Article Title: Limited plasticity in gene expression for providing or receiving parental care under different temperatures

Short Running Title: Plasticity in gene expression influencing parenting

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ABSTRACT

Parenting is thought to evolve to buffer offspring from variable, unpredictable, and challenging environmental conditions. In the subsocial carrion beetle, *Nicrophorus orbicollis*, stressful temperatures during parenting do not affect parental behavior despite imposing steep fitness costs to parents. Here, we ask if plasticity of gene expression underpins this behavioral stability or facilitates independent compensation by larvae. To test this we characterized gene expression of parents and offspring before and during active parenting under benign (20°C) and stressful (24°C) temperatures to identify genes of parents and offspring associated with thermal response, parenting/being parented, and gene expression plasticity associated with behavioral stability of parental care. The main effects of thermal and social condition each shaped patterns of gene expression in females, males, and larvae. In addition, we implicated 79 genes in females as 'buffering' parental behavior across environments. The majority of these underwent significant changes in expression in actively parenting mothers at the benign temperature, but not at the stressful temperature. Our results suggest that neither genetic programs for parenting nor their effects on offspring gene expression are fundamentally different under stressful conditions, and that behavioral stability is associated primarily with the maintenance of existing genetic programs rather than replacement or supplementation. Thus, while selection for compensatory gene expression could expand the range of thermal conditions parents will tolerate, without expanding the toolkit of genes involved selection is unlikely to lead to adaptive changes of function.

Keywords: Insects, Parental Care, Transcriptomics, Parent-Offspring, Climate Change

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 even beyond those that promoted its evolution (Clutton-Brock, 1991; Royle, Smiseth, & Kölliker, 2012; Wilson, 1975). 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 for offspring to compensate when conditions exceed those that parents can or are willing to buffer. In some cases, this phenotypic plasticity may be immediately detectable as a change in behavior. For example, many birds compensate for hot breeding season conditions by extending their time and effort at the nest, sometimes risking dehydration to maintain stable incubation conditions for embryos (McClintock, Hepp, & Kennamer, 2014; Clauser & McRae, 2016; Sharpe, Bayter, & Gardner, 2021), while offspring have been shown to upregulate metabolic and immune pathways in response to the removal of a caring parent (Körner et al., 2020; Mashoodh, Westoby, & Kilner, 2021; Ziadie, Ebot-Ojong, McKinney, & Moore, 2019) or exposure to pathogens (Körner et al., 2020). However, when parents and offspring are pushed to their physiological limits, maintaining a phenotype stability (i.e., continuing to provide care at the same level) is likely just as important for ensuring offspring success as is altering behavior.

Transcriptomic approaches are useful for examining gene expression plasticity beneath the surface of phenotypes because gene expression is inherently plastic, and gene expression profiles can undergo profound changes while maintaining stable higher-level phenotypes (Eberwine & Kim, 2015; Nijhout, Best, & Reed, 2019; Rivera et al., 2021); that is, many ‘genetic programs’ can give rise to identical phenotypes. While most research in this area has

70 focused on the role of plastic gene expression in maintaining physiological performance and
71 stabilizing morphological traits under stress (Oleksiak, Roach, & Crawford, 2005; Cheviron,
72 Whitehead, & Brumfield, 2008; Cheviron & Brumfield, 2011; Peck et al. 2015), compensatory
73 molecular mechanisms are also expected to underpin stability of complex behaviors across
74 fluctuating environments (Fischer, Hauber, & Bell, 2021). This perspective is valuable to the
75 study of parental care because parenting evolves to minimize environmental fluctuations and
76 involves transformational changes of gene expression (Parker et al. 2015; Ray et al. 2016;
77 Bukhari et a. 2019; Fischer et al. 2019; Lopes & de Brujn), and the ultimate configuration of
78 expressed genes associated with care may differ depending on environment. Because parental
79 care is inherently a social exchange and phenotypic plasticity can stem from donors and/or
80 recipients of care, a comprehensive test of these predictions should profile the gene expression of
81 parents and offspring simultaneously (Butler & Maruska 2021).

82 Here, we performed RNA-seq on a subsocial burying beetle, *Nicrophorus orbicollis*, to
83 explore if plasticity of gene expression helps buffer families against environmental stressors.
84 There is a strong basis for studying the genetics of parenting and development of burying beetles
85 (Benowitz, McKinney, Cunningham, & Moore, 2017; Cunningham et al., 2019; Jacobs et al.,
86 2016; Palmer et al., 2016; Parker et al., 2015; Won et al., 2018), yet the genes responsible for
87 mounting responses to major environmental stressors while beetles are parenting/being parented
88 remain uncharacterized. Such mechanisms are particularly relevant in the context of rapid
89 environmental change, as they could provide targets of selection in populations unable to
90 behaviorally avoid stressful conditions. Even less understood is the extent to which application
91 of a secondary stressor may modify core parental responses; i.e., how plasticity in behavioral
92 gene expression buffers higher level phenotypes.

93 Higher temperatures during parenting for *N. orbicollis* impose steep reproductive and
94 survival costs; however, females and males do not change their parental care behavior, type, or
95 intensity (Moss and Moore, 2021). Whether behavioral stability of parents results from gene
96 expression changes that alter or supplement the genetic program for parental care, or whether
97 gene expression changes in offspring may independently compensate for thermal stress, is not
98 known. Here, we address these possibilities while controlling for any confounding effects of
99 differences in care. We examined the effects of two thermal environments to characterize
100 'thermal response' genes (20°C or 24°C, the same as that investigated in Moss and Moore, 2021)
101 and parent-offspring interactions to characterize 'parenting/being parented' genes (before or
102 during active parenting), comparing responses of mothers, fathers, and offspring to understand
103 the similarity of each's response. Our prediction was that thermal stressors and parental care will
104 each elicit independent gene expression responses and that these will differ between family
105 members (represented by the main effects of the statistical model). Further, we predicted that
106 some genes would show distinct patterns of expression change in response to the combination of
107 stressors to stabilize the behaviors across environments ("buffering genes" represented by the
108 interaction term of the statistical model); specifically, higher temperatures should significantly
109 modify expression of core genes for parenting/being parented or elicit distinct changes in gene
110 expression during family interactions if genetic plasticity facilitates behavioral stability across
111 environments.
112
113
114 **MATERIALS AND METHODS**
115

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). Temperatures at the upper end of this range (23–25°C) are highly challenging for parents and developing offspring, with families forced to breed under these conditions suffering shortened adult and larval lifespans and reduced clutch size larval mass (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 excretions, 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 larvae of *N. orbicollis* have relatively high starvation tolerance (mean time to starvation: ~17 hours), upon hatching their capacity to self-feed from a prepared carcass is extremely limited, and they rely on at least three hours of post-hatching parental care for survival (Capodeanu-Nägler et al. 2018). This post-hatching stage is likely to be the most energetically demanding stage of care for parents as it is when attendance to offspring is highest and the stage at which we see the most sex differences of parental behavior (Moss & Moore, 2021).

Study Design

138 We used *N. orbicollis* beetles that had been collected locally and reared in the laboratory
139 for one generation at the University of Georgia, as described in Moss and Moore (2021).
140 Different beetles were used to generate behavioral (Moss and Moore 2021) and transcriptomic
141 datasets (this study). Briefly, adults were housed individually in plastic containers (9 cm
142 diameter, 4 cm deep; Eco Products, Boulder, CO, USA) filled with potting soil and fed organic
143 ground beef twice weekly *ad libitum.* Stock breeding took place at room temperature (20 ±
144 0.5°C), and resulting offspring were divided evenly between two incubators starting three days
145 into pupation for a thermal acclimation period prior to breeding. These incubators were
146 programmed to long-day light conditions (reverse light:dark 14:10) on ramping temperature
147 cycles to simulate a diurnal range: the first fluctuating between 21 and 20°C and the second
148 between 25 and 24°C. Because breeding takes place underground in relatively thermostable
149 conditions, however, only the lower end of the diurnal range of each thermal treatment (20°C
150 and 24°C) was used for breeding. Virgin, non-sibling beetles (aged ≥ 14 days post-eclosion)
151 were paired in plastic boxes (17.2 x 12.7 x 6.4 cm; Pioneer Plastics, Dixon, KY, USA) filled
152 with approximately 2 cm of moistened soil and a 40–45 g thawed mouse carcass (RodentPro,
153 Evansville, IN, USA) and moved to either a constant dark temperature-controlled room (20°C) or
154 incubator (24°C) corresponding to their acclimation environment. Boxes were monitored twice
155 daily for the presence of eggs, and after 2–3 days eggs were collected into petri dishes containing
156 damp filter paper and stored at their respective treatment temperatures. Eggs were checked every
157 four hours between 0800 and 1700 until hatching, which occurs within two days after laying.
158 In addition to manipulating the thermal environments in which care took place, we also
159 generated within-temperature treatment controls to disentangle the effects of "active" parenting
160 (the stage at which parents and larvae interact socially, which we refer to as parenting or direct

161 parental care) from a general parenting state which includes behavior such as carcass preparation
162 that does not require social interactions and begins before larvae are present. Thus, we sampled
163 at two time points: 1) post-larval hatching but before larvae and parents interacted (≤ 16 hours
164 old larvae); and 2) after the first 24 hours of direct parental care. These larvae were collected
165 directly from the petri dish immediately when found to have hatched, with parents collected
166 simultaneously from the carcass they were preparing. Larvae of families for parenting samples,
167 by contrast, were transferred to brood balls attended by parents when found to have hatched.
168 These families were left undisturbed for 24 hours so that direct parenting could take place before
169 collecting both parents and offspring (24–40 hours old larvae) for analysis. Parents in both
170 treatments had initiated indirect parental care involving preparation of a brood ball for larval
171 feeding. Timing of sampling parents before interactions coincided with the circadian switch to
172 active parenting (Oldekop, Smiseth, Piggins, & Moore, 2007).
173
174 *Sample Collection, Preparation, and RNA Sequencing*
175 Our design yielded ten families at 20°C (five before parenting, five during parenting) and
176 nine families at 24°C (four before parenting, five during parenting). We collected the heads of
177 adults, consisting of brain and associated "fat body" tissue, as in previous studies (Parker et al
178 2015). This allowed us to capture gene expression in the two tissue types that influence behavior
179 in insects. Because of the size of larvae at hatching and because we had no *a-priori* predictions
180 for the specific tissues affected by interactions with adults, we collected whole bodies. Larval
181 samples therefore consisted of seven to ten larvae for families before parenting and two to four
182 larvae for families during parenting. Individual adult samples, both before and during parenting,
183 were age-matched. Samples were flash frozen in liquid nitrogen and stored at -80°C until RNA

184 extraction. Extractions were performed following the Qiagen RNAeasy Lipid kit protocols
185 (Qiagen, Venlo, the Netherlands). Sequencing libraries were prepared from 1.3 µg total RNA
186 using the Illumina TruSeq mRNA Stranded Library Kit (Illumina, Inc., San Diego, CA, USA)
187 according to the manufacturer's protocol with standard Illumina adapters and primers.
188 Sequencing was carried out on a NovaSeq 6000 platform with a 150 bp paired-end protocol,
189 targeting 40M read pairs per sample.
190
191 *Read Mapping, Transcriptome Assembly, and Annotation*
192 Reads were trimmed using Trimmomatic (Version 0.39; Bolger et al. 2014) to remove
193 adapter sequences with default parameters, except low-quality bases (Phred <15 at the leading
194 and trailing ends and from 4-base sliding windows), and short reads (<36 bp). Reads were
195 corrected using RCorrector with default parameters (Version 1.0.3.1; Song and Florea 2015).
196 Quality was assessed using FastQC (Version 0.11.9;
197 http://www.bioinformatics.babraham.ac.uk/projects/fastqc/; Supplementary Table 1). Paired-end
198 reads were mapped against a draft reassembly of the WGS data of Benowitz et al. (2017) of *N.*
199 *orbicollis* genome (NCBI BioProject PRJNA371654; Supplementary Methods 1). Read mapping
200 was performed using HISAT2 with default parameters (Version 2.1.0; Kim et al. 2015). A
201 reference-guided transcriptome was assembled using StringTie (Version 2.1.1; Pertea et al. 2015)
202 and redundant transcripts were removed using CD-HIT (Version 4.8.1; Li and Godzik 2006)
203 with a sequence similarity cut-off value of 95%. Finally, we assessed the completeness of the
204 assembly using BUSCO (Insecta dataset; Version 5.0.0; Simão et al. 2015).
205 Annotation of contigs was performed using a combination of approaches. Briefly, the
206 longest isoform of each gene was screened against the annotated and published genome of the

207 closely related burying beetle *N. vespilloides* (NCBI Assembly Accession No.
208 GCF_001412225.1; Cunningham et al., 2015) using Magic-BLAST (Version 1.5.0; Boratyn et
209 al. 2019). Transcripts were also fed through the 'dammit' pipeline (http://dib-
210 lab.github.io/dammit/), which identifies candidate coding regions in transcripts with
211 Transdecoder (Version 2.0.1; https://hpc.ilri.cgiar.org/transdecoder-software) and annotates
212 them, drawing from multiple databases. We used the *N. vespilloides* proteome (Cunningham et
213 al., 2015) as a user database to search against and also retained any hits to known protein
214 domains in Pfam-A (Version 32.0; El-Gebali et al. 2019) and orthologs in OrthoDB (Version 10;
215 Kriventseva et al. 2019). Orthologous genes were retrieved from the OrthoDB database by
216 searching cluster IDs at the level Polyphaga. Gene ontology (GO) terms for annotated genes
217 were assigned by using Pfam accession numbers and GO terms of OrthoDB assignment. GO
218 terms were also supplemented using eggNOG-mapper (Version 5.0; Huerta-Cepas et al. 2019)
219 and OrthoDB searches at the Order (Insecta) level. Unique GO terms from any source were
220 retained for a gene.
221
222 *Transcript Quantification and Filtering*
223 We estimated transcript abundances for each sample separately using StringTie. A
224 transcript count matrix was extracted with the prepDE.py script provided with StringTie and
225 imported into R (Version 4.0.3; R Core Development Team 2019) along with sample library
226 sizes (calculated using Picardtools, Version 2.21.6; http://picard.sourceforge.net) and transcript
227 lengths. We computed the fragments per kilobase per million (FPKM) matrix with the package
228 *countToFPKM* (Version 1.0; Alhendi 2019). For each family member and social state (before or
229 during parenting), we retained transcripts that were expressed >2 FPKM in more than half of

230 samples and then combined all unique transcripts across groups in downstream analyses (Bloch
231 et al., 2018). Finally, we used *IsoformSwitchAnalyzeR* (Version 1.12.0; Vitting-Seerup and
232 Sandelin 2019) to convert transcript-level counts into gene-level counts.
233
234 *Differential Expression Analysis*
235 We performed differential expression analysis using *DESeq2* (Bioconductor Version
236 1.30.0; Love et al. 2014). To cluster samples, we first applied variance stabilizing transformation
237 using DESeq2 and performed a principal components analysis (PCA) using R's built-in 'prcomp'
238 function. Visualization of the clustering was done using *DESeq2*'s 'pcaplot' function and
239 extracted principal components were regressed on each grouping factor (family member,
240 temperature, parenting, and the statistical interactions among these factors) *via* analysis of
241 variance (ANOVA using R). Significant differences among family members in the full model
242 ANOVA led us to analyze males, females, and larvae separately. For each family member,
243 differential expression was estimated using parametric dispersion. We used the likelihood ratio
244 test (LRT) implemented in *DESeq2* to compare the goodness-of-fit of several models for each
245 family member to estimate differential gene expression. Two tests were used to capture gene
246 expression changes in response to main effects. The first compared a full model fitted with both
247 temperature and parenting (temperature + parenting) to a reduced model fitted only with
248 parenting to identify differentially expressed genes mutually affected by temperature across
249 parenting states (henceforth, 'thermal response' genes). The second compared the same full
250 model to a reduced model fitted only with temperature to produce a list of differentially
251 expressed genes mutually affected by parenting across thermal environments (henceforth,
252 'parenting' genes). The *p*-values that resulted from these tests were adjusted for multiple testing

253 using the Benjamini-Hochberg correction (Benjamini & Hochberg, 1995) and genes with
254 adjusted $p < 0.05$ were considered statistically significantly differentially expressed genes.
255 To assess similarity of gene expression responses to the main effects of temperature and
256 parenting across family members, we extracted lists of overlapping genes from the intersect of
257 male, female, and larval parenting/being parented and thermal response gene sets. To determine
258 whether overlap was significantly greater than expected by chance given variable input sizes, we
259 further carried out randomization tests. Briefly, 10,000 gene sets were randomly generated for
260 males, females, and larvae by sampling from the full list of expressed genes in that family
261 member a number equivalent to the size of the gene set being simulated. These random gene sets
262 were used to calculate a null distribution of overlap values, and significance was calculated as
263 the proportion exceeding the observed overlap for each pairwise comparison. While overlap of
264 the most highly significant genes between two sets lends itself to intuitive interpretation, use of
265 fixed thresholds can be overly stringent and lead to false negatives. Therefore, we implemented a
266 complementary threshold-free approach: rank-rank hypergeometric overlap (RRHO) analysis.
267 This analysis ranks DE genes according to their significance level along with the magnitude and
268 direction of change to make qualitative assessments of concordance between complete gene sets
269 independent of *p*-value cutoff. Results were visualized using the stratified method of the R
270 package *RRHO2* (Version 1.0; Cahill et al. 2018), with genes ranked according to the
271 recommended metric of -log10(*p*-value)*sign($\log_2$ fold change).
272 Finally, we assessed whether the genetic program for parenting/being parented was
273 altered or supplemented under the addition of thermal stress in two ways. First, we performed a
274 differential expression analysis using the LRT approach to identify genes showing distinct
275 responses to parenting/being parented (substantial differences in magnitude or different signs) in

276 the presence of thermal stress (henceforth, 'buffering' genes). To obtain this list, we compared a
277 full model fitted with a statistical interaction term (temperature x parenting) to a reduced model
278 fitted only with the main effects (temperature + parenting). To further characterize patterns of
279 differential gene expression across social and temperature groups, we performed an ANOVA
280 followed by post-hoc contrasts (Tukey HSD) at the level of each gene in the buffering set,
281 comparing the magnitude of expression and sign of the response at 24°C vs 20°C. Second, we
282 compared differential expression of parenting/being parented genes between the two thermal
283 environments. Even if changes of expression of these genes are similar enough across thermal
284 environments to be captured as main effects, the magnitude of the response may be enhanced or
285 dampened due to thermal stress. To test this possibility, we estimated log2fold changes of
286 expression of parenting/being parented genes separately for each temperature treatment using the
287 'ashr' shrinkage estimator with false discovery rate correction (Stephens 2017) and fit a
288 regression to these data. A slope different than one allows rejection of the null hypothesis that
289 responses are generally similar in magnitude across thermal treatments and provides a more
290 holistic comparison of the treatments.
291
292 *Functional Enrichment Analysis and Candidate Genes*
293 We queried the differentially expressed gene sets and the overlapping gene sets for
294 enriched Gene Ontology terms using topGO (Version 2.42.0; Alexa and Rahnenfuhrer 2020)
295 searching for enrichment in all three gene ontology categories: biological process, molecular
296 function, and cellular component. The nodeSize parameter was set to 10 to remove GO terms
297 with fewer than ten annotated genes and only terms with >1 significant gene were retained.
298 Significance was estimated using a Fisher's exact test following the program's suggested

299 protocol. To examine the directionality of change of enriched pathways, Z-scores were
300 calculated following the formula of Walter, Sánchez-Cabo, & Ricote (2015): number of
301 downregulated genes annotated subtracted from the number of upregulated genes annotated for
302 the term divided by the square-root of the total number of genes annotated for the term.
303
304
305 **RESULTS**
306 Our final transcriptome assembly contained 91% of single-copy conserved nucleotide
307 orthologs (Complete: 90.5% [Single Copy: 76.4%, Duplicated: 14.1%], Fragmented: 4.2%,
308 Missing: 5.3%) and a total of 12,406 genes.
309
310 *Thermal and Social Conditions Shape Gene Expression of Family Members*
311 We visualized differences in gene expression among male parents, female parents, and
312 offspring using principal components analysis. Parents and offspring separated strongly along
313 two primary axes (Fig 1A), which together explained 77.8% of total variance in gene expression
314 across all samples (PC1: 73.08%; PC2: 4.76%; Table 1). Family member (parent vs larvae)
315 clearly separated along PC1, while separation along PC2 was driven primarily by parenting
316 (Table 1). The interactive effect of family member and parenting and family member and
317 temperature were significantly associated with PC2 (Table 1; Fig 1B). Visualizing family
318 members separately with their own dispersions further clarified how global gene expression
319 differed among groups. Female gene expression displayed considerable overlap between thermal
320 environments but was most divided with respect to parenting, particularly in the 20°C
321 environment (Fig 1C). Male gene expression differentiated most strongly with temperature (Fig

322 1D). In larvae, behavioral groups formed distinct clusters, with parenting accounting for far
323 greater among-group differences than temperature (Fig 1E).
324
325 *Plasticity of Gene Expression Underpins Thermal Response, Parenting/Being Parented, and*
326 *Buffering*
327 We used differential expression analysis to characterize plastic changes in gene
328 expression associated with the main effects of temperature and parenting, as well as their
329 interaction. We identified genes showing both shared (overlapping) and distinct responses to
330 main effects across family members, consistent with variation in physiology and behavior
331 between sexes and life stages. There was also gene expression variation associated with
332 buffering of behavioral responses, where family members expressing the same behaviors in
333 different environments showed different patterns of gene expression. These included subtle
334 expression variation of parenting/being parented genes across temperature treatments, as well as
335 some changes that occurred specifically in response to the interaction of temperature and
336 parenting.
337
338 *i. Thermal Response Genes*
339 Temperature induced a stronger gene expression response in males (n = 418 differentially
340 expressed genes; Supplementary Table 2) and larvae (n = 487 differentially expressed genes;
341 Supplementary Table 3) than in females (n = 284 differentially expressed genes; Supplementary
342 Table 4). However, male, female, and larval responses to temperature showed statistically
343 significant overlap in terms of gene identity (male-female: $P < 0.001$; female-larvae: $P < 0.001$;
344 male-larvae: $P = 0.006$; Fig 2A). Global concordance was pronounced between males and

345 females (Fig 2C) – both in genes that were up-regulated and down-regulated in response to high
346 temperature – whereas this signal was weak when comparing females to larvae (Fig 2D) and
347 males to larvae (Fig 2E).
348
349 *ii. Parenting Genes*
350 With the parenting main effect, the highest number of differentially expressed genes was
351 associated with being parented, with 7,189 genes differentiating larvae before and during
352 interactions with parents (Supplementary Table 5). Adults had fewer differentially expressed
353 genes than larvae, and within adults there were sex differences. Fewer differentially expressed
354 genes were detected in males after the arrival of larvae (n = 63; Supplementary Table 6) than in
355 females (n =189; Supplementary Table 7). Despite this, females and males showed statistically
356 significant overlap of gene identities while parenting (Fig 2B; $P = 0.044$). When considering all
357 expressed genes, concordance was striking but directional, with overlap between the sexes
358 concentrated in genes downregulated in both during larval interaction (Fig 2F). Females
359 interacting with larvae and larvae interacting with parents shared more differentially expressed
360 genes in common (n = 115) than parenting males and parented larvae (n = 28), although this did
361 not reach statistical significance ($P = 0.059$) and neither comparison showed global signatures of
362 concordance of gene expression (Fig 2G–H).
363
364 *iii. Buffering Genes*
365 To evaluate the role of gene expression plasticity in facilitating parental buffering and
366 offspring compensation under stressful environments, we performed a transcriptome-wide survey
367 for genes showing distinct responses to parenting/being parented at 24°C versus 20°C, or a

368 significant interaction between temperature and parenting. More genes met this criterion and
369 were differentially expressed in females (n = 79; Supplementary Table 8) than in males (n = 4;
370 Supplementary Table 9) or larvae (n = 26; Supplementary Table 10). To test whether genes
371 showing plasticity indeed showed stronger and broader expression changes at the more stressful
372 temperature, the 79 buffering genes in females were subjected to closer examination
373 (Supplementary Table 11). At the benign temperature (20°C), most genes (n = 57) showed
374 increases of expression under active parenting, whereas at the higher temperature (24°C) only
375 five genes showed any change in expression. Hence, most buffering genes fell into two
376 categories: gene expression levels before parenting were equivalent between thermal treatments
377 (n = 35) or gene expression levels started out significantly higher at 24°C than at 20°C (n = 32;
378 e.g., Apolipophorin-III; Fig 3C). Because plasticity was temperature-dependent, gene expression
379 levels during parenting were either significantly lower at 24°C (n = 29) or did not differ between
380 thermal environments (n = 45), and only five genes showed significantly higher expression levels
381 during parenting in the warmer treatment.
382 We next compared gene expression responses to parenting/being parented across
383 temperature treatments. Among the core genes for parenting/being parented (genes that were
384 differentially expressed between parenting states across temperatures), differential expression
385 was weaker at 24°C relative to 20°C in females (slope = 0.514; Fig3A, B) and males (slope =
386 0.357), whereas the slope of this regression in larvae was close to one (slope = 0.976), suggesting
387 that responses of larvae were generