of variation influencing behavioural changes.

We used RSEM (v1.2.20; default settings; Li and Dewey, 2011) with BowTie2 (v2.2.9; default settings; Langmead and Salzberg, 2012) to map and quantify reads against the *N. vespilloides* Official Gene Set (OGS) v1.0 transcriptome (Cunningham et al., 2015). To better assess the completeness of the

Nv OGS v1.0 before mapping, we used the updated BUSCO gene set (v2.0; default settings; Simao et al., 2015) with the Arthropoda Hidden Markov Models (2,675 HMM gene models). This gene set is defined as gene models that are present in 90% of the searched species as single-copy orthologs. We found that 2,607 (97.5%) genes were present with 2,484 (92.8%) as complete gene. Of the complete genes, 2,183 were single-copy orthologs and 301 were duplicated. A further 123 genes were fragmented.

Differential expression was estimated using both a parametric and non-parametric differential gene expression analysis to find genes that individually exhibited strong responses to our manipulation. These two methods find differential expression based on different biological signals and so can identify different sets of genes between contrasts of interest. For each analysis, we performed three contrasts (Table 1b).

We imported the expected read count per gene from RSEM into the DESeq2 package (v1.12.3; default settings; Love et al., 2014) using the tximport package (v1.0.3; Soneson et al., 2015) of R (v3.3.1; R Core Team, 2016). Following the suggested workflow of DESeq2, we preformed overall sample quality control by visual inspected for and removed two outlier samples (one Flexible care, one Non-Flexible care) after completing quality control by visual inspection of a principal component analysis (PCA) plot using the data without regard to any factor in the study design. Statistical significance was assessed after a Benjamini-Hochberg (BH) correction of *P*-values (Benjamini and Hochberg, 1995). We used NOISeq (2.16.0; Tarazona et al., 2015) to test for differential gene expression as a non-parametric method. Following the suggested workflow of NOISeq, we preformed overall sample quality control by visual inspected for and removed one outlier sample (flexible care) after completing quality control by visual inspection of samples on PCA plot using trimmed mean of M-values (TMM) standardized data without regard to any factor in the study design, as per program guidelines. Each analysis was conducted using TMM standardized data, filtering out genes with counts per million reads (CPM) < 1, correcting for gene length, substituting zero gene counts with 0.01 to avoid undefined gene counts, and with 20 permutations using the NOISeqBIO function. Statistical significant was assessed after a BH correction of *P*-values. We used all the unique differentially expressed gene from either DESeq2 and NOISeq gene list (i.e., the union of the two gene lists) for each of the three contrasts to test for enrichments of all three categories of Gene Ontology (GO) terms: biological process, molecular function, and cellular component. We used the AgriGO webserver to test for enriched GO terms (Du et al., 2010). We performed a Singular Enrichment Analysis (SEA) using Complete GO terms and a hypergeometric test with a BH correction. The complete list of GO terms assigned to all *N. vespilloides* genes was used as the background for the enrichment test.

Because genes usually act within a network, and whole networks can exhibit responses to a manipulation even if the individual genes within the network do not, we also performed a weighted gene co-expression network analysis (WGCNA). This technique also allows for the centrality of a gene to a network to be estimated with the assumption that genes deeply connected within a network are of increased overall importance because changing their expression influences many other genes. We again looked for associations with our three contrasts and the expression of gene modules between these contrasts. We used the WGCNA package of R (Storey, 2002; Langfelder and Horvath, 2008) to perform a weighted gene co-expression network analysis using default guidelines and parameters. We used the Variance Stabilized Transformation that was blind to the study design from DESeq2, with the same two outlier samples removed, as input data with genes with <10 reads in 20 samples removed, as per programs

suggestion. We converted the correlation matrix of variance stabilized transformed values (DESeq2's default transformation) to a signed adjacency matrix with an exponent of 10 and a minimum module size of 30. We tested for an association between modules and traits of interest using the biweight mid-correlation (bicor) function with a robustY setting, as per program guidelines for our data types. Modules significantly associated with traits were assessed for enrichment of GO terms as described above.

