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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.

47 **IMPACT STATEMENT**

48 Parenting is thought to evolve to buffer offspring from harsh or inhospitable conditions.
49 However, once evolved, does parenting continue to buffer against new environmental challenges
50 such as might occur under global warming? One way organisms achieve different phenotypes
51 without evolving different genes for each trait is by changing timing or extent of gene
52 expression. Changes in gene expression influencing physiological traits such as hypoxia have
53 been shown to facilitate adaptation to changing environments. It is less clear if changes in gene
54 expression are also important for behavioral plasticity in complex social traits, such as parental
55 care. In this study, we ask whether families of the subsocial burying beetle, *Nicrophorus*
56 *orbicollis*, show altered expression of parent or offspring genes that may help compensate for
57 reduced offspring success under global warming. To test the extent that gene expression
58 underlies behavioral plasticity in parenting and offspring responses to parenting, we characterize
59 the gene expression profiles of whole families (males, females, and larvae) with and without
60 parent-offspring interactions under two thermal conditions: 20°C and 24°C. We found that heat
61 stress predominantly induced downregulation of genes, consistent with a constrained acclimation
62 response. However, when temperature was considered in conjunction with parent-offspring
63 interactions, genetic plasticity was extremely limited. That is, the genes underlying parent-
64 offspring interactions did not show strong signatures of differential expression under more
65 stressful thermal conditions. Given that we previously found no effects of temperature on
66 parental behavior, we suggest that constrained, not plastic gene expression, underpins stability of
67 parent-offspring interactions and limits the buffering capacity for family interactions under
68 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

72 **INTRODUCTION**

73 Parental care evolves to buffer offspring against environmental stress and once evolved,
74 can be instrumental for ensuring success across a range of ecological conditions, beyond those
75 that promoted its evolution (Wilson 1975; Clutton-Brock 1991; Royle *et al.* 2012). In the face of
76 rapidly changing environments, phenotypic plasticity in family interactions could contribute to
77 species persistence by allowing parents to invest more when conditions are poor and offspring to
78 compensate when conditions exceed those that parents can or are willing to buffer. In some
79 cases, this plasticity may be immediately detectable as a change in behavior. For example, many
80 birds compensate for harsh breeding season conditions by increasing the density of food loads
81 delivered to chicks (Grissot *et al.* 2019) or by extending incubation times (McClintock *et al.*
82 2014; Sharpe *et al.* 2021), while offspring have been shown to upregulate metabolic and immune
83 pathways in response to the removal of a caring parent (Ziadie *et al.* 2019; Körner *et al.* 2020;
84 Mashoodh *et al.* 2021) or exposure to pathogens (Körner *et al.* 2020). However, when parents
85 and offspring are pushed to their physiological limits, maintaining a stable phenotype is likely
86 just as important for ensuring offspring success as is altering behavior.

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

95 consistency of phenotype despite changes in environmental conditions (Fischer *et al.* 2021). In
96 this way, studying transcriptional profiles could shed light on the potential mechanisms of
97 adaptation without requiring knowledge of a specific phenotype (Cheviron & Brumfield 2011).
98 This perspective is valuable to the study of parental care because both parents and offspring
99 facing environmental stress should be favored to maintain cooperative interactions but could
100 differ in their capacities to do so dependent on personal costs. However, very few studies have
101 profiled gene expression in parents and offspring simultaneously to test whether plasticity at the
102 transcriptional level helps to stabilize parent-offspring interactions under environmental stress or
103 whether the 'genetic programs' for these complex traits are themselves constrained.
104 In this study, we performed RNA-seq on a subsocial burying beetle, *Nicrophorus*
105 *orbicollis*, to explore if plasticity of gene expression may help buffer biparental families against
106 future temperature rise. We previously showed that female and male *N. orbicollis* do not change
107 parenting behaviors in response to temperature manipulation (Moss & Moore 2021); however,
108 whether this behavioral stability results from constraint or plasticity of underlying genetic
109 mechanisms remains unclear. Moreover, the potential for offspring to independently compensate
110 for thermal stress has not been studied in this system. Here, we can address this possibility while
111 controlling for any confounding effects of differences in care. There is a strong basis for studying
112 the genetics of parenting and development of burying beetles (Parker *et al.* 2015; Jacobs *et al.*
113 2016; Palmer *et al.* 2016; Benowitz *et al.* 2017a; Won *et al.* 2018; Cunningham *et al.* 2019), yet
114 the factors that influence the expression of genes underlying parent-offspring interactions remain
115 uncharacterized. Even less understood is the extent to which expression of care-related genes is
116 modulated in response to environmental stress. Here we examined the effects of parent-offspring
117 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

