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Article Title: Patterns of parent and offspring gene expression reflect canalization, not plasticity, in response to environmental stress

Short Running Title: Stress constrains genetics of family interactions

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ABSTRACT

Parenting buffers offspring from hostile environments, but it is not clear how or if the genes that underlie parenting change their expression under environmental stress. We recently demonstrated that for the subsocial carrion beetle, *Nicrophorus orbicollis*, temperature during parenting does not affect parenting phenotypes. Here, we ask if transcriptional changes associated with parenting are likewise robust to environmental stress. The absence of a transcriptional response for parenting under stress would suggest that the genetic ‘programs’ for parenting and being parented are canalized. Conversely, a robust transcriptional response would suggest that plasticity of underlying gene expression is critical for maintaining behavioral stability, and that these mechanisms provide a potential target for selection in the face of environmental change. We test these alternatives by characterizing gene expression of parents and offspring with and without parent-offspring interactions under a benign and a stressful temperature. We found that parent-offspring interactions elicit distinct transcriptional responses of parents and larvae irrespective of temperature. We further detected robust changes of gene expression in beetles breeding at 24°C compared to 20°C irrespective of family interaction. However, no strong interaction between parent-offspring interaction and temperature was detected for either parents or larvae. We therefore conclude that canalization, not plasticity of gene expression, most likely explains the absence of behavioral plasticity under thermal stress. This result suggests that species may not have the genetic variation needed to respond to all environmental change, especially for complex phenotypes.

IMPACT STATEMENT

Parenting is thought to evolve to buffer offspring from harsh or inhospitable conditions. However, once evolved, does parenting continue to buffer against new environmental challenges such as might occur under global warming? One way organisms achieve different phenotypes without evolving different genes for each trait is by changing timing or extent of gene expression. Changes in gene expression influencing physiological traits such as hypoxia have been shown to facilitate adaptation to changing environments. It is less clear if changes in gene expression are also important for behavioral plasticity in complex social traits, such as parental care. In this study, we ask whether families of the subsocial burying beetle, *Nicrophorus orbicollis*, show altered expression of parent or offspring genes that may help compensate for reduced offspring success under global warming. To test the extent that gene expression underlies behavioral plasticity in parenting and offspring responses to parenting, we characterize the gene expression profiles of whole families (males, females, and larvae) with and without parent-offspring interactions under two thermal conditions: 20°C and 24°C. We found that heat stress predominantly induced downregulation of genes, consistent with a constrained acclimation response. However, when temperature was considered in conjunction with parent-offspring interactions, genetic plasticity was extremely limited. That is, the genes underlying parent-offspring interactions did not show strong signatures of differential expression under more stressful thermal conditions. Given that we previously found no effects of temperature on parental behavior, we suggest that constrained, not plastic gene expression, underpins stability of parent-offspring interactions and limits the buffering capacity for family interactions under environmental stress. Insights from this study deepen our understanding of the interplay between

69 genetic and phenotypic plasticity in complex behavioral traits and how traits such as parenting
70 may limit responses to novel environmental challenges.
71

INTRODUCTION

Parental care evolves to buffer offspring against environmental stress and once evolved, can be instrumental for ensuring success across a range of ecological conditions, beyond those that promoted its evolution (Wilson 1975; Clutton-Brock 1991; Royle *et al.* 2012). In the face of rapidly changing environments, phenotypic plasticity in family interactions could contribute to species persistence by allowing parents to invest more when conditions are poor and offspring to compensate when conditions exceed those that parents can or are willing to buffer. In some cases, this plasticity may be immediately detectable as a change in behavior. For example, many birds compensate for harsh breeding season conditions by increasing the density of food loads delivered to chicks (Grissot *et al.* 2019) or by extending incubation times (McClintock *et al.* 2014; Sharpe *et al.* 2021), while offspring have been shown to upregulate metabolic and immune pathways in response to the removal of a caring parent (Ziadie *et al.* 2019; Körner *et al.* 2020; Mashoodh *et al.* 2021) or exposure to pathogens (Körner *et al.* 2020). However, when parents and offspring are pushed to their physiological limits, maintaining a stable phenotype is likely just as important for ensuring offspring success as is altering behavior.

Transcriptomic studies have much to contribute to our understanding of the interplay between genetic plasticity and phenotypic plasticity. Gene expression is inherently plastic, and transcriptional profiles can undergo profound changes to maintain stable higher-level phenotypes (Eberwine & Kim 2015; Nijhout *et al.* 2019; Rivera *et al.* 2021). For instance, research on adaptation to high-altitude hypoxia has revealed that sparrows living at high altitude in the Peruvian Andes constitutively upregulate nearly 200 metabolic and biosynthesis genes relative to counterparts inhabiting lower elevation sites (Cheviron *et al.* 2008). Similarly, plasticity of molecular mechanisms underlying behavior could fulfil crucial compensatory functions, ensuring

consistency of phenotype despite changes in environmental conditions (Fischer *et al.* 2021). In this way, studying transcriptional profiles could shed light on the potential mechanisms of adaptation without requiring knowledge of a specific phenotype (Cheviron & Brumfield 2011). This perspective is valuable to the study of parental care because both parents and offspring facing environmental stress should be favored to maintain cooperative interactions but could differ in their capacities to do so dependent on personal costs. However, very few studies have profiled gene expression in parents and offspring simultaneously to test whether plasticity at the transcriptional level helps to stabilize parent-offspring interactions under environmental stress or whether the ‘genetic programs’ for these complex traits are themselves constrained.

In this study, we performed RNA-seq on a subsocial burying beetle, *Nicrophorus orbicollis*, to explore if plasticity of gene expression may help buffer biparental families against future temperature rise. We previously showed that female and male *N. orbicollis* do not change parenting behaviors in response to temperature manipulation (Moss & Moore 2021); however, whether this behavioral stability results from constraint or plasticity of underlying genetic mechanisms remains unclear. Moreover, the potential for offspring to independently compensate for thermal stress has not been studied in this system. Here, we can address this possibility while controlling for any confounding effects of differences in care. There is a strong basis for studying the genetics of parenting and development of burying beetles (Parker *et al.* 2015; Jacobs *et al.* 2016; Palmer *et al.* 2016; Benowitz *et al.* 2017a; Won *et al.* 2018; Cunningham *et al.* 2019), yet the factors that influence the expression of genes underlying parent-offspring interactions remain uncharacterized. Even less understood is the extent to which expression of care-related genes is modulated in response to environmental stress. Here we examined the effects of parent-offspring interactions, thermal environment, and family membership on gene expression in a 2 x 2 x 3

factorial design to determine if any genes underlying parent-offspring interactions modify their expression depending on temperature.

METHODS

Study System

Nicrophorus orbicollis is a biparental carrion beetle that breeds on small vertebrate carcasses in woodlands ranging from southern Canada to northern Texas. At the southeastern edge of this distribution, daily mean temperatures as low as 18°C and as high as 25°C may arise over the course of the long summer breeding season (Moss & Moore 2021). As in most members of the genus, *N. orbicollis* expresses a large repertoire of pre- and post-hatching care behaviors (Eggert & Müller 1997; Scott 1998). Carcass preparation begins with the removal of hair and liberal application of anal secretions, which suppress microbial growth on the prepared ‘brood ball.’ Females oviposit throughout the soil surrounding the brood ball, and within 2–3 days these eggs hatch and larvae migrate through the soil to the brood ball attended by their parents. At this stage, parents commence directly provisioning begging young *via* regurgitation. While both males and females are capable of the full repertoire of care behaviors, parenting is sex-biased with females typically residing longer on the brood ball (Scott & Traniello 1990; Trumbo 1991; Scott 1998; Benowitz & Moore 2016) and performing a disproportionate share of the larval provisioning (Moss & Moore 2021).

Study Design

We used *N. orbicollis* beetles that had been reared in the laboratory for one generation at the University of Georgia, as described in Moss and Moore (2021). Briefly, adults were housed individually in plastic containers (9 cm diameter, 4 cm deep; Eco Products, Boulder, CO, USA) filled with potting soil and fed organic ground beef twice weekly *ad libitum*. Stock breeding took place at room temperature ($20 \pm 0.5^\circ\text{C}$), and resulting offspring were divided evenly between two incubators starting three days into pupation for a thermal acclimation period prior to breeding. These incubators were programmed to long-day light conditions (reverse light:dark 14:10) on ramping temperature cycles to simulate a diurnal range: the first fluctuating between 21 and 20°C and the second between 25 and 24°C . Because breeding takes place underground in relatively thermostable conditions, however, only the lower end of the diurnal range of each thermal treatment (20°C and 24°C) was used for breeding. Virgin, non-sibling beetles (aged ≥ 14 days post-eclosion) were paired in plastic boxes (17.2 x 12.7 x 6.4 cm; Pioneer Plastics, Dixon, KY, USA) filled with approximately 2 cm of moistened soil and a 40–45 g thawed mouse carcass (RodentPro, Evansville, IN, USA) and moved to either a constant dark temperature-controlled room (20°C) or incubator (24°C) corresponding to their acclimation environment. Boxes were monitored twice daily for the presence of eggs, and after 2–3 days eggs were collected into petri dishes containing damp filter paper and stored at their respective treatment temperatures. Eggs were checked every 4 hours between 0800 and 1700 until hatching (within 2 days of collection).

In addition to manipulating the thermal environments in which care took place, we also generated within-temperature treatment controls to disentangle the specific effects of parent-offspring interaction from a general parenting states. Thus, we sampled at two time points: 1) post-larval hatching but before larvae and parents interacted (no parent-offspring interactions);

and 2) after 24 hours of parental care (interacting – full biparental care). While larvae of *N. orbicollis* have relatively high starvation tolerance (mean time to starvation: ~17 hours), as first instars their capacity to self-feed from a prepared carcass is extremely inefficient and they rely on at least three hours of post-hatching parental care for survival (Capodeanu-Nägler et al. 2018). Therefore, larvae of families without parent-offspring interaction were sampled directly from the petri dish immediately upon discovery (within 18 hours of hatching), with parents sampled simultaneously. Larvae of families with parent-offspring interaction, by contrast, were transferred to brood balls attended by parents upon discovery (within 18 hours of hatching) and families were left undisturbed for 24 hours before sampling (when larvae were in their second instar; Fetherston et al. 1990, Scott 1998).

Sample Collection, Preparation, and RNA Sequencing

Our design yielded ten families at 20°C (five without parent-offspring interactions, five with parent-offspring interactions) and nine families at 24°C (four without parent-offspring interactions, five with parents-offspring interactions). To characterize transcriptomic signatures associated with parent-offspring interactions, adults heads were collected and, due to their small size, whole larvae were collected into liquid nitrogen and stored at -80°C until RNA extraction. Larval samples consisted of 7–10 first instar (≤ 18 hours old) larvae for families without parent-offspring interaction and 2–4 second instar (24–48 hours old) larvae for families with parent-offspring interaction. Extractions were performed following the Qiagen RNeasy Lipid kit protocols (Qiagen, Venlo, the Netherlands). Sequencing libraries were prepared from 1.3 μ g total RNA using the Illumina TruSeq mRNA Stranded Library Kit (Illumina, Inc., San Diego, CA, USA) according to the manufacturer’s protocol with standard Illumina adapters and primers.

Sequencing was carried out on a NovaSeq 6000 platform with a 150 bp paired-end protocol, targeting 40M read pairs per sample.