We also established orthology between Amel OGS3.2 (Elsik et al., 2014) and Nicve OGS1.0 using OrthoFinder (default setting using diamond; v2.1.2; Emms and Kelly, 2015) to assess the overlap of differentially expressed "direct caring" genes between Shpigler et al (2018) and this study.

MethylC-seq Preparation and Differential Gene- and Cytosine-Methylation Analysis

We used MethylC-seq to estimate levels of cytosine methylation associated with different behavioural states. We prepared MethylC-seq libraries following Urich et al. (2015) targeting 200 ng of gDNA as input per library. Six individuals, three each from sample group 1 & 2 (Table 1a), that we used for RNA-seq were haphazardly chosen for whole genome bisulfite sequencing. Libraries were quality controlled with the above RNA-seq protocol. We sequenced six adult samples with a 150bp single-end (SE) protocol using Illumina's NextSeq500 with a High-Output flow cell at the University of Georgia's Georgia Genomics Facility (Table S2).

We followed the protocol of Cunningham et al. (2015) to determine the methylation status of individual cytosines and genes that was used to survey the methylome of larval *N. vespilloides*. Briefly, we used the methylpy analysis pipeline (Schultz et al., 2015) that checks reads for adapter contamination and quality score trimming with cutadapt (v1.9dev), maps with Bowtie1 (v1.1.1; parameters: -S -k 1 -m 1 --chunkmbs 3072 --best --strata -o 4 -e 80 -l 20 -n 0), removes PCR duplicate reads with Picard (v2.4.1; default settings; broadinstitute.github.io/picard), and uses a BH corrected binomial test against the sample specific non-conversion rate of fully unmethylated lambda gDNA to call methylated cytosines. Cytosines within a region of interest (here, CDS) were aggregated and a BH corrected binomial test against the mean percentage of methylated cytosines per gene is used to call methylated genes. To estimate how conserved gene methylation status is between adult and larval life history stages, we re-analysed the six adult samples from this study and the three larvae samples from Cunningham et al. (2015; NCBI BioProject: PRJNA283826) together. To address the influence of different sequencing coverage between these samples, we restricted our analysis to genes that had at least five CpGs covered with at least three mapped reads; Cunningham et al., 2015 within the CDS regions for all nine samples (i.e., we only assessed genes with sufficient amounts of information from all samples to reduce the influence of noise from low-coverage CpGs and coverage differences between samples). A BH corrected binomial test determined the methylation status of each gene within each sample using the mean percent of methylated CpGs of all samples across all genes as the null probability. Genes identified as methylated in all adult samples and unmethylated in all larval samples were defined as adult-specific methylated genes, and vice-versa. We defined the overlap as the union of adult methylated genes compared with the union of the larval methylated genes.

We estimated differential cytosine methylation amongst the two adult behavioural states (flexible care vs. nonflexible no-care) in two different ways (qualitative and quantitative) at the gene (Patalano et

al., 2015) and individual nucleotide (Libbrecht et al., 2016) levels. Our analysis was designed within an exploratory framework to capture any signal of individual cytosine or gene methylation status associated with social behaviour. For the qualitative analysis at the gene level, we assessed how many genes were consistently methylated or non-methylated in one sample group while having the opposite methylation status in other sample group. The quantitative analysis was a BH-corrected *t*-test of the proportion of methylated cytosines across a gene or a BH-corrected *t*-test of weighted methylation level across a gene (# of methylated reads/all reads mapped to a cytosine; Schultz et al., 2012) with at least 10 mapped cytosines (12,627 genes meet the minimum coverage threshold; Patalano et al., 2015).

For the qualitative analysis at the nucleotide level, we assessed how many cytosines were methylated or non-methylated in one sample group while having the opposite methylation status in the other sample group. The quantitative analysis was a BH-corrected *t*-test of the weighted methylation level (# of methylated reads/all reads mapped to a cytosine) for every cytosine that was mapped in all adult samples with at least five reads.