141 We used *N. orbicollis* beetles that had been reared in the laboratory for one generation at
142 the University of Georgia, as described in Moss and Moore (2021). Briefly, adults were housed
143 individually in plastic containers (9 cm diameter, 4 cm deep; Eco Products, Boulder, CO, USA)
144 filled with potting soil and fed organic ground beef twice weekly *ad libitum.* Stock breeding took
145 place at room temperature (20 ± 0.5°C), and resulting offspring were divided evenly between
146 two incubators starting three days into pupation for a thermal acclimation period prior to
147 breeding. These incubators were programmed to long-day light conditions (reverse light:dark
148 14:10) on ramping temperature cycles to simulate a diurnal range: the first fluctuating between
149 21 and 20°C and the second between 25 and 24°C. Because breeding takes place underground in
150 relatively thermostable conditions, however, only the lower end of the diurnal range of each
151 thermal treatment (20°C and 24°C) was used for breeding. Virgin, non-sibling beetles (aged ≥
152 14 days post-eclosion) were paired in plastic boxes (17.2 x 12.7 x 6.4 cm; Pioneer Plastics,
153 Dixon, KY, USA) filled with approximately 2 cm of moistened soil and a 40–45 g thawed mouse
154 carcass (RodentPro, Evansville, IN, USA) and moved to either a constant dark temperature-
155 controlled room (20°C) or incubator (24°C) corresponding to their acclimation environment.
156 Boxes were monitored twice daily for the presence of eggs, and after 2–3 days eggs were
157 collected into petri dishes containing damp filter paper and stored at their respective treatment
158 temperatures. Eggs were checked every 4 hours between 0800 and 1700 until hatching (within 2
159 days of collection).
160 In addition to manipulating the thermal environments in which care took place, we also
161 generated within-temperature treatment controls to disentangle the specific effects of parent-
162 offspring interaction from a general parenting states. Thus, we sampled at two time points: 1)
163 post-larval hatching but before larvae and parents interacted (no parent-offspring interactions);

164 and 2) after 24 hours of parental care (interacting – full biparental care). While larvae of *N.*
165 *orbicollis* have relatively high starvation tolerance (mean time to starvation: ~17 hours), as first
166 instars their capacity to self-feed from a prepared carcass is extremely inefficient and they rely
167 on at least three hours of post-hatching parental care for survival (Capodeanu-Nägler et al. 2018).
168 Therefore, larvae of families without parent-offspring interaction were sampled directly from the
169 petri dish immediately upon discovery (within 18 hours of hatching), with parents sampled
170 simultaneously. Larvae of families with parent-offspring interaction, by contrast, were
171 transferred to brood balls attended by parents upon discovery (within 18 hours of hatching) and
172 families were left undisturbed for 24 hours before sampling (when larvae were in their second
173 instar; Fetherston et al. 1990, Scott 1998).
174
175 *Sample Collection, Preparation, and RNA Sequencing*
176 Our design yielded ten families at 20°C (five without parent-offspring interactions, five
177 with parent-offspring interactions) and nine families at 24°C (four without parent-offspring
178 interactions, five with parents-offspring interactions). To characterize transcriptomic signatures
179 associated with parent-offspring interactions, adults heads were collected and, due to their small
180 size, whole larvae were collected into liquid nitrogen and stored at -80°C until RNA extraction.
181 Larval samples consisted of 7–10 first instar (≤ 18 hours old) larvae for families without parent-
182 offspring interaction and 2–4 second instar (24–48 hours old) larvae for families with parent-
183 offspring itneraction. Extractions were performed following the Qiagen RNAeasy Lipid kit
184 protocols (Qiagen, Venlo, the Netherlands). Sequencing libraries were prepared from 1.3 µg total
185 RNA using the Illumina TruSeq mRNA Stranded Library Kit (Illumina, Inc., San Diego, CA,
186 USA) according to the manufacturer's protocol with standard Illumina adapters and primers.