Read Mapping, Transcriptome Assembly, and Annotation

Reads were trimmed using Trimmomatic (Version 0.39; Bolger et al. 2014) to remove adapter sequences with default parameters, except low-quality bases (Phred <15 at the leading and trailing ends and from 4-base sliding windows), and short reads (<36 bp). Reads were corrected using RCorrector with default parameters (Version 1.0.3.1; Song and Florea 2015). Quality was assessed using FastQC (Version 0.11.9; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>; Supplementary Table 1). Paired-end reads were mapped against a draft reassembly of the WGS data of Benowitz et al. (2017) of *N. orbicollis* genome (NCBI BioProject PRJNA371654; Supplementary Methods 1). Read mapping was performed using HISAT2 with default parameters (Version 2.1.0; Kim et al. 2015). A reference-guided transcriptome was assembled using StringTie (Version 2.1.1; Pertea et al. 2015) and redundant transcripts were removed using CD-HIT (Version 4.8.1; Li and Godzik 2006) with a sequence similarity cut-off value of 95%. Finally, we assessed the completeness of the assembly using BUSCO (Insecta dataset; Version 5.0.0; Simão et al. 2015).

Annotation of contigs was performed using a combination of approaches. Briefly, the longest isoform of each gene was screened against the annotated and published genome of the closely related burying beetle, *N. vespilloides* (NCBI Assembly Accession No. GCF_001412225.1; Cunningham et al., 2015) using Magic-BLAST (Version 1.5.0; Boratyn et al. 2019). Transcripts were also fed through the ‘dammit’ pipeline (<http://dib-lab.github.io/dammit/>), which identifies candidate coding regions in transcripts with

Transdecoder (Version 2.0.1; <https://hpc.ilri.cgiar.org/transdecoder-software>) and annotates them, drawing from multiple databases. We used the *N. vespilloides* proteome (Cunningham *et al.* 2015) as a user database to search against and also retained any hits to known protein domains in Pfam-A (Version 32.0; El-Gebali *et al.* 2019) and orthologs in OrthoDB (Version 10; Kriventseva *et al.* 2019). Orthologous genes were retrieved from the OrthoDB database by searching cluster IDs at the level Polyphaga. Gene ontology (GO) terms for annotated genes were assigned by using Pfam accession numbers and GO terms of OrthoDB assignment. GO terms were also supplemented using eggNOG-mapper (Version 5.0; Huerta-Cepas *et al.* 2019) and OrthoDB searches at the Order (Insecta) level. Unique GO terms from any source were retained for a gene.

Transcript Quantification and Filtering

We estimated transcript abundances for each sample separately using StringTie. A transcript count matrix was extracted with the prepDE.py script provided with StringTie and imported into R (Version 4.0.3; R Core Development Team 2019) along with sample library sizes (calculated using Picardtools, Version 2.21.6; <http://picard.sourceforge.net>) and transcript lengths. We computed the fragments per kilobase per million (FPKM) matrix with the package *countToFPKM* (Version 1.0; Alhendi 2019). For each family member and social state (with or without parent-offspring interactions), we retained transcripts that were expressed >2 FPKM in more than half of samples and then combined all unique transcripts across groups in downstream analyses (Bloch *et al.* 2018). Finally, we used *IsoformSwitchAnalyzeR* (Version 1.12.0; Vitting-Seerup and Sandelin 2019) to convert transcript-level counts into gene-level counts.

Differential Expression Analysis

We performed differential expression (DE) analysis using *DESeq2* (Bioconductor Version 1.30.0; Love et al. 2014). To cluster samples, we first applied variance stabilizing transformation using *DESeq2* and performed a principal components analysis (PCA) using R's built-in 'prcomp' function. Visualization of the clustering was done using *DESeq2*'s 'pcaplot' function and extracted principal components were regressed on each grouping factor (family member, temperature, social interaction, and the statistical interactions among these factors) *via* analysis of variance (ANOVA carried out in R stats). Significant differences among family members in the full model ANOVA led us to analyze males, females, and larvae separately. For each cohort, differential expression was estimated using parametric dispersion. We used the likelihood ratio test (LRT) implemented in *DESeq2* to compare the goodness-of-fit of several models for each cohort to estimate differential gene expression. The first test compared a full model fitted with both temperature and social interaction (temperature + social interaction) to a reduced model fitted only with temperature to produce a list of DEGs mutually affected by social interaction across thermal environments. The second test compared the same full model to a reduced model fitted only with social interaction to identify DEGs mutually affected by temperature across social interactions. Our final test compared a full model fitted with a statistical interaction term (temperature x social interaction) to a reduced model fitted only with the main effects (temperature + social interaction), which allowed us to identify genes whose response to social interaction differed between the thermal environments. The *p*-values that resulted from these tests were adjusted for multiple testing using the Benjamini-Hochberg correction (Benjamini & Hochberg 1995) and genes with adjusted $p < 0.05$ were considered statistically significantly differentially expressed genes (DEGs).

To assess similarity of transcriptomic responses to temperature and social interaction across family members, we extracted DEG overlap lists from the intersect of male, female, and larval DEG sets. To determine whether overlap was significantly greater than expected by chance given variable input sizes, we further carried out randomization tests. Briefly, 10,000 gene sets were randomly generated for males, females, and larvae by sampling from the full list of expressed genes in that cohort a number equivalent to the size of the DEG set being simulated. These random gene sets were used to calculate a null distribution of overlap values, and significance was calculated as the proportion exceeding the observed overlap for each pairwise comparison. While overlap of the most highly significant genes between two sets lends itself to intuitive interpretation, use of fixed thresholds can be overly stringent and lead to false negatives. Therefore, we implemented a complementary threshold-free approach: rank-rank hypergeometric overlap (RRHO) analysis. This analysis ranks DE genes according to their significance level along with the magnitude and direction of change to make qualitative assessments of concordance between sets (i.e., coordinated shifts in the same direction) without requiring that values attain the same magnitude in all family members. Results were visualized using the stratified method of the R package *RRHO2* (Version 1.0; Cahill et al. 2018), with genes ranked according to the recommended metric of $-\log_{10}(p\text{-value}) * \text{sign}(\log_2 \text{ fold change})$.

Functional Enrichment Analysis and Candidate Genes

Finally, we queried the DEG sets and the overlapping DEG sets for enriched Gene Ontology terms using topGO (Version 2.42.0; Alexa and Rahnenfuhrer 2020) searching for enrichment in all three gene ontology categories: biological process, molecular function, and cellular component. Significance was estimated using a Fisher's exact test following the

program's suggested protocol and visualized using the R package 'GOplot' (Walter *et al.* 2015) to examine the magnitude and directionality of change of enriched pathways.

RESULTS

Our final transcriptome assembly contained 91% of single-copy conserved nucleotide orthologs (Complete: 90.5% [Single Copy: 76.4%, Duplicated: 14.1%], Fragmented: 4.2%, Missing: 5.3%) and a total of 12,406 genes.

Gene Expression Among Family Members

We visualized differences in gene expression among male parents, female parents, and offspring using principal components analysis. Parents and offspring separated strongly along two primary axes (Fig 1A), which together explained 77.8% of total variance in gene expression across all samples (PC1: 73.08%; PC2: 4.76%; Table 1). Family member (parent vs larvae) clearly separated along PC1, while separation along PC2 was driven primarily by social interaction (Table 1). The interactive effect of family member and social interaction and family member and temperature were significantly associated with PC2 (Table 1; Fig 1B). Visualizing cohorts separately with their own dispersions further clarified how global gene expression differed among groups. Female gene expression displayed considerable overlap between thermal environments but was most divided with respect to social interaction, particularly in the 20°C environment (Fig 1C). Male gene expression differentiated most strongly with temperature (Fig 1D). In larvae, behavioral groups formed distinct clusters, with social interaction accounting for far greater among-group differences than temperature (Fig 1E).

Figure 1: Principal components analysis (PCA) of normalized transcriptomic profiles across the three family member cohorts (females, males, and larvae) and four treatment groups (20°C:without parent-offspring interactions, 20°C:with parent-offspring interactions, 24°C:without parent-offspring interactions, and 24°C:with parent-offspring interactions). (A) Sample clustering along the first two principal components of the full model PCA (~Family Member + Temperature + Social Interaction + Family Member*Temperature*Social Interaction). Ellipses are drawn for 95% confidence intervals. (B) Normalized expression

profiles of the top 500 genes loading onto PC1 and PC2 as heatmaps, with each column representing an individual sample (grouped by family member) and each row representing a gene (clustered in a dendrogram by expression similarity). Cell colors represent normalized count values, such that the visual distinctiveness of clusters of samples corresponds to the magnitude of difference in their gene expression along PC1 and PC2. (C–E) Clustering of female, male, and larval samples, respectively, when analysis is repeated using only those samples. Illustration of *N. orbicollis* family in Fig 1A by K.E. Kollars.

Table 1: ANOVA of principal components of overall gene expression.

Variable	PC1 (73.08% variance)		PC2 (4.76% variance)	
	F _{2,55}	P	F _{2,55}	P
Family Member	5346.224	<0.0001	0.624	0.540
Temp	4.047	0.050	7.073	0.011
Social Interaction	27.238	<0.0001	88.311	<0.0001
Family Member x Temperature	0.499	0.611	11.406	<0.0001
Family Member x Social Interaction	4.852	0.012	92.516	<0.0001
Temperature x Social Interaction	2.139	0.151	0.925	0.341
Family Member x Temperature x Social Interaction	1.653	0.203	1.287	0.286

Differential Gene Expression among family members

Differential expression (DE) analysis largely recapitulated the results of the PCA visualization for each family member, The highest number of differentially expressed genes (DEGs) was associated with being parented, with 7,189 DEGs differentiating larvae with and without interacting parents (Supplementary Table 2). Adults showed fewer changes in gene expression, but there were also underlying sex differences: biparental males responded less to the presence of larvae ($n = 63$ DEG; Supplementary Table 3) than biparental females ($n = 189$ DEG; Supplementary Table 4). Despite this, females and males had a statistically significant overlap of gene identities while parenting (Fig 2A; $P = 0.044$). When considering all expressed genes, concordance was striking but directional, with overlap between the sexes concentrated in genes downregulated in both during larval interaction (Fig 2B). Females interacting with larvae and larvae interacting with parents shared more DEGs in common ($n = 115$) than parenting males and parented larvae ($n = 28$), but this was not statistically significant ($P = 0.059$) and neither comparison showed global signatures of concordance of gene expression (Fig 2C–D).

Differential Gene Expression under different Temperature Regimes

Temperature induced a stronger transcriptional response in males ($n = 418$ DEGs; Supplementary Table 5) and larvae ($n = 487$ DEGs; Supplementary Table 6) than in females ($n = 284$ DEGs; Supplementary Table 7). Despite differing numbers of DEGs, male, female, and larval responses to temperature showed statistically significant overlap in terms of gene identity (male-female: $P < 0.001$; female-larvae: $P < 0.001$; male-larvae: $P = 0.006$; Fig 2E). Global concordance was pronounced between males and females (Fig 2F) – both in genes that were up-

regulated and down-regulated in response to high temperature – whereas this signal was weak when comparing females to larvae (Fig 2G) and males to larvae (Fig 2H).

Differential Gene Expression for the Interaction of Social Interaction and Temperature

The interaction between temperature and social interaction was more explanatory of within-group differences in females (n = 79 DEGs; Supplementary Table 8) than in males (n = 4 DEGs; Supplementary Table 9) or larvae (n = 26 DEGs; Supplementary Table 10). Overall, however, very little gene expression was explained by the interaction of both treatments.

Figure 2: Concordance in differential gene expression between family members as estimated by direct overlap of differentially expressed genes (DEGs; top panel) and rank-rank hypergeometric

overlap (RRHO; bottom panel). Numbers of shared DEGs associated with social interactions are shown in Venn diagrams for females, males, and larvae (A); and (E) the contrast between 20°C and 24°C. The bottom panel shows the results of the RRHO analysis of all 12,406 genes for each of the respective contrasts (with vs without parent-offspring interactions: B–D; and 20°C vs 24°C: F–H). The x (family member 1) and y (family member 2) axes of each plot correspond to the significance of differential expression (ranked $-\log_{10}(p\text{-values})$) multiplied by the signed $\log_2$ fold difference in gene expression for each family member-specific analysis. White boundaries demarcate the switch from down- to up-regulation between behavioral groups for each family member. Pixel color corresponds to the value of the $-\log_{10}(p\text{-value})$ from the differential expression analysis, such that hot spots in the plot designate the strength and directionality of concordance in gene expression between family members.

Functional Enrichment Analysis

Examination of DEG lists produced by within-cohort parent-offspring interaction contrasts revealed both shared and distinct enriched functional characteristics across females, males, and larvae. Actively parenting females showed significant positive enrichment in a range of metabolic, catabolic, and biosynthetic functions compared to their respective non-interacting controls (Supplementary Table 11; Fig S1). Seventy-one of the 95 GO terms that were enriched in female DEGs were also significantly enriched in the 115 DEGs that females shared with larvae, particularly terms related to the metabolism of organic, carboxylic, and fatty acids (Supplementary Table 12). Conversely, analysis of GO terms in males revealed significant downregulation of non-overlapping GO terms (e.g., larval cuticle patterning; Supplementary

Table 13; Fig S2). Larval functional pathways were predominantly upregulated in response to interactions with parents and spanned many categories, from cellular organization and differentiation to whole organism development (Supplementary Table 14; Fig S3).

Compared to the 20°C group, females, males, and larvae in the 24°C group predominantly showed downregulation of functional pathways. The most significantly enriched GO terms in parents were related to chitin and carbohydrate metabolism (Supplementary Table 15–16; Figs S4–5), whereas larvae showed the most enrichment for cellular housekeeping functions (e.g., ribosome biogenesis and rRNA processing; Supplementary Table 17; Fig S6).

Finally, we examined functional responses to parent-offspring interaction under the two temperatures. In females interacting with larvae, we found 70 significantly enriched GO terms annotations for genes that were differentially expressed between 20°C and 24°C, with 61 related to oxidative and catabolic activities, in particular fatty acid oxidation (Supplementary Table 18). These pathways comprised genes that were significantly downregulated, on average (Fig 3B). Males similarly showed enrichment in pathways related to lipid transport and localization (Supplementary Table 19). Larval DEGs were enriched for a total 15 GO terms (Supplementary Table 20), the most significant of which (larval serum protein complex and synaptic target inhibition) were upregulated in interacting larvae responding to heat stress (Fig 3C).

Figure 3: Visual summary of genes showing significant differential expression associated with parent-offspring interactions for each family member under the different temperature (20°C versus 24°C). (A) Venn diagram showing the number of significantly differentially expressed genes (DEGs) in each cohort (females, males, and larvae) and their overlap; and bubble plots showing functionally enriched gene ontology (GO) terms in the (B) female and (C) larval DEG set. For B and C, the negative logarithm of each term's p-value is plotted against its z-score ((number of upregulated genes annotated for the term – number of downregulated genes annotated for the term)/square-root of the number of genes annotated for the term) to illustrate the overall magnitude and directionality of change in the functional pathway (<0 = downregulation, >0 = upregulation). The size of each dot represents the number of genes annotated for that term and color represents functional category: biological process, cellular component, or molecular function. Highly enriched terms for each cohort are labeled.

DISCUSSION

If the function of parental care is to buffer offspring against environmental stress and plasticity of gene expression allows families to optimize dynamics for their current environment, then responses to an ecologically relevant stressor (i.e., temperature) should have a transcriptional signature. Our study provides a first test of this prediction in a subsocial insect with biparental care. We have shown that parent-offspring interaction and thermal environment shape patterns of gene expression in *N. orbicollis* females, males, and larvae, and that many responses are distinct to specific family members. However, we found limited evidence for genetic plasticity. That is, neither parents nor offspring appear to moderate transcriptional responses to parent-offspring interaction dependent on thermal environment. Taken with our previous findings of limited behavioral plasticity of *N. orbicollis* parents exposed to high temperatures (Moss & Moore 2021), this implies that behavioral stability under stress emerges from constrained, rather than plastic gene expression. The capacity of parenting to buffer environmental stress is limited.

With our study, we provide a first glimpse into the genes underlying parent-offspring and offspring-parent interactions in a subsocial insect with biparental care. We characterized the gene expression profiles of family members before and 24 hours after larvae colonized the breeding resource. Not surprisingly, we found a larger transcriptional response in offspring than in parents. Thousands of differentially expressed genes (DEGs) were detected in larvae spanning many functional categories, consistent with the very rapid development and growth occurring at this early stage (Won *et al.* 2018) and the strong influence of parenting (Capodeanu-Nägler *et al.* 2018; Mashoodh *et al.* 2021). In comparison to the dramatic changes that occur in larvae once they are accepted by parents, the behavioral switch required for parents to accept their larvae and

begin active parenting is relatively subtle, occurring more than 50 hours into care (Oldekop *et al.* 2007). In line with past brain transcriptomic studies suggesting that the modification of behavior within a state (i.e., parenting) involves a much smaller subset of the genes required to transition between states (Bell *et al.* 2016; Benowitz *et al.* 2017a; Cunningham *et al.* 2019), changes in gene expression associated with the switch to active parenting in our study were subtle compared to, for example, the changes in expression associated with the transition from non-parenting to parenting states in *N. vespilloides* (up to 650 DEGs; Parker *et al.* 2015, Cunningham *et al.* 2017).

As in previous work on the related *N. vespilloides* (Parker *et al.* 2015), males and females parenting together showed strong concordance in overall gene expression patterns, but the magnitude of transcriptional response in terms of number of significantly differentially expressed genes (DEGs) was greatly reduced in males. Moreover, differences in functional enrichment of male and female DEG sets and their overlap with offspring implies that the transcriptomic architecture underlying parent-offspring interactions may be sex specific. For example, the top functionally enriched categories in actively parenting mothers (e.g., lipid and carboxylic acid metabolism) were also enriched in the overlap between females and offspring – a trend not detected in fathers. We propose that genes shared between mothers and offspring may play a role in feeding and nutrient exchange (i.e., mouth-to-mouth regurgitation), given that mothers in biparental pairs were seen to perform a disproportionate share of provisioning in our behavioral experiment (Moss & Moore 2021). If we are correct, it would suggest that there are substantial sex-specific aspects of how parental care is manifested at the level of gene expression even when care is predominantly biparental (Benowitz & Moore 2016).

In addition to being a social exchange, parental investment and offspring development are sensitive to environmental quality as poor conditions can impose competing physiological

demands. To observe the physiological pathways involved in this response, we sampled families from a benign (20°C) and a harsh (24°C) thermal environment across the breeding cycle. While our previous work showed that *N. orbicollis* do not modify their behavior in response to temperature (Moss and Moore 2021), closer examination at the level of gene expression revealed robust changes in adults and larvae. In parents, the predominant transcriptomic response to heat stress was downregulation, particularly of pathways involved in cuticle formation and carbohydrate metabolism (Figs S4–S5). Similarly, larvae showed downregulation of many genes involved in cellular housekeeping functions (Fig S6). A recent study comparing the acclimation response of *Daphnia* to a medium-temperature range versus a high-temperature range suggests that overwhelming downregulation under extreme heat stress may reflect an inability to mount systemic responses, which is more feasible in a lower temperature range (Becker *et al.* 2018). Taken together, the thermal conditions simulated in our experiment clearly present a physiological challenge to all family members, but the paths to mitigating these challenges may differ dependent on social role.

Finally, we asked if parents and offspring responded differently to social interaction depending on their thermal environment. Our prediction was that if plasticity in gene expression is important for maintaining behavioral stability, then we should detect many genes showing significant differential expression only when accounting for the interaction of temperature and social condition. Overall, few transcripts satisfied this condition in any family member, which implies that gene expression, like behavior, is constrained in its response to environmental stress. The largest transcriptional response occurred in heat stressed actively parenting mothers; however, closer inspection of the 79 DEGs *via* functional enrichment analysis revealed significant downregulation of pathways involved in fatty acid oxidation and lipid processing (Fig

3B). Lipids are the principal macronutrient used for fueling energetically intensive behavior in insects (e.g., flight: Canavoso et al. 2003), yet previous efforts to link post-hatching care (i.e., presumed to be the costliest form of care) to increased lipid metabolism in burying beetles have turned up no relationship (Benowitz *et al.* 2017b). Here, we suggest that females restrict the use of stored energy even more in an energetically demanding environment, implying that the combined stressors of temperature and parenting could directly mediate trade-offs between current and future reproduction. Offspring of stressed mothers in turn upregulated genes involved in forming the larval serum protein complex – a structure that sequesters amino acids early in development for the synthesis of adult proteins (Chrysanthis *et al.* 1994). Whether this transcriptional response by larvae may help compensate for constrained parental investment remains to be seen. What is clear is that parental responses are unlikely to stabilize behaviors or enhance offspring success, and thus the scope for climate change adaptation to proceed *via* selection on the transcriptional architecture underlying care would appear limited.

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DATA ACCESSIBILITY: All sequencing data is deposited in NCBI GenBank under the BioProject ID PRJA71530. Raw sequence reads are deposited in the Sequence Read Archive (SRA) under submission SUB9220525 and Accession numbers SAMN18349415–SAMN18349472 and the assembled transcriptome is deposited in the Transcriptome Shotgun Assembly (TSA) Sequence Database under submission SUB9312680. Supporting data and R scripts are deposited as an Open Science Framework project: osf.io/ztyqa.

References

- Alexa, A. & Rahnenfuhrer, J. (2020). topGO: Enrichment analysis for gene ontology. *R Packag. version 2.42.0*.
- Alhendi, A. (2019). countToFPKM: Convert counts to fragments per kilobase of transcript per million (FPKM). *R Packag. version 3.1.0*.
- Becker, D., Reydelet, Y., Lopez, J.A., Jackson, C., Colbourne, J.K., Hawat, S., *et al.* (2018). The

transcriptomic and proteomic responses of *Daphnia pulex* to changes in temperature and food supply comprise environment-specific and clone-specific elements. *BMC Genomics*, 19, 1–30.

Bell, A.M., Bukhari, S.A. & Sanogo, Y.O. (2016). Natural variation in brain gene expression profiles of aggressive and nonaggressive individual sticklebacks. *Behaviour*, 153, 1723–1743.

Benjamini, Y. & Hochberg, Y. (1995). *Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing*. *J. R. Stat. Soc. B*.

Benowitz, K.M., McKinney, E.C., Cunningham, C.B. & Moore, A.J. (2017a). Relating quantitative variation within a behavior to variation in transcription. *Evolution (N. Y.)*, 71, 1999–2009.

Benowitz, K.M., McKinney, E.C., Roy-Zokan, E.M., Cunningham, C.B. & Moore, A.J. (2017b). The role of lipid metabolism during parental care in two species of burying beetle (*Nicrophorus* spp.). *Anim. Behav.*, 129, 143–149.

Benowitz, K.M. & Moore, A.J. (2016). Biparental care is predominant and beneficial to parents in the burying beetle *Nicrophorus orbicollis* (Coleoptera: Silphidae). *Biol. J. Linn. Soc.*, 119, 1082–1088.

Bloch, N.I., Corral-López, A., Buechel, S.D., Kotrschal, A., Kolm, N. & Mank, J.E. (2018). Early neurogenomic response associated with variation in guppy female mate preference. *Nat. Ecol. Evol.* 2018 211, 2, 1772–1781.

Bolger, A.M., Lohse, M. & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, 30, 2114–2120.

Boratyn, G.M., Thierry-Mieg, J., Thierry-Mieg, D., Busby, B. & Madden, T.L. (2019). Magic-

BLAST, an accurate RNA-seq aligner for long and short reads. *BMC Bioinformatics*, 20, 405.

Cahill, K.M., Huo, Z., Tseng, G.C., Logan, R.W. & Seney, M.L. (2018). Improved identification of concordant and discordant gene expression signatures using an updated rank-rank hypergeometric overlap approach. *Sci. Reports 2018 81*, 8, 1–11.

Canavoso, L.E., Jouni, Z.E., Karnas, K.J., Pennington, J.E. & Wells, M.A. (2003). Fat metabolism in insects. *Annu. Rev. Nutr.*, 21, 23–46.

Capodeanu-Nägler, A., Prang, M.A., Trumbo, S.T., Vogel, H., Eggert, A.K., Sakaluk, S.K., *et al.* (2018). Offspring dependence on parental care and the role of parental transfer of oral fluids in burying beetles. *Front. Zool.*, 15, 33.

Cheviron, Z.A. & Brumfield, R.T. (2011). Genomic insights into adaptation to high-altitude environments. *Hered. 2012 1084*, 108, 354–361.

Cheviron, Z.A., Whitehead, A. & Brumfield, R.T. (2008). Transcriptomic variation and plasticity in rufous-collared sparrows (*Zonotrichia capensis*) along an altitudinal gradient. *Mol. Ecol.*, 17, 4556–4569.

Chou, H., Jima, D.D., Funk, D.H., Jackson, J.K., Sweeney, B.W. & Buchwalter, D.B. (2020). Transcriptomic and life history responses of the mayfly *Neocloeon triangulifer* to chronic diel thermal challenge. *Sci. Reports 2020 101*, 10, 1–11.

Chrysanthis, G., Kaliafas, A.D. & Mintzas, A.C. (1994). Biosynthesis and tissue distribution of four major larval serum proteins during development of *Ceratitis capitata* (Diptera). *Insect Biochem. Mol. Biol.*, 24, 811–818.

Clutton-Brock, T.H. (1991). *The evolution of parental care*. Princeton University Press, Princeton, New Jersey, U.S.A.

576 Cunningham, C.B., Badgett, M.J., Meagher, R.B., Orlando, R. & Moore, A.J. (2017).
577 Ethological principles predict the neuropeptides co-opted to influence parenting. *Nat.*
578 *Commun.*, 81, 4225.

579 Cunningham, C.B., Ji, L., McKinney, E.C., Benowitz, K.M., Schmitz, R.J. & Moore, A.J.
580 (2019). Changes of gene expression but not cytosine methylation are associated with male
581 parental care reflecting behavioural state, social context and individual flexibility. *J. Exp.*
582 *Biol.*, 222, 1–9.

583 Cunningham, C.B., Ji, L., Wiberg, R.A.W., Shelton, J., McKinney, E.C., Parker, D.J., *et al.*
584 (2015). The genome and methylome of a beetle with complex social behavior, *Nicrophorus*
585 *vespilloides* (coleoptera: Silphidae). *Genome Biol. Evol.*, 7, 3383–3396.

586 Eberwine, J. & Kim, J. (2015). Cellular Deconstruction: Finding Meaning in Individual Cell
587 Variation. *Trends Cell Biol.*, 25, 569–578.

588 Eggert, A.-K. & Müller, J.K. (1997). Biparental care and social evolution in burying beetles:
589 lessons from the larder. In: *The Evolution of Social Behavior in Insects and Arachnids* (eds.
590 Choe, J.C. & Crespi, B.I.). Cambridge University Press, Cambridge, Massachusetts, USA,
591 pp. 216–236.

592 El-Gebali, S., Mistry, J., Bateman, A., Eddy, S.R., Luciani, A., Potter, S.C., *et al.* (2019). The
593 Pfam protein families database in 2019. *Nucleic Acids Res.*, 47, D427–D432.

594 Fetherston, I.A., Scott, M.P. & Traniello, J.F.A. (1990). Parental care in burying beetles: The
595 organization of male and female brood-care behavior. *Ethology*, 85, 177–190.

596 Fischer, E.K., Hauber, M.E. & Bell, A.M. (2021). Back to the basics? Transcriptomics offers
597 integrative insights into the role of space, time and the environment for gene expression and
598 behaviour. *Biol. Lett.*, 17, 20210293.

Grissot, A., Araya-Salas, M., Jakubas, D., Kidawa, D., Boehnke, R., Błachowiak-Samołyk, K., *et al.* (2019). Parental coordination of chick provisioning in a planktivorous arctic seabird under divergent conditions on foraging grounds. *Front. Ecol. Evol.*, 0, 349.

Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernández-Plaza, A., Forslund, S.K., Cook, H., *et al.* (2019). EggNOG 5.0: A hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Res.*, 47, D309–D314.

Jacobs, C.G.C., Steiger, S., Heckel, D.G., Wielsch, N., Vilcinskas, A. & Vogel, H. (2016). Sex, offspring and carcass determine antimicrobial peptide expression in the burying beetle. *Sci. Reports 2016 61*, 6, 1–8.

Kim, D., Langmead, B. & Salzberg, S.L. (2015). HISAT: A fast spliced aligner with low memory requirements. *Nat. Methods*, 12, 357–360.

Körner, M., Vogelweith, F., Libbrecht, R., Foitzik, S., Feldmeyer, B. & Meunier, J. (2020). Offspring reverse transcriptome responses to maternal deprivation when reared with pathogens in an insect with facultative family life. *Proc. R. Soc. B*, 287.

Kriventseva, E. V., Kuznetsov, D., Tegenfeldt, F., Manni, M., Dias, R., Simão, F.A., *et al.* (2019). OrthoDB v10: Sampling the diversity of animal, plant, fungal, protist, bacterial and viral genomes for evolutionary and functional annotations of orthologs. *Nucleic Acids Res.*, 47, D807–D811.

Li, W. & Godzik, A. (2006). Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics*, 22, 1658–1659.

Love, M.I., Huber, W. & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.*, 15, 550.

Mashoodh, R., Westoby, J. & Kilner, R. (2021). Evolved changes in DNA methylation in response to the sustained loss of parental care in the burying beetle. *bioRxiv*.

McClintock, M.E., Hepp, G.R. & Kennamer, R.A. (2014). Plasticity of incubation behaviors helps Wood Ducks (*Aix sponsa*) maintain an optimal thermal environment for developing embryos. *Auk*, 131, 672–680.

Moss, J.B. & Moore, A.J. (2021). Constrained flexibility of parental cooperation limits adaptive responses to harsh conditions. *Evolution (N. Y.)*, 75, 1835–1849.

Nijhout, H.F., Best, J.A. & Reed, M.C. (2019). Systems biology of robustness and homeostatic mechanisms. *Wiley Interdiscip. Rev. Syst. Biol. Med.*, 11, e1440.

Oldekop, J.A., Smiseth, P.T., Piggins, H.D. & Moore, A.J. (2007). Adaptive switch from infanticide to parental care: How do beetles time their behaviour? *J. Evol. Biol.*, 20, 1998–2004.

Palmer, W.J., Duarte, A., Schrader, M., Day, J.P., Kilner, R. & Jiggins, F.M. (2016). A gene associated with social immunity in the burying beetle *Nicrophorus vespilloides*. *Proc. R. Soc. B Biol. Sci.*, 283, 20152733.

Parker, D.J., Cunningham, C.B., Walling, C.A., Stamper, C.E., Head, M.L., Roy-Zokan, E.M., *et al.* (2015). Transcriptomes of parents identify parenting strategies and sexual conflict in a subsocial beetle. *Nat. Commun.*, 6, 8449.

Pertea, M., Pertea, G.M., Antonescu, C.M., Chang, T.C., Mendell, J.T. & Salzberg, S.L. (2015). StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat. Biotechnol.*, 33, 290–295.

R Core Development Team. (2019). R: A language and environment for statistical computing. *R Found. Stat. Comput.*

645 Rivera, H.E., Aichelman, H.E., Fifer, J.E., Kriefall, N.G., Wuitchik, D.M., Wuitchik, S.J.S., *et*
646 *al.* (2021). A framework for understanding gene expression plasticity and its influence on
647 stress tolerance. *Mol. Ecol.*, 30, 1381–1397.

648 Royle, N.J., Smiseth, P.T. & Kölliker, M. (2012). *The Evolution of Parental Care*. Oxford
649 University Press, Oxford, U.K.

650 Scott, M.P. (1998). The ecology and behavior of burying beetles. *Annu. Rev. Entomol.*, 43, 595–
651 618.

652 Scott, M.P. & Traniello, J.F.A. (1990). Behavioural and ecological correlates of male and female
653 parental care and reproductive success in burying beetles (*Nicrophorus spp.*). *Anim. Behav.*,
654 39, 274–283.

655 Sharpe, L.L., Bayter, C. & Gardner, J.L. (2021). Too hot to handle? Behavioural plasticity during
656 incubation in a small, Australian passerine. *J. Therm. Biol.*, 98, 102921.

657 Simão, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E. V. & Zdobnov, E.M. (2015).
658 BUSCO: Assessing genome assembly and annotation completeness with single-copy
659 orthologs. *Bioinformatics*, 31, 3210–3212.

660 Song, L. & Florea, L. (2015). Rcorrector: Efficient and accurate error correction for Illumina
661 RNA-seq reads. *Gigascience*, 4, 48.

662 Trumbo, S.T. (1991). Reproductive benefits and the duration of paternal care in a biparental
663 burying beetle, *Nicrophorus orbicollis*. *Behaviour*, 117, 82–105.

664 Vitting-Seerup, K. & Sandelin, A. (2019). IsoformSwitchAnalyzeR: analysis of changes in
665 genome-wide patterns of alternative splicing and its functional consequences.
666 *Bioinformatics*, 35, 4469–4471.

667 Walter, W., Sánchez-Cabo, F. & Ricote, M. (2015). GOplot: an R package for visually

combining expression data with functional analysis. *Bioinformatics*, 31, 2912–2914.

Wilson, E.O. (1975). *Sociobiology: The New Synthesis*. Belknap, Cambridge, U.K.

Won, H.I., Schulze, T.T., Clement, E.J., Watson, G.F., Watson, S.M., Warner, R.C., *et al.*

(2018). De novo assembly of the burying beetle *Nicrophorus orbicollis* (Coleoptera:

Silphidae) transcriptome across developmental stages with identification of key immune

transcripts. *J. Genomics*, 6, 41.

Zhao, L., Wit, J., Svetec, N. & Begun, D.J. (2015). Parallel gene expression differences between

low and high latitude populations of *Drosophila melanogaster* and *D. simulans*. *PLOS*

Genet., 11, e1005184.

Ziadie, M.A., Ebot-Ojong, F., McKinney, E.C. & Moore, A.J. (2019). Evolution of personal and

social immunity in the context of parental care. *Am. Nat.*, 193, 296–308.