Results

Behavioural Analysis

In the sample where males were induced to shift from no-care to direct care (Table 1, sample 1), the percentage of observed time spent directly feeding larvae shifted from 0 (with female; Day 1) to 28.3 ± 0.4 (after female removal; Day 2). In samples where females weren't removed but males direct care was observed (Table 1, sample 4), males spent 34.0 ± 5.5% of the observation period on direct care in Day 1, and 35.9 ± 4.1% of the observation period directly caring for larvae on Day 2. These results recapitulate those of Smiseth et al. (2005).

There was no statistically significant difference in the proportion of direct care (DC) and indirect care (IC) for males that expressed care with and then also without a partner (Day 1: DC – 34.0%, IC – 4.6%; Day 2: DC – 35.9%, IC – 3.5; DC: $t_{23.5}$= -0.22, P = 0.826; IC: t_{24}=0.269, P = 0.79). There was also no statistically significant difference in the proportion of direct care (DC) and indirect care (IC) for males that expressed care (Day 2: Biparental Care – DC – 35.9%, IC – 0.35%; Flexible Care – DC – 28.3%, IC – 5.3%; DC: $t_{25.2}$ = 1.010, P = 0.32; IC: $t_{24.2}$ = -0.522, P = 0.61).

Differentially Expressed Genes and Gene Co-Expression Networks

To identify differentially expressed genes and gene co-expression networks associated with changes of behaviour in different contexts, we investigated gene expression between three contrasts: behavioural state, social context, and individual flexibility (Table 1b; Table S3). For the behavioural state contrast, we found 522 total differentially expressed genes using parametric analysis (177 genes up-regulated and 345 genes down-regulated contrasting caring to non-caring), 150 differentially expressed genes using non-parametric analysis (138 genes up-regulated and 12 genes down-regulated; the union of two sets is 552 genes; Fig. 1), and seven co-expressed gene modules using WGCNA (Modules 1, 2, 5, 7, 8, 9, 10; Table 2). For the social context contrast, we found 97 differentially expressed genes using parametric analysis (45 genes up-regulated and 52 genes down-regulated comparing without partner to with partner), zero

genes differentially expressed using non-parametric analysis, and one co-expressed gene module using WGCNA (Module 5; Table 2). For the individual flexibility contrast, we found 17 differentially expressed genes using parametric analysis (eight genes up-regulated and nine genes down-regulated comparing flexible to non-flexible), three differentially expressed genes using non-parametric analysis (all up-regulated; union of two sets is 19 genes), and three co-expressed gene modules using WGCNA (Modules 1, 9, 10; Table 2). High number of down-regulated gene is consistent with the pattern of caring to post-care (Parker et al., 2015). As expected, there was little overlap between the differentially expressed genes between the contrasts suggesting that we could cleanly dissect each effect (Fig 2).

Functional Categories of Genes using Gene Ontology (GO) Analysis

We next used gene ontology (GO) analysis to examine the potential functions or functional categories of the genes and gene co-expression networks associated with each contrast. We found 77 GO terms enriched for the behavioural state contrast, with glutamine family amino acid metabolism, cellular aromatic compound metabolism, carboxylic acid metabolism, oxoacid metabolism, cellular amino acid biosynthetic processes, and organic acid metabolism being the most significantly associated (all P = 0.0063, Table S4). Only two of the seven gene co-expression networks associated with the behavioural state contrast had significant GO enrichment. Module 7 was enriched for terms related to mitochondria, cell envelope, and organelle envelope (all P = 0.037), whereas Module 9 was enriched for terms related to cellular amino acid metabolism, carboxylic acid metabolism, oxoacid metabolism, organic acid metabolism, and small molecule biosynthetic processes (all P = 0.019). Genes differentially expressed associated with variation due to difference of social context were enriched for GO terms related to only three terms; ion binding, cation binding, and metal-ion binding (all P = 0.011). The one gene co-expression network associated with social context had no significant GO enrichments. The differentially expressed genes of the individual flexibility contrast were not enriched for any GO terms. Of the three gene co-expression networks associated with the individual flexibility contrast, only Module 9 had enriched GO terms (see above).

Gene and Cytosine Methylation

We investigated differences of gene or cytosine methylation to assess its relationship with flexibility in expressing care, focusing on a comparison of individuals that changed from no-care to care and those that never changed (Table 1a; sample 1 versus sample 2). This comparison should capture any mechanism associated with changes of behavioural state or individually flexibility. The genes methylated in reproductive adults overlapped highly with methylated genes in *N. vespilloides* larvae (99.4%; Fig 3). However, we found that only 2.1% of conserved adult methylated genes were also differentially expressed in any of our three contrasts (Fig 4, showing largest overlap contrast; Table S5).

We next asked whether any methylation changes at the gene level were associated with individual flexibility of adults. We found no association between the total number of methylated genes and changes of behaviour (t_4 = 0.714, P = 0.515). We then asked if methylation of individual genes differentiates these samples. We found 17 genes displaying a qualitative difference in methylation status. However, two methods of quantitative gene methylation analysis, percent of methylated cytosines and weighted

methylation level, showed that zero and one gene, respectively, differed between flexibly expressed care and non-flexible no-care males.

It could be possible that methylation differences of individual cytosines (rather than across the entire gene body) are responsible for producing phenotypic differences. Therefore, we examined whether methylation of individual cytosines was associated with flexibly expressed care. Qualitatively, we found 460 cytosines with differing methylation status between the two groups. A permutation analysis of our samples showed that 510.5 ± 307.0 (mean ± SD) cytosines differed in methylation status. Therefore, 460 cytosines are no more than expected by chance, and provide little evidence that individual cytosine methylation is associated with behavioural state or individual flexibility. Furthermore, quantitative analysis of cytosine weighted methylation level showed only a single nucleotide (out of 56,753 methylated cytosines that had coverage in all samples) significantly associated with behavioural state or individual flexibility.

Discussion

Gene Expression and Differing Forms of Plasticity

Our results suggest a hierarchy of associations with gene expression during socially responsive parental care. Greater differences of gene expression were induced by manipulating behavioural states (directly caring vs. non-caring), fewer associated with social context, and least associated with individual variation in expressing a behaviour. The first result is consistent with the large body of studies showing differences between many behavioural states are strongly associated with gene expression differences and to a lesser extent with other factors (Zayed and Robinson, 2012; Cardoso et al., 2015; Parker et al., 2015; Toth and Rehan, 2017; Tripp et al., 2018). Better understanding why some factors are more impactful to transcriptional architecture will require more studies using factorial designs to discover general patterns. The more flexible, and therefore rapid, the behavioural change the fewer gene expression changes involved. This suggests that increased flexibility requires fewer changes of gene expression, perhaps because fewer phenotypic components are changing.

By going beyond broad state comparisons, we show that transcriptional architecture depends on the context under which behaviour is expressed. Most studies that compare differences in caring and non-caring states have compared different castes or developmental states in Hymentoptera (Toth and Rehan, 2017). Such transitions are typically long-lasting, and can include confounds from developmental change. It is unclear how to compare such studies and the present study, which is more concerned with rapid changes and switches that can be reversed. A more comparable study is that of Shpigler et al. (2018), which compared young nurses present with queen larvae to initiate alloparental care. Shpigler et al. (2018) sampled the mushroom body of the brain of nurses at 30, 60, and 120 minutes after care was initiated followed by RNA-seq. We examined the overlap of the genes identified by Shpigler et al. (2018) by establishing one-to-one orthology and then asked about the overlap between our 552 Caring DEG and their 1268 DEG from any sampling point. We found 49 orthology groups that contained genes that were differentially expressed in both gene sets (Table S6). We suggest this small overlap has to do with the difference of the sampling points, although it may also reflect the restricted brain region sampled in Shpigler et al. (2018). The latter hypothesis seems less likely, however, because our samples included

mushroom bodies as well as other part of the brain. Instead, we may have missed the genes causing a "change of behaviour" and sampled the "stable expression of behaviour" genes. We are clearly at a point where we need more functional studies of genes associated with parenting. This further highlights the message that considering the context, rapidity, and type of change is important.

Comparisons can also be made with a recent studies using *N. vespilloides*. Genes previous found to be differentially expressed in *N. vespilloides* when transitioning from a completely non-parenting to parenting state; such as *vitellogenin 1*, *vitellogenin 2*, and their receptor (*vg1*, *vg2*, *vgr*; Roy Zokan et al. 2015) and *neuropeptide f* and its receptor, *npfr* (Cunningham et al. 2016); were not involved in the subtler plasticity examined here. Benowitz et al. (2017) sampled *N. vespilloides* and *Nicrophorus orbicollis* males and females that contrasted the transcriptional architecture of high vs low direct care expressed by parents in a 2 x 2 x 2 design. This study found very subtle differences of transcription associate with the level of direct care expressed by parents. Here we found very clear differences of gene expression when comparing males providing direct care to those that did not. In this way, we align much more with an earlier study of *N. vespilloides* by Parker et al. (2015) that compares very large differences of behaviours/social context (virgin to direct care to post-care) and finds very clear differences of gene expression. Our current study reaffirms the suggestion of Benowitz et al. (2017) that transcription variation underpinning the phenotypic variation within a behaviour (high vs low direct care) is much subtler than the transcriptional variation that underpins complete changes of behaviours (high vs no direct care). We also suggest this highlights how impactful small changes of behaviour can be to transcriptional architecture.

When we assessed the functional categorization of the differentially expressed genes and gene co-expression networks, we found an abundance of metabolic related categories. Despite the abundance of GO terms related to metabolism, we do not expect these genes to reflect the energetic cost of parenting because we only sampled brains. Instead, we suggest that metabolic genes might be co-opted for a social function in *N. vespilloides*, as is argued elsewhere (Zayed and Robinson, 2012; Rittschof et al., 2014; Wu et al., 2014; Cunningham et al., 2016; Fischer and O'Connell, 2017). Alternatively, metabolic genes may be involved in neurotransmitter synthesis (Livingstone and Tempel, 1983), as many neurotransmitter pathways influence parental care (Mileva-Seitz et al., 2016). One potentially interesting candidate gene found in both the list of differentially expressed genes and as a hub gene in the gene co-expression network (Module 9) associated with caring is NK homeobox 7 (*nk7*). This gene was also one of the only genes showing evidence for positive selection in the *N. vespilloides* genome (Cunningham et al., 2015), and thus multiple lines of evidence suggest it is an important regulator of parental care behaviour. The differentially expressed genes associated with differences of social context related to ion binding, which might be associated with ion-gated channels in the brain that modulate neural activity (Simms and Zamponi, 2013). Thus, these channels may represent a candidate pathway mediating effects of the social context on behaviour. Individual flexibility of behaviour produced a clear gene expression signal associated, but the types of gene underlying this phenotype are difficult to classify. The gene co-expression network associated with flexibility is more strongly associated with caring than with individual flexibility *per se*. Individual flexibility in ants and bees is associated with morphological changes in the brain (Gronenberg et al., 1995; Groh et al., 2006), and thus we expected to detect genes annotated with neurotropic activity or neuron axon manipulation. That we made no such observation suggests that gross morphological changes in the brain might only be seen in species that make permanent

or developmental changes between behavioural states (Cardoso et al., 2015). It is also possible that we sampled males too late to capture the genes involved in causal changes in behavioural change, especially the immediate early genes that respond within minutes to hours to a stimulus (Cardoso et al., 2015).

Cytosine Methylation is Not Associated with Plastic Parental Care

There is little evidence to suggest that methylation at the individual gene or individual cytosine level is associated with behavioural state or individual flexibility of male direct care of *N. vespilloides*. There were few differences at the gene or individual cytosine level between the two samples compared (Flexible Caring vs. Non-Flexible Non-Caring). Furthermore, very few (2.1%) of the adult methylated genes were also genes that were differentially expressed for any of the three contrasts of gene expression. Adult methylated genes were also highly overlapping with larval methylated genes, which indicates that gene methylation is stable across broad life history stages (and generations) encompassing widespread behavioural and physiological changes.

Our results are informative because we assessed a transient behaviour, extending the range of behaviours that cytosine methylation has been thought to influence. Our results also fall in line with other studies of social insects demonstrating few differences of cytosine methylation between different behavioural states (Patalano et al., 2015; Libbrecht et al., 2016). Not all social Hymenoptera even have active DNA methylation systems (Standage et al., 2016). Therefore, cytosine methylation does not appear to be a general mechanism to regulate behavioural changes of insects (Patalano et al., 2015; Libbrecht et al., 2016; Bewick et al., 2017; Glastad et al., 2017), but it remains possible that it might regulate socially responsive gene expression of any one species, such as in honey bees and long-term changes of aggression after territorial challenges (Herb et al., 2018).

Conclusion

Using the socially responsive and naturally variable male parental care of the subsocial beetle *Nicrophorus vespilloides*, we made a series of comparisons to understand the influence of behavioural states, social context, and individual flexibility on transcriptional architecture of a transient social behaviour. We found clear signals of gene expression after manipulating behavioural state (directly caring vs. non-caring), social context (with or without a female mate), and to a much lesser extent with an individual's ability to rapidly change behaviour. This suggests a complex and hierarchical influence on the transcriptional architecture of parenting behaviour by males. Research on behavioural transitions has long examined the role of single molecules, such as neuropeptides and hormones. Thus, it is perhaps unsurprising that an individual's ability to change behaviour might involve fewer changes of gene expression. While differential gene expression has for many genes long been associated with changes of long-term or permanent behaviour (Zayed and Robinson, 2012; Cardoso et al., 2015), this study further supports that gene expression is also associated with more rapid changes of behaviour responding to immediate environments. Contrary to previous predictions regarding epigenetic control of behaviour (Cardoso et al., 2015; Yan et al., 2015), we find no support for an association between cytosine methylation and the expression of direct parental care or individual flexibility and conclude that rapid changes of cytosine methylation is not the mechanism underpinning the rapid behavioural transitions for this species. This leads to the conclusion that, contrary to some predictions, rapid gene expression

affecting behaviour may be regulated by standard processes of transcriptional control. Our work suggests that studying the genetic underpinnings of behavioural flexibility, a key attribute that defines behaviour as a unique phenotype (Bailey et al., 2018), should consider the extent that the behavioural change is transient.

Data Availability: Data associated with this project are available at NCBI BioProject PRJNA375005. Genomic resources for *N. vespilloides* are now collated at an i5k Workspace at the National Agricultural Library of the USDA (i5k.nal.usda.gov/nicrophorus-vespilloides).

Conflict of Interest. We have no conflicts of interest to declare.

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Author Contributions. CBC and AJM conceived the idea of the project. CBC, RJS, AJM designed the experiment. CBC, KMB, ECM performed the behavioural observations. CBC, ECM processed the samples. CBC, LJ performed data analysis, with assistance from RJS and AJM. CBC, KMB, AJM drafted the manuscript, which was edited by all authors.

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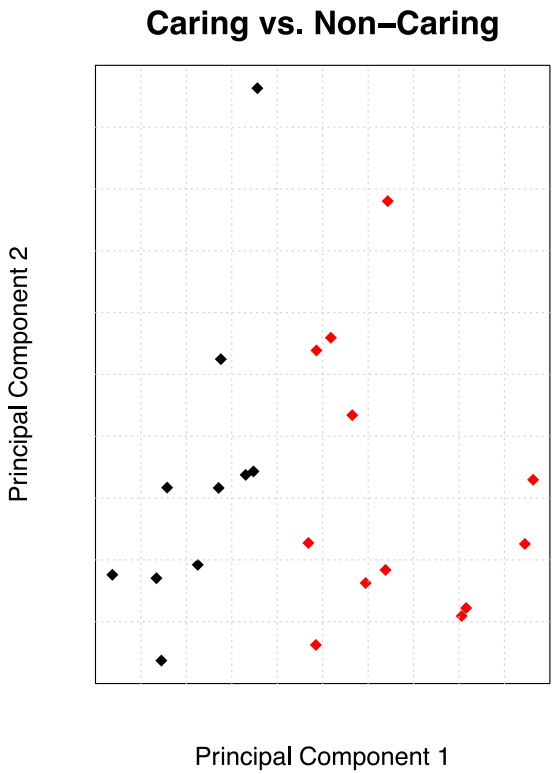
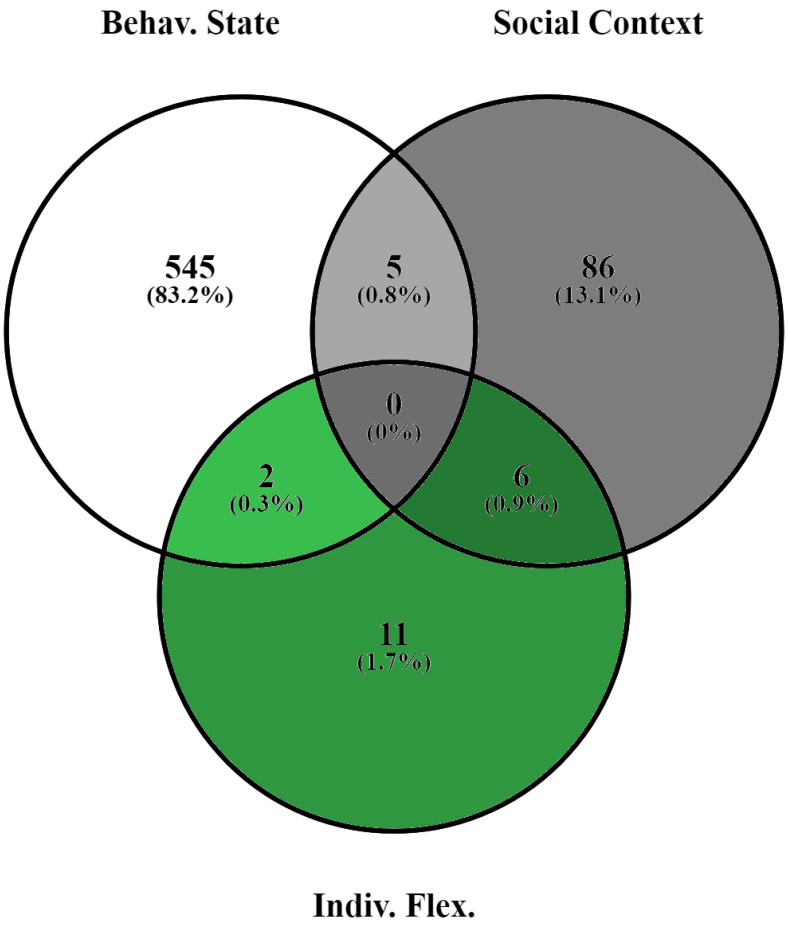


Figure 1. . Principal component analysis of gene expression with individual samples coloured by Behavioural State; caring (black, n = 10) vs. non-caring (red, n = 12) normalized with DESeq2. The graph clearly shows component one as an axis of separation for this contrast.



827
828 Figure 2. Venn diagram showing the overlap of significantly differentially expressed genes between the
829 three contrasts analysed; Behavioural State, Social Context, Individual Flexibility.
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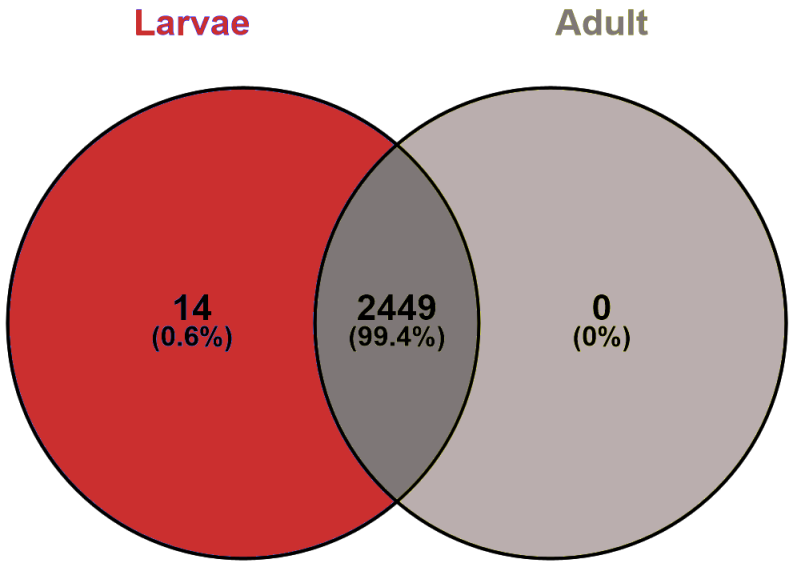

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832 Figure 3. Venn diagram showing the large overlap between the methylated genes of adults and the
833 methylated genes of larvae, using only genes that had high sequencing coverage amongst all samples to
834 adjust for differences of sequencing depth between adult and larval samples.
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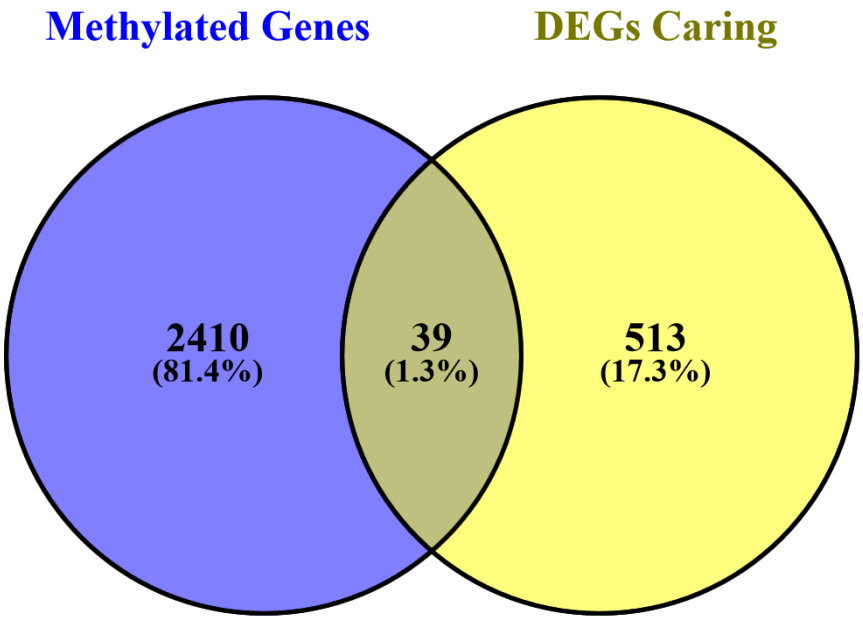

837
838
839 Figure 4. Venn diagram showing the overlap between methylated adult genes and the differentially
840 expressed genes (DEGs) between the caring vs. non-caring contrast.

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Table 1. Experimental design. Four different sample groups collected, reflecting differences in male social context (presence or absence of the female parent) or parental behaviour (expressed or not expressed) on two days of observation. From these, three different contrasts were made.

Table 1a. Samples collected.

Sample	Caring Day 1	Caring Day 2	Social Context* (Day 2)	Flexible	Phenotype Description
1	No	Yes	Mate absent	Yes	Flexible care
2	No	No	Mate absent	No	Non-flexible no-care
3	No	No	Mate present	No	Biparental no-care
4	Yes	Yes	Mate present	No	Biparental care

*Females were always paired with males on Day 1.

Table 1b. Specific sample groups contrasted

Factors	Samples Contrasted
Behavioural State	1+4 versus 2+3
Social Context	1+2 versus 3+4
Individual	1 versus 2+3+4

844
845
846

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847
848

Table 2. Modules of Co-Expressed Genes and their correlation with Behavioural State, Social Context, and Individual Flexibility in the context of parental care by male *N. vespilloides*.

Module No.	No. of Genes	Behavioural State	Social Context	Individual Flexibility
0	1501	-0.044	-0.084	0.0013
1	2113	**-0.74**	-0.085	**-0.51**
2	1850	**0.48**	-0.025	0.13
3	1401	0.013	-0.029	-0.24
4	933	-0.23	-0.25	0.12
5	609	**-0.49**	**-0.44**	-0.05
6	470	0.096	0.19	0.17
7	133	**0.42**	0.35	0.33
8	161	**0.45**	0.35	0.16
9	111	**0.73**	0.093	**0.44**
10	57	**0.92**	0.0087	**0.51**

Statistically significant correlations after BH-correction for multiple testing are in bold.

849
850
851