Title: Changes of gene expression but not cytosine methylation are associated with behavioural plasticity of parental care

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Abstract

Standard evolutionary theory emphasizes that heritable genetic sequence changes drive evolutionary change. This approach has been questioned recently for its ability to explain and incorporate some biological phenomena, such as phenotypic plasticity and epigenetics. Individual plasticity in behaviour, being an extreme form of phenotypic plasticity, has been one of the challenges posed and thus examining the mechanisms underlying behavioural plasticity provides a test case for an extended evolutionary synthesis. To provide such a test, we contrasted gene expression during male parental care in *Nicrophorus vespilloides* under different conditions. Parental care in *N. vespilloides* includes regurgitation of pre-digested food to offspring, and males typically do not provide care. However, plastic male care equivalent to females can be induced by removing a female. Our experimental design allowed contrasts that isolated effects associated with parenting, social conditions, or behavioural plasticity. We investigated differences of both gene expression and cytosine methylation. We found a hierarchy of influences on gene expression with those associated with a transition to parenting showing the most gene expression changes, then gene expression changes associated with different social contexts, and lastly gene expression underlying behavioural plasticity. The genes methylated were nearly identical amongst larvae and adult samples. Further, changes of cytosine methylation were not associated with changes of gene expression in behavioural plasticity. Even in the most plastic of traits, behaviour, genetically programmed alteration of gene expression is a more robust explanation than other non-genetic explanations, and thus standard evolutionary explanations appear to be sufficient for even extreme forms of phenotypic plasticity.

Introduction

There is a current debate amongst evolutionary biologists about whether standard evolutionary theory following from the Modern Synthesis (MS), emphasizing changes of allele frequencies over time (Fisher 1930, Haldane 1932, Wright 1932), is sufficiently comprehensive. The rapidity of adaptive responses of organisms to their environment, the role of developmental plasticity in generating novel phenotypes, and the ubiquity of epigenetics influencing traits associated with fitness benefits has led to the suggestion that we need an extended evolutionary synthesis (EES), which posits that these mechanisms drive rather than simply reflect evolution (Pigliucci 2007; Laland et al., 2011; Laland et al., 2015). EES suggests that new phenotypes arise in response to novel environments, rather than new mutations, and these are then constructive aspects of evolution. Fundamental to EES theory is that non-genetic inheritance mechanisms, i.e., transmission of phenotypic change across generations without changes to DNA sequences, contribute to similarity amongst related individuals (Laland et al. 2015). Genetic assimilation and accommodation arises secondarily to the phenotype produced through plastic response to novel environments (West-Eberhard 2003; Lande 2009).

One of the most extreme forms of phenotypic plasticity in response to novel environments, and therefore one of the challenges to MS, is behavioural plasticity as animals often adaptively change behaviour within seconds or minutes in response to changes of the social or abiotic environment (West-Eberhard, 2003). Behaviour can appear infinitely flexible, with animals displaying novel behaviour in novel environments (West-Eberhard 2003). Differential gene expression is often associated with adaptive plastic behavioural changes (Zayed and Robinson, 2012; Cardoso et al., 2015); however, less information is available on novel expression of behaviour. The MS and EES both predict this plasticity is adaptive, but differ in the mechanism by which the plasticity associated with changes of gene expression is achieved. The MS suggests that behavioural plasticity is a programmed switch to a new transcriptional state, owing to evolution's use of changes of gene regulation (Linksvayer and Wade, 2005; Wray, 2007; Albert and Kruglyak, 2015) and near universal pleiotropy (Wright, 1968). The EES suggests that behavioural plasticity is not completely explained by gene sequences but must also be accompanied by gene-independent yet heritable changes (Pigliucci, 2007; Laland et al., 2011; Laland et al., 2015). Distinguishing between these two requires an examination of non-genetic inheritance underpinning novel or uncommon behaviours.

Cytosine methylation is a strong candidate for a non-genetic mechanism regulating behavioural plasticity. Although other epigenetic mechanisms can affect gene expression, cytosine methylation is one that is expected and has been shown to accompany changes in behaviour (Cardoso et al., 2015; Yan et al., 2015) as 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). Furthermore, many studies report cytosine methylation associated with behavioural plasticity in a range of insects, including different hymenopterans (Kucharski et al., 2008; Lyko et al., 2010; Lockett et al., 2011; Bonasio et al., 2012; Herb et al., 2012; Foret et al., 2012; Amarasinghe et al., 2014; Kucharski et al., 2016) and an orthopteran (Wang et al., 2014). However, the generality of the role of cytosine methylation for behavioural plasticity is unclear (Toth and Rehan, 2017). Its association with behavioural plasticity is not ubiquitous, as cytosine methylation does not associate with individuals expressing different behaviours in several

hymenopterans (Patalano et al., 2015; Libbrecht et al., 2016) nor with the evolution of social behaviour of insects in general (Bewick et al. 2017). However, if behavioural plasticity in insects is more consistent with predictions stemming from the EES, cytosine methylation remains a prime candidate for epigenetic influences on plasticity.

In this study, we ask if plasticity of male parenting behaviour in the subsocial beetle *Nicrophorus vespilloides* is consistent with predictions from the EES by examining whether cytosine methylation is required to produce rapid gene expression changes. This species has several characteristics that make it amenable to test EES predictions. First, we have a sequenced and annotated genome for *N. vespilloides*, and there is gene body methylation in this species (Cunningham et al. 2015). Parenting in *N. vespilloides* has maternal (parental) effects on offspring (Lock et al. 2004, 2007), and thus should influence inclusive inheritance. Parenting is plastic in males but not females (Smiseth et al. 2005), typically expressed under female-only uniparental conditions or biparentally, with male-only care rarely found (Parker et al. 2015). Even during biparental care, males may not participate in the feeding of the offspring but instead provide indirect forms of care (Smiseth et al. 2005). However, consistent with conditions necessary to test EES predictions (Laland et al. 2015), male-only care can be induced (Smiseth et al. 2005) yet parental effects of care are equal for males or females (Parker et al. 2015). Different levels of parenting are inherited, although with different levels of heritability and patterns of genetic correlations in males and females (Walling et al. 2008). This plasticity, as a rare trait expressed in response to an uncommon environment, makes male parenting a useful model for the investigation of non-genetic mechanisms of heritability that could lay the groundwork for genetic assimilation.

We compared males that either did or did not express parental care consistently to males that showed a plastic response to mate loss and care for offspring to look for evidence that non-genetic mechanisms explained the plasticity of male behaviour. We controlled for extant variation in care (individuals that always cared or did not care), and additional effects of altering the social context (presence and absence of females), by making multiple contrasts (Table 1). We predicted to see genes previously identified as being involved with regulating social interactions, such as neuropeptides (Cunningham et al., 2016; 2017), neurotropic factors influencing the remodelling of the brain (e.g, *bdnf*; Cunha et al., 2010), or other gene regulating gene expression (Cardoso et al., 2015), to also be associated with plasticity. We then estimated the association of cytosine methylation underpinning variation by comparing whole genome bisulfite sequencing data from males that showed plastic care to males that never cared for despite their mate being removed. We predicted that data consistent with EES would indicate that the plasticity we see is regulated by cytosine methylation. We found many differences of gene expression between caring and non-caring males, fewer expression differences due to changing social contexts, but few cytosine methylation changes were associated with differences of expression of these socially responsive genes.

Methods

Parenting in *N. vespilloides*

Parenting behaviour of *N. vespilloides* is multifaceted, easily observed, and reliably scored (Smiseth et al. 2004, 2005; Walling et al. 2008), and parenting is extensive and elaborate in burying beetles including

direct provisioning of regurgitated food to begging offspring (Eggert and Müller, 1997; Scott, 1998). Adults search for and bury a small vertebrate carcass on which they feed and rear offspring. Parents provide both indirect and direct care. Before young 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. The latter form of indirect care also occurs after young 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 enzymes into the larval cavity to provide pre-digested food for larvae in the cavity. Parenting 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 provide more direct care to offspring 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 males switching to direct care (Smiseth et al., 2005).

Experimental Design and Behavioural Observations

We obtained beetles from an outbred colony of *N. vespilloides* maintained at the University of Georgia, which is augmented with new families yearly. Our breeding and parenting setup followed the protocol of Smiseth et al. (2005). Unrelated female and male pairs 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 after either removing the female mate of males that showed no direct care or leaving the pair intact for males that showed either care or no care on Day 1.

Our experimental design yielded groups of males with four different experiences provide three contrasts (Table 1). Three factors defined our groups. First, we considered the behaviour observed on Day 1 and Day 2. There were three possible outcomes. Males could care both days, not care both days, or switch from no care to care. Males do not care on Day 1 and then not care on Day 2. Second, males could be categorized as having shown care or not, regardless of consistency between days. Third, males could have experienced the loss of their female mate on day two (Table 1a). Thus, we have four samples (arbitrarily numbered): sample 1 contained males that showed plastic care when the female was removed, sample 2 contained males that never cared even if the female was removed, sample 3 contained males that never cared and the female was not removed, and sample 4 contained males that that always cared and the female was not removed. Other samples are not available as males do not show care on Day 2 after no care on Day 1 in the presence of females, etc. To maximize the power of our molecular study, we only used samples that showed "pure" phenotypes; that is, consistently high care or consistently no-care throughout our observation periods. Given these four groups we could then make three contrasts (Table 1b): care versus no-care (samples 1 + 4 versus 2 +3), which tests for associations with parental care regardless of social context; effects of social context changes (samples 1 + 2 versus 3 + 4), which tests for effects of changing social contexts regardless of the behaviour exhibited; and behavioural plasticity

(sample 1 versus 2 + 3 + 4), which tests for changes in individual behaviour regardless of social context or starting behaviour. Taken together, then, these three contrasts lead to a description of gene expression and epigenetics unique to behavioural plasticity.

Differential gene expression

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). 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 (Supplementary Table 1).

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).

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 2607 (97.5%) genes were present with 2484 (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). We removed two outlier samples (one plastic care, one nonplastic 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, as per program guidelines. Statistical significant 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 using a non-parametric method. We removed one outlier sample (plastic 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. Analysis for each contrast was conducted using TMM standardized data, filtering 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. The union of the sets of genes that were differentially expressed for any contrast were tested for enrichments of Gene Ontology (GO) terms.

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 performed a weighted gene co-expression network analysis. 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 assess for enrichment of GO terms.

We used AgriGO webserver to test for enriched GO terms for each of the comparisons (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.

Differential gene 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. Individuals from sample group 1 & 2 (Table 1a) were haphazardly chosen for sequencing. Libraries were quality controlled with the above RNA-seq protocol. We sequenced six adult

samples with a 150bp single-end (SE) protocol using to Illumina's NextSeq500 with a High-Output flow cell at the University of Georgia's Georgia Genomics Facility (Supplementary File 1).

We followed the protocol outlined in Cunningham et al. (2015) to determine the methylation status of individual cytosines and genes. 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 compared adult samples to larvae samples from Cunningham et al. (2015). To address the influence of different sequencing coverage between these samples, we restricted our analysis to genes that had at least five high coverage CpGs (at least three mapped reads) 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 methyatled 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 (plastic care vs. nonplastic 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 (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 and Discussion

Behavioural Analysis

We manipulated the presence of a female mate to influence male care. In the sample (Table 1, sample 1) where males shifted from no-care to care, the % of time spent directly caring for larvae ranged from 0.0 ± 0.0 (with female; Day 1) to 28.3 ± 0.4 (after female removal; Day 2). In samples where females weren't removed but males cared (Table 1, sample 4), males spent 34.0 ± 5.5 % of the observation period on care in Day 1, and 35.9 ± 4.1 % of the observation period caring for larvae on Day 2. This recapitulated the results of Smiseth et al. (2005).

Gene expression

We wanted to identify the genes associated with behavioural plasticity after a change of social context. We therefore investigated gene expression between three contrasts using our samples, parenting, social context, and behavioural plasticity using three methods (Table 1b): parametric differential expression analysis (DESeq2), non-parametric differential expression analysis (NOISeq), and weighted gene co-expression network analysis (WGCNA; Table 2). For the care – no care contrast we found 522 total differentially expressed genes using parametric analysis (Fig 1), 150 differentially expressed genes using non-parametric analysis (union of two sets is 552 genes), and seven co-expressed gene modules using WGCNA. For the social context contrast, we found 97 differentially expressed genes using parametric analysis, zero genes differentially expressed using non-parametric analysis, and one co-expressed gene module. For the behavioural plasticity contrast, we found 17 differentially expressed genes using parametric analysis, three differentially expressed genes using non-parametric analysis (union of two sets is 19 genes), and three co-expressed gene modules. As expected, there was little overlap between the differentially expressed genes between the contrasts suggesting we were able to cleanly dissect each effect (Fig 2; Supplementary File 1).

All three methods suggest that the greatest gene expression differences are associated with the differences between caring and non-caring males. This result illustrates the difference of plasticity or variation in stable expression within a population, and provides continuing support for the idea that discrete changes in behaviour are related to robust changes in gene expression (Zayed and Robinson 2012; Parker et al. 2015). However, our other two contrasts (social context and behavioural plasticity) examine whether changes in gene expression can be associated with factors independent of the behavioural status of the male. While we find fewer gene expression differences, the answer in both cases is yes. Gene expression is responsive to the presence or absence of a social partner, and differs between plastic and non-plastic individuals. Thus, variability in gene expression appears to be not only associated with actual changes in behaviour, but also with responses to social context and individual plasticity.

We next used gene ontology (GO) analysis to examine potential functions for the genes and networks associated with our three contrasts. Genes differentially expressed for the Caring contrast (combining both methods) were enriched for 77 GO terms, notable categories being glutamine family amino acid metabolism, cellular aromatic compound metabolism, carboxylic acid metabolism, oxoacid metabolism, cellular amino acid biosynthetic processes, and organic acid metabolism (all $P = 0.0063$, Supplementary File 1). Only two of the seven WGCNA modules associated with the Caring contrast had significant GO slim 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 $P = 0.019$). Despite the abundance of GO terms related to metabolism associated with caring, we do not expect these genes to reflect the energetic component of parenting because our samples were brains only. 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; 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 parenting (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 network (Module 9) associated with caring is NK homeobox 7 (*nk7*). This gene was also one of the only genes showing positive selection in the *N. vespilloides* genome (Cunningham et al. 2015), and thus multiple lines of evidence suggest it may be an important regulator of parenting behaviour.

Genes differentially expressed under different social contexts were enriched for GO terms related to only three terms; ion binding, cation binding, and metal-ion binding (all $P = 0.011$). The single gene network associated with social context had no significant GO enrichments. Ion binding genes 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.

The differentially expressed genes were not enriched for any GO terms. Of the three gene networks associated with behavioural plasticity, only Module 9 had enriched GO terms (see above). However, this module is more strongly associated with caring than with behavioural plasticity. Thus, despite a clear gene expression signal associated with plasticity, the types of gene underlying this phenotype are difficult to classify. Behavioural plasticity 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. The fact 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 changing their transcriptional state, especially the immediate early genes that respond within minutes to hours to a stimulus (Cardoso et al. 2015).

Overall, our results suggest that gene expression is associated with behavioural plasticity, and that differential expression shows a hierarchy of differences from fixed state differences, to responses to social change, and to individual plasticity in behaviour. Another way to describe this is that the more stable the behaviour, the more likely it is to be influenced by gene expression. These results are consistent with the large body of studies showing behavioural change is associated with gene expression changes (Zayed and Robinson, 2012; Cardoso et al., 2015; Toth and Rehan, 2017). Nevertheless, differences of gene expression are clearly associated with behavioural plasticity.

Gene and Cytosine Methylation

We investigated differences of cytosine or gene methylation to assess its relationship with behavioural plasticity, focusing on a comparison of individuals that changed from no care to care and those that never changed, as this is the most conservative comparison regarding behavioural plasticity. The genes methylated in reproductive adults overlapped highly with methylated genes in *N. vespilloides* larvae (99.4%), indicating that gene methylation is stable across broad life history stages (and generations) encompassing widespread behavioural and physiological changes (Fig 3). Thus, this fulfils the criteria for a mechanism that could be associated with epigenetic influences. However, we found that only 2.1% of conserved adult methylated genes were also differentially expressed in any of our three contrasts, suggesting that cytosine methylation does not playing a major role in producing gene expression changes (Fig 4, showing largest overlap contrast; Supplementary File 1).

We next asked whether any methylation changes of adults at the gene level were associated with behavioural plasticity. We found no association between the total number of methylated genes and behavioural plasticity ($t_4 = 0.714$, $P = 0.515$). Looking more closely at whether 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, % methylated cytosines and weighted methylation level, showed zero and one genes, respectively, that differed between plastic care and nonplastic no-care males. Therefore, we argue that there is no evidence to suggest that methylation on the gene level is associated with behavioural plasticity.

It is possible, however, that methylation differences of individual cytosines (rather than across the entire gene body) might be responsible for producing phenotypic differences. Therefore, we examined whether methylation of individual cytosines was associated with plastic care. Qualitatively, we found 460 cytosines with differing methylation statuses between the two groups. However, a permutation analysis of our samples showed that 510.5 ± 307.0 (mean ± SD) cytosines differed in methylation status. Therefore, these 460 cytosines are clearly no more than expected by chance, and provide no evidence that individual cytosine methylation is associated with plasticity. Furthermore, quantitative analysis of cytosine weighted methylation level showed only a single nucleotide (out of 65,280) significantly associated with behavioural plasticity.

Extended Discussion

Proponents of the EES suggest that phenotypic plasticity presents a challenge to standard evolutionary theory because phenotypic evolution may precede genetic evolution, leading to genetic assimilation, especially when the new phenotype is novel in response to atypical environments (Laland et al. 2015). If this is correct, then factors beyond DNA sequence should play an outsized role in regulating plastic traits. Here, we examine whether such a hypothesized mechanism, cytosine methylation, plays a role in regulating male parental behaviour in the subsocial beetle *Nicrophorus vespilloides*.

We performed a series of comparisons where different combinations of potential influences on male parenting behaviour were manipulated or measured to isolate different influences on different forms of plasticity, looking for overlap. We find clear signals of genetic influences associated with behavioural plasticity, as gene expression is clearly associated with parenting, changes in social experience, and behavioural plasticity. Because we observed differential gene expression within each comparison, by

extension gene expression influences plasticity in parental care whatever the stimulus for the plasticity. The observation of gene expression differences associated with plastic behaviour is, in itself, consistent with either the EES or the Modern Synthesis (MS). It is the causal factors underlying these expression changes that will ultimately distinguish these theories.

In order to examine whether male *N. vespilloides* behavioural plasticity may be underpinned by factors beyond DNA sequence, we measured levels of cytosine methylation between plastic and non-plastic individuals. Cytosine methylation is a strong candidate mechanism for genetic assimilation because it is environmentally sensitive (Angers et al., 2010) and heritable (Yan et al. 2015). However, while *N. vespilloides* has an active methylation system (Cunningham et al. 2015), we find no support for an association between cytosine methylation and expression of parenting, falling in line with a growing body of literature in social insects demonstrating few differences of cytosine methylation between different behavioural states (Patalano et al. 2015; Libbrecht et al. 2016). Moreover, not all social Hymenoptera even have active DNA methylation systems (Standage et al. 2016). Thus, while it remains possible that cytosine methylation will play a role in regulating socially responsive gene expression in any one species, it does not appear that cytosine methylation is a general mechanism to regulate behavioural changes in insects (Patalano et al. 2015; Libbrecht et al. 2016; Bewick et al. 2017). Furthermore, even in honey bees were many studies have reported associations between cytosine methylation and behaviour, recent research suggests that *cis*-regulatory transcription factors are strongly associated with dynamic changes in behaviour in response to social cues (Shpigler et al. 2017).

Our data on parenting of this subsocial burying beetle (Coleoptera) add to the growing evidence in eusocial Hymenoptera suggesting that non-genetic processes are not necessary to produce complex patterns of behavioural plasticity. In combination, all these results support the idea that behavioural plasticity can be explained as a programmed response to environmental variation encoded directly by DNA sequence, and therefore fits more neatly within the classical paradigm of evolutionary genetics laid out during the Modern Synthesis.

Data Availability: Data associated with this project are available at NCBI under BioProject PRJNA375005.

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Competing Interests. The authors declare no competing financial interests.

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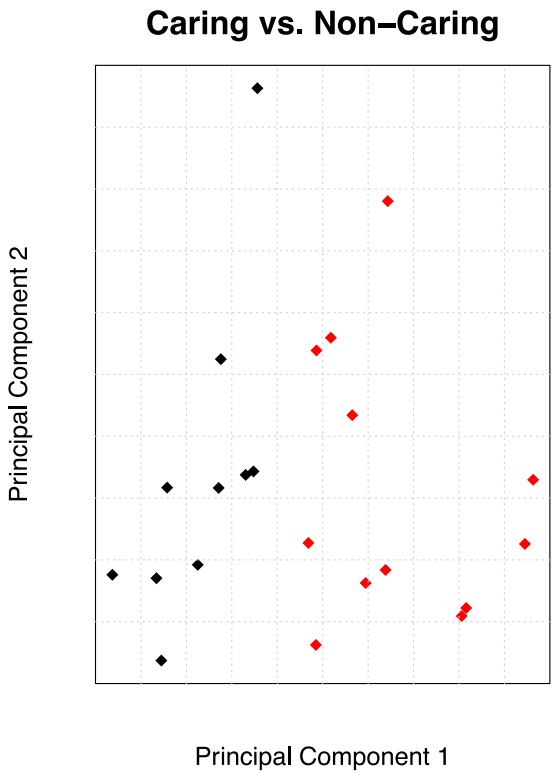

Figure 1. Principal component analysis of gene expression with samples coloured by caring (black) vs. non-caring (red) contrast. The graph clearly shows component one as an axis of separation for this contrast.

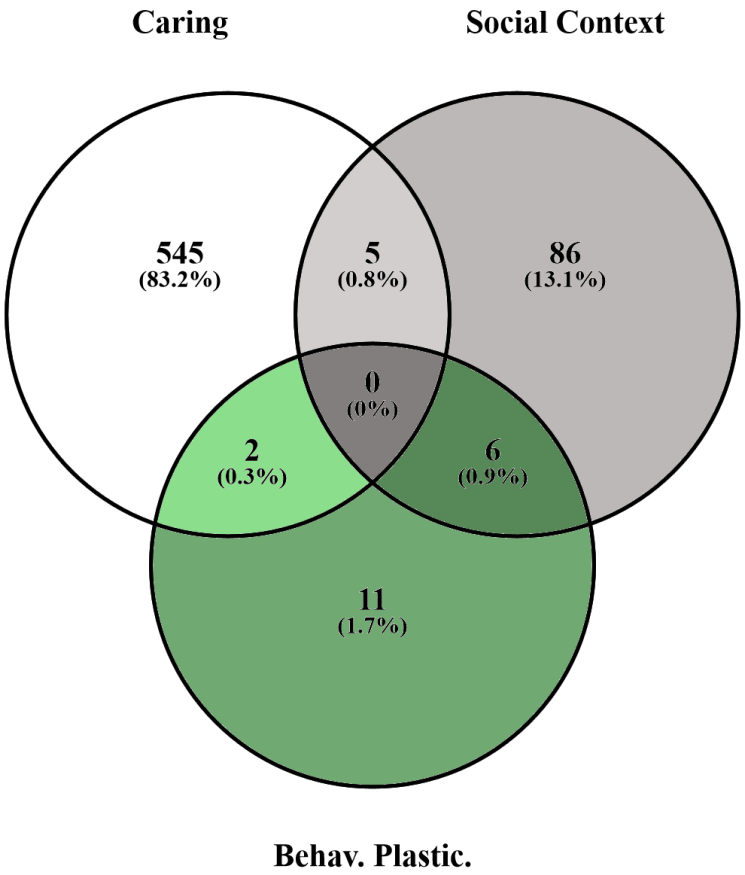

Figure 2. Venn diagram showing the overlap of significantly differentially expressed genes between the three contrasts analysed; Parenting, Social Context, Behavioural Plasticity.

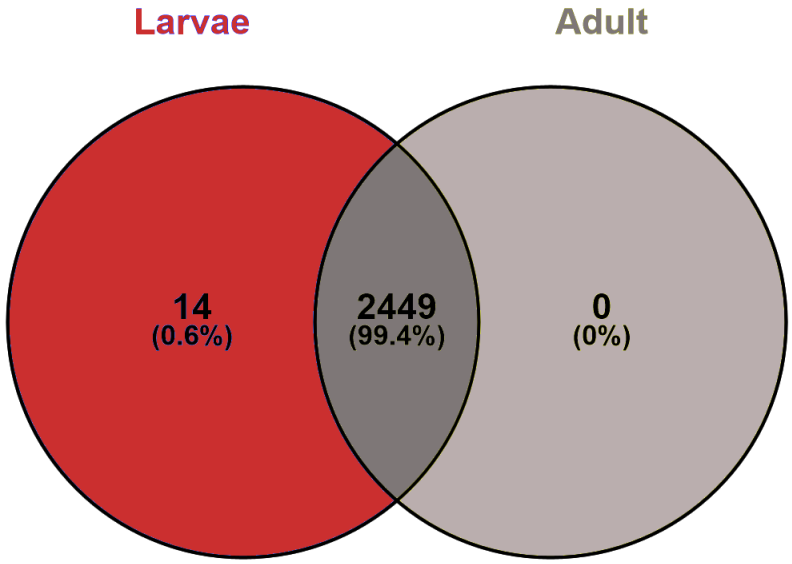

Figure 3. Venn diagram showing the large overlap between the methylated genes of adults and the methylated genes of larvae, using only genes that had high sequencing coverage amongst all samples to adjust for differences of sequencing depth between adult and larval samples.

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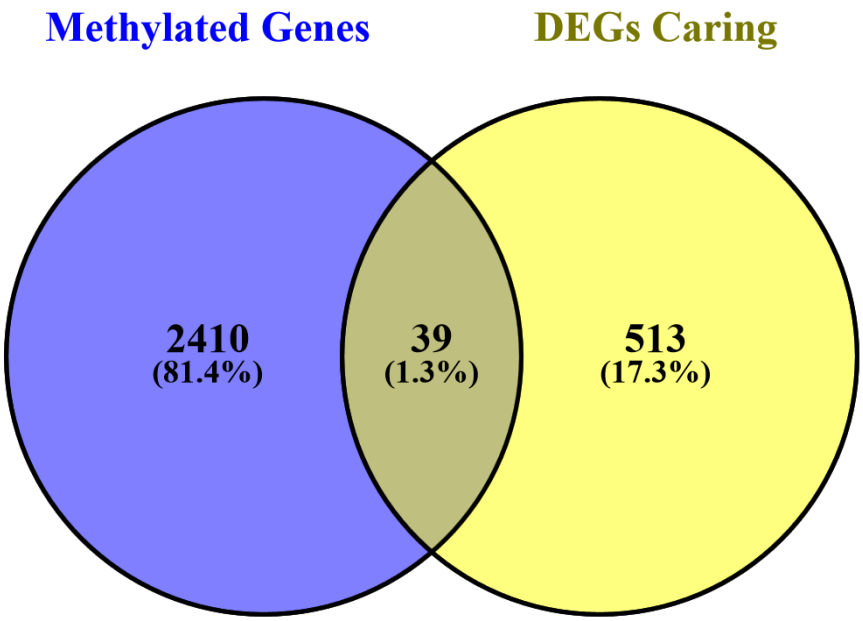

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735 Figure 4. Venn diagram showing the overlap between methylated adult genes and the differentially
736 expressed genes (DEGs) between the caring vs. non-caring contrast.

Table 1. Experimental design. Four different sample groups collected, reflecting differences in male social context or parental behaviour on two days of observation. From these, three different contrasts were made.

Table 1a. Samples collected.

Sample Group	Exhibit Caring (Day 1)	Exhibit Caring (Day 2)	Social Context* (Day 2)	Plastic Behaviour	Phenotype Description
1	No	Yes	Mate absent	Yes	Plastic care
2	No	No	Mate absent	No	Nonplastic 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

Contrast	Type of Plasticity	Samples in Group 1	Samples in Group 2
Parenting	Care versus no-care	1+4	2+3
Social Context	Environmental	1+2	3+4
Behavioural Plasticity	Individual	1	2+3+4

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Table 2. Modules of Co-Expressed Genes and their correlation with Caring, Social Context, and Behavioural Plasticity

Module No.	No. of Genes	Caring	Social Context	Behavioural Plasticity
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 bolded.

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