187 Sequencing was carried out on a NovaSeq 6000 platform with a 150 bp paired-end protocol,
188 targeting 40M read pairs per sample.
189
190 *Read Mapping, Transcriptome Assembly, and Annotation*
191 Reads were trimmed using Trimmomatic (Version 0.39; Bolger et al. 2014) to remove
192 adapter sequences with default parameters, except low-quality bases (Phred <15 at the leading
193 and trailing ends and from 4-base sliding windows), and short reads (<36 bp). Reads were
194 corrected using RCorrector with default parameters (Version 1.0.3.1; Song and Florea 2015).
195 Quality was assessed using FastQC (Version 0.11.9;
196 http://www.bioinformatics.babraham.ac.uk/projects/fastqc/; Supplementary Table 1). Paired-end
197 reads were mapped against a draft reassembly of the WGS data of Benowitz et al. (2017) of *N.*
198 *orbicollis* genome (NCBI BioProject PRJNA371654; Supplementary Methods 1). Read mapping
199 was performed using HISAT2 with default parameters (Version 2.1.0; Kim et al. 2015). A
200 reference-guided transcriptome was assembled using StringTie (Version 2.1.1; Pertea et al. 2015)
201 and redundant transcripts were removed using CD-HIT (Version 4.8.1; Li and Godzik 2006)
202 with a sequence similarity cut-off value of 95%. Finally, we assessed the completeness of the
203 assembly using BUSCO (Insecta dataset; Version 5.0.0; Simão et al. 2015).
204 Annotation of contigs was performed using a combination of approaches. Briefly, the
205 longest isoform of each gene was screened against the annotated and published genome of the
206 closely related burying beetle, *N. vespilloides* (NCBI Assembly Accession No.
207 GCF_001412225.1; Cunningham et al., 2015) using Magic-BLAST (Version 1.5.0; Boratyn et
208 al. 2019). Transcripts were also fed through the 'dammit' pipeline (http://dib-
209 lab.github.io/dammit/), which identifies candidate coding regions in transcripts with

210 Transdecoder (Version 2.0.1; https://hpc.ilri.cgiar.org/transdecoder-software) and annotates
211 them, drawing from multiple databases. We used the *N. vespilloides* proteome (Cunningham *et*
212 *al.* 2015) as a user database to search against and also retained any hits to known protein
213 domains in Pfam-A (Version 32.0; El-Gebali et al. 2019) and orthologs in OrthoDB (Version 10;
214 Kriventseva et al. 2019). Orthologous genes were retrieved from the OrthoDB database by
215 searching cluster IDs at the level Polyphaga. Gene ontology (GO) terms for annotated genes
216 were assigned by using Pfam accession numbers and GO terms of OrthoDB assignment. GO
217 terms were also supplemented using eggNOG-mapper (Version 5.0; Huerta-Cepas et al. 2019)
218 and OrthoDB searches at the Order (Insecta) level. Unique GO terms from any source were
219 retained for a gene.
220
221 *Transcript Quantification and Filtering*
222 We estimated transcript abundances for each sample separately using StringTie. A
223 transcript count matrix was extracted with the prepDE.py script provided with StringTie and
224 imported into R (Version 4.0.3; R Core Development Team 2019) along with sample library
225 sizes (calculated using Picardtools, Version 2.21.6; http://picard.sourceforge.net) and transcript
226 lengths. We computed the fragments per kilobase per million (FPKM) matrix with the package
227 *countToFPKM* (Version 1.0; Alhendi 2019). For each family member and social state (with or
228 without parent-offspring interactions), we retained transcripts that were expressed >2 FPKM in
229 more than half of samples and then combined all unique transcripts across groups in downstream
230 analyses (Bloch *et al.* 2018). Finally, we used *IsoformSwitchAnalyzeR* (Version 1.12.0; Vitting-
231 Seerup and Sandelin 2019) to convert transcript-level counts into gene-level counts.
232

233 *Differential Expression Analysis*

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

256 To assess similarity of transcriptomic responses to temperature and social interaction
257 across family members, we extracted DEG overlap lists from the intersect of male, female, and
258 larval DEG sets. To determine whether overlap was significantly greater than expected by
259 chance given variable input sizes, we further carried out randomization tests. Briefly, 10,000
260 gene sets were randomly generated for males, females, and larvae by sampling from the full list
261 of expressed genes in that cohort a number equivalent to the size of the DEG set being simulated.
262 These random gene sets were used to calculate a null distribution of overlap values, and
263 significance was calculated as the proportion exceeding the observed overlap for each pairwise
264 comparison. While overlap of the most highly significant genes between two sets lends itself to
265 intuitive interpretation, use of fixed thresholds can be overly stringent and lead to false negatives.
266 Therefore, we implemented a complementary threshold-free approach: rank-rank hypergeometric
267 overlap (RRHO) analysis. This analysis ranks DE genes according to their significance level
268 along with the magnitude and direction of change to make qualitative assessments of
269 concordance between sets (i.e., coordinated shifts in the same direction) without requiring that
270 values attain the same magnitude in all family members. Results were visualized using the
271 stratified method of the R package *RRHO2* (Version 1.0; Cahill et al. 2018), with genes ranked
272 according to the recommended metric of -log10(*p*-value)*sign($\log_2$ fold change).
273
274 *Functional Enrichment Analysis and Candidate Genes*
275 Finally, we queried the DEG sets and the overlapping DEG sets for enriched Gene
276 Ontology terms using topGO (Version 2.42.0; Alexa and Rahnenfuhrer 2020) searching for
277 enrichment in all three gene ontology categories: biological process, molecular function, and
278 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).

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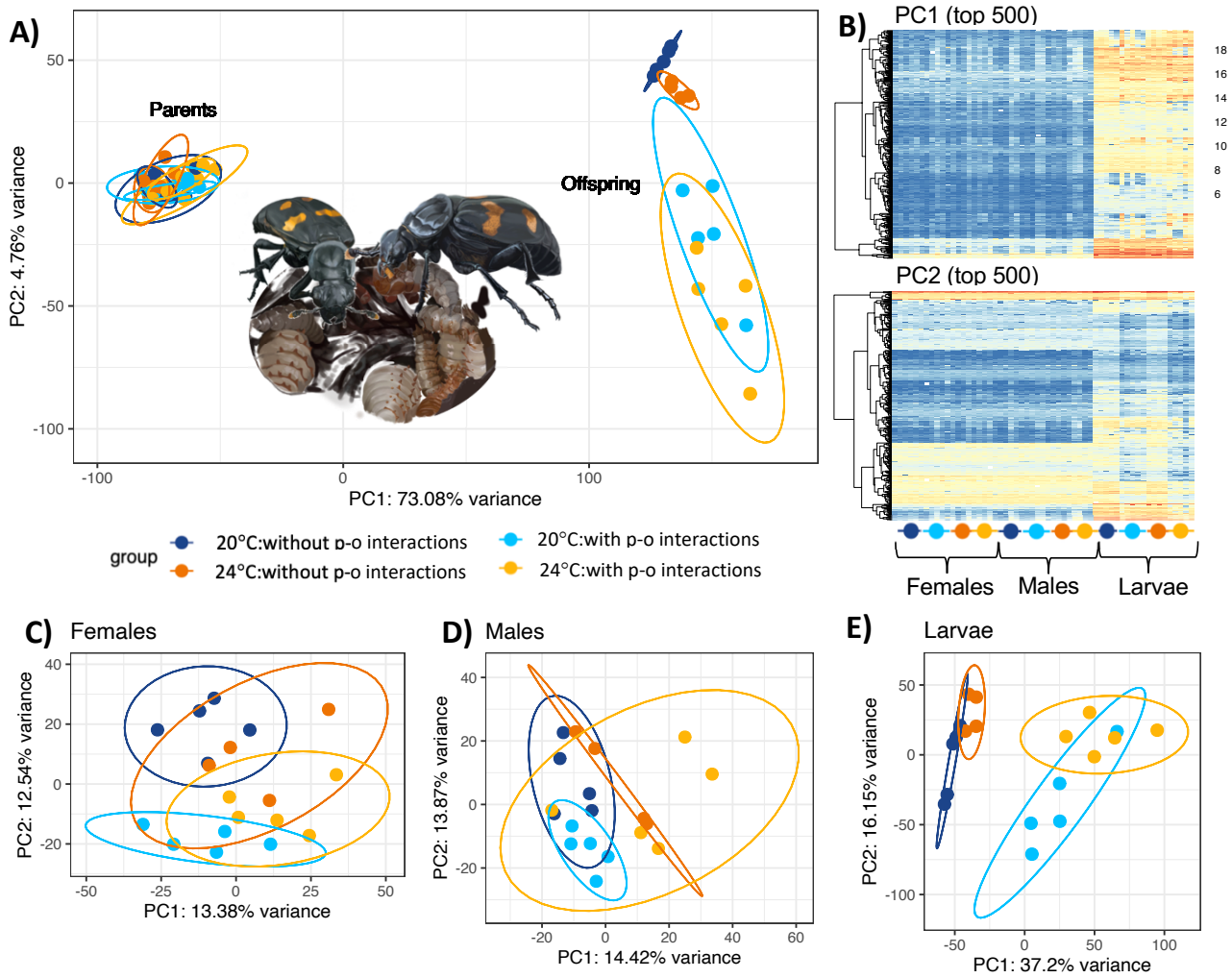

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304 Figure 1: Principal components analysis (PCA) of normalized transcriptomic profiles across the

305 three family member cohorts (females, males, and larvae) and four treatment groups

306 (20°C:without parent-offspring interactions, 20°C:with parent-offspring interactions,

307 24°C:without parent-offspring interactions, and 24°C:with parent-offspring interactions). (A)

308 Sample clustering along the first two principal components of the full model PCA (~Family

309 Member + Temperature + Social Interaction + Family Member*Temperature*Social

310 Interaction). Eclipses are drawn for 95% confidence intervals. (B) Normalized expression

311 profiles of the top 500 genes loading onto PC1 and PC2 as heatmaps, with each column
312 representing an individual sample (grouped by family member) and each row representing a gene
313 (clustered in a dendrogram by expression similarity). Cell colors represent normalized count
314 values, such that the visual distinctiveness of clusters of samples corresponds to the magnitude of
315 difference in their gene expression along PC1 and PC2. (C–E) Clustering of female, male, and
316 larval samples, respectively, when analysis is repeated using only those samples. Illustration of
317 *N. orbicollis* family in Fig 1A by K.E. Kollars.
318
319
320
321 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

322
323
324

325 *Differential Gene Expression among family members*

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

339

340 *Differential Gene Expression under different Temperature Regimes*

341 Temperature induced a stronger transcriptional response in males (n = 418 DEGs;
342 Supplementary Table 5) and larvae (n = 487 DEGs; Supplementary Table 6) than in females (n =
343 284 DEGs; Supplementary Table 7). Despite differing numbers of DEGs, male, female, and
344 larval responses to temperature showed statistically significant overlap in terms of gene identity
345 (male-female: $P < 0.001$; female-larvae: $P < 0.001$; male-larvae: $P = 0.006$; Fig 2E). Global
346 concordance was pronounced between males and females (Fig 2F) – both in genes that were up-

347 regulated and down-regulated in response to high temperature – whereas this signal was weak
348 when comparing females to larvae (Fig 2G) and males to larvae (Fig 2H).
349
350 *Differential Gene Expression for the Interaction of Social Interaction and Temperature*
351 The interaction between temperature and social interaction was more explanatory of
352 within-group differences in females (n = 79 DEGs; Supplementary Table 8) than in males (n = 4
353 DEGs; Supplementary Table 9) or larvae (n = 26 DEGs; Supplementary Table 10). Overall,
354 however, very little gene expression was explained by the interaction of both treatments.
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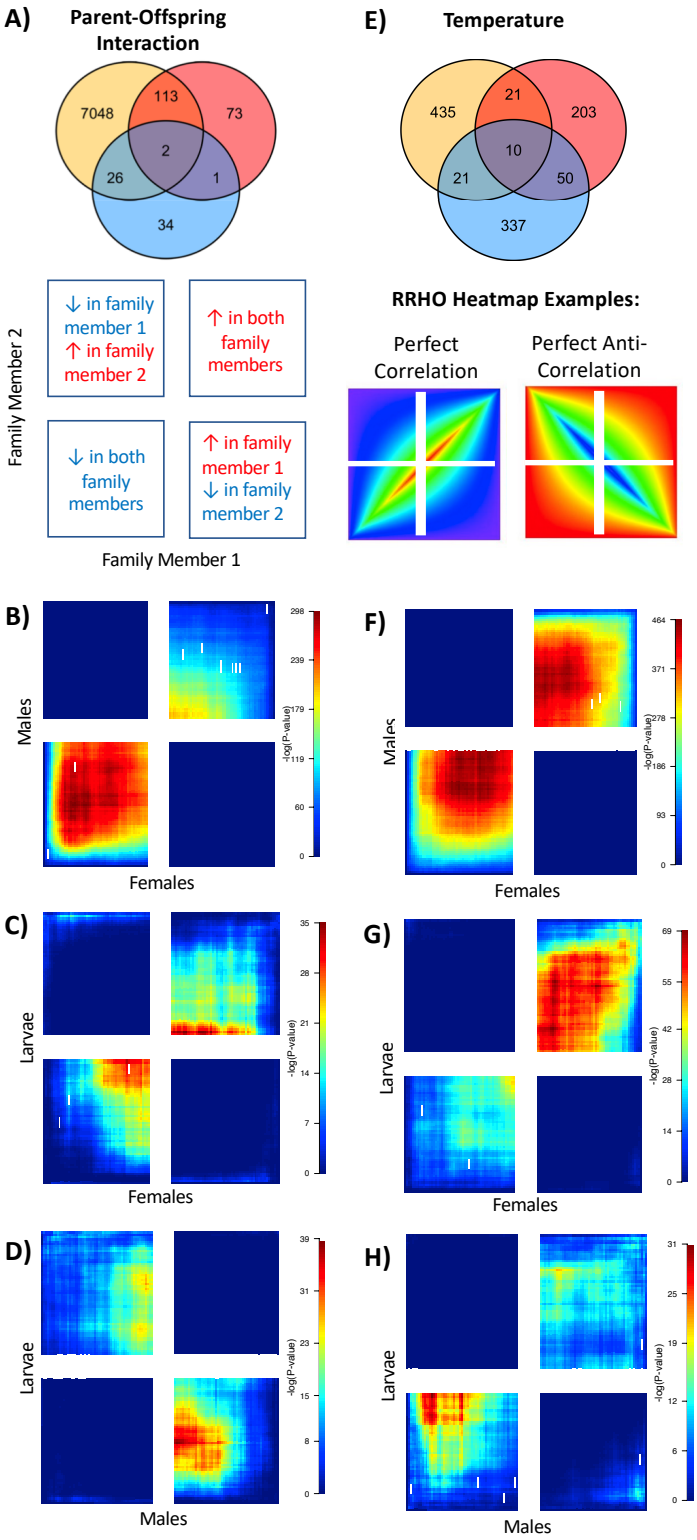

Figure 2: Concordance in differential gene expression between family members as estimated by direct overlap of differentially expressed genes (DEGs; top panel) and rank-rank hypergeometric

360 overlap (RRHO; bottom panel). Numbers of shared DEGs associated with social interactions are
361 shown in Venn diagrams for females, males, and larvae (A); and (E) the contrast between 20°C
362 and 24°C. The bottom panel shows the results of the RRHO analysis of all 12,406 genes for each
363 of the respective contrasts (with vs without parent-offspring interactions: B–D; and 20°C vs
364 24°C: F–H). The x (family member 1) and y (family member 2) axes of each plot correspond to
365 the significance of differential expression (ranked -log10(*p*-values)) multiplied by the signed
366 log2fold difference in gene expression for each family member-specific analysis. White
367 boundaries demarcate the switch from down- to up-regulation between behavioral groups for
368 each family member. Pixel color corresponds to the value of the -log10(*p*-value) from the
369 differential expression analysis, such that hot spots in the plot designate the strength and
370 directionality of concordance in gene expression between family members.
371
372
373 *Functional Enrichment Analysis*
374 Examination of DEG lists produced by within-cohort parent-offspring interaction
375 contrasts revealed both shared and distinct enriched functional characteristics across females,
376 males, and larvae. Actively parenting females showed significant positive enrichment in a range
377 of metabolic, catabolic, and biosynthetic functions compared to their respective non-interacting
378 controls (Supplementary Table 11; Fig S1). Seventy-one of the 95 GO terms that were enriched
379 in female DEGs were also significantly enriched in the 115 DEGs that females shared with
380 larvae, particularly terms related to the metabolism of organic, carboxylic, and fatty acids
381 (Supplementary Table 12). Conversely, analysis of GO terms in males revealed significant
382 downregulation of non-overlapping GO terms (e.g., larval cuticle patterning; Supplementary

383 Table 13; Fig S2). Larval functional pathways were predominantly upregulated in response to
384 interactions with parents and spanned many categories, from cellular organization and
385 differentiation to whole organism development (Supplementary Table 14; Fig S3).
386 Compared to the 20°C group, females, males, and larvae in the 24°C group
387 predominantly showed downregulation of functional pathways. The most significantly enriched
388 GO terms in parents were related to chitin and carbohydrate metabolism (Supplementary Table
389 15–16; Figs S4–5), whereas larvae showed the most enrichment for cellular housekeeping
390 functions (e.g., ribosome biogenesis and rRNA processing; Supplementary Table 17; Fig S6).
391 Finally, we examined functional responses to parent-offspring interaction under the two
392 temperatures. In females interacting with larvae, we found 70 significantly enriched GO terms
393 annotations for genes that were differentially expressed between 20°C and 24°C, with 61 related
394 to oxidative and catabolic activities, in particular fatty acid oxidation (Supplementary Table 18).
395 These pathways comprised genes that were significantly downregulated, on average (Fig 3B).
396 Males similarly showed enrichment in pathways related to lipid transport and localization
397 (Supplementary Table 19). Larval DEGs were enriched for a total 15 GO terms (Supplementary
398 Table 20), the most significant of which (larval serum protein complex and synaptic target
399 inhibition) were upregulated in interacting larvae responding to heat stress (Fig 3C).
400
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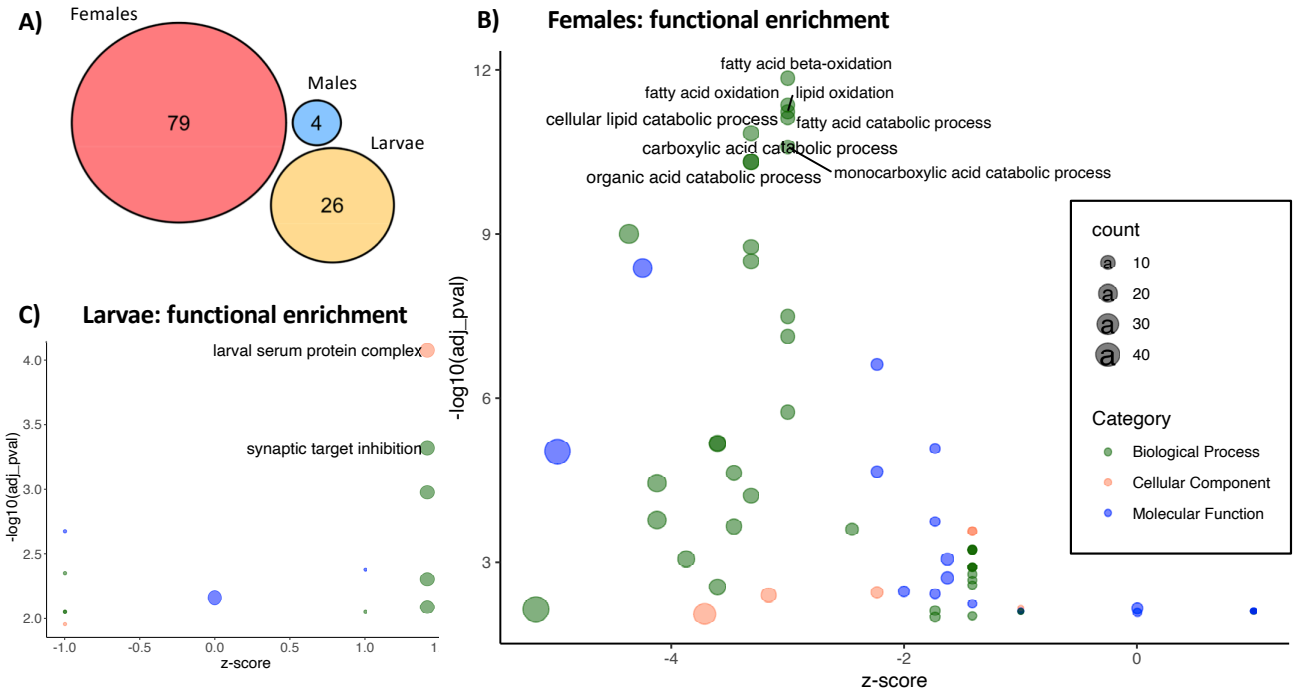

Figure 3: Visual summary of genes showing significant differential expression associated with parent-offspring interactions for each family member under the different temperature (20°C versus 24°C). (A) Venn diagram showing the number of significantly differentially expressed genes (DEGs) in each cohort (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.

416 **DISCUSSION**

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

430 With our study, we provide a first glimpse into the genes underlying parent-offspring and
431 offspring-parent interactions in a subsocial insect with biparental care. We characterized the gene
432 expression profiles of family members before and 24 hours after larvae colonized the breeding
433 resource. Not surprisingly, we found a larger transcriptional response in offspring than in
434 parents. Thousands of differentially expressed genes (DEGs) were detected in larvae spanning
435 many functional categories, consistent with the very rapid development and growth occurring at
436 this early stage (Won *et al.* 2018) and the strong influence of parenting (Capodeanu-Nägler *et al.*
437 2018; Mashoodh *et al.* 2021). In comparison to the dramatic changes that occur in larvae once
438 they are accepted by parents, the behavioral switch required for parents to accept their larvae and

439 begin active parenting is relatively subtle, occurring more than 50 hours into care (Oldekop *et al.*
440 2007). In line with past brain transcriptomic studies suggesting that the modification of behavior
441 within a state (i.e., parenting) involves a much smaller subset of the genes required to transition
442 between states (Bell *et al.* 2016; Benowitz *et al.* 2017a; Cunningham *et al.* 2019), changes in
443 gene expression associated with the switch to active parenting in our study were subtle compared
444 to, for example, the changes in expression associated with the transition from non-parenting to
445 parenting states in *N. vespilloides* (up to 650 DEGs; Parker et al. 2015, Cunningham et al. 2017).
446 As in previous work on the related *N. vespilloides* (Parker et al. 2015), males and females
447 parenting together showed strong concordance in overall gene expression patterns, but the
448 magnitude of transcriptional response in terms of number of significantly differentially expressed
449 genes (DEGs) was greatly reduced in males. Moreover, differences in functional enrichment of
450 male and female DEG sets and their overlap with offspring implies that the transcriptomic
451 architecture underlying parent-offspring interactions may be sex specific. For example, the top
452 functionally enriched categories in actively parenting mothers (e.g., lipid and carboxylic acid
453 metabolism) were also enriched in the overlap between females and offspring – a trend not
454 detected in fathers. We propose that genes shared between mothers and offspring may play a role
455 in feeding and nutrient exchange (i.e., mouth-to-mouth regurgitation), given that mothers in
456 biparental pairs were seen to perform a disproportionate share of provisioning in our behavioral
457 experiment (Moss & Moore 2021). If we are correct, it would suggest that there are substantial
458 sex-specific aspects of how parental care is manifested at the level of gene expression even when
459 care is predominantly biparental (Benowitz & Moore 2016).
460 In addition to being a social exchange, parental investment and offspring development
461 are sensitive to environmental quality as poor conditions can impose competing physiological

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

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

485 3B). Lipids are the principal macronutrient used for fueling energetically intensive behavior in
486 insects (e.g., flight: Canavoso et al. 2003), yet previous efforts to link post-hatching care (i.e.,
487 presumed to be the costliest form of care) to increased lipid metabolism in burying beetles have
488 turned up no relationship (Benowitz *et al.* 2017b). Here, we suggest that females restrict the use
489 of stored energy even more in an energetically demanding environment, implying that the
490 combined stressors of temperature and parenting could directly mediate trade-offs between
491 current and future reproduction. Offspring of stressed mothers in turn upregulated genes
492 involved in forming the larval serum protein complex – a structure that sequesters amino acids
493 early in development for the synthesis of adult proteins (Chrysanthis *et al.* 1994). Whether this
494 transcriptional response by larvae may help compensate for constrained parental investment
495 remains to be seen. What is clear is that parental responses are unlikely to stabilize behaviors or
496 enhance offspring success, and thus the scope for climate change adaptation to proceed *via*
497 selection on the transcriptional architecture underlying care would appear limited.
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499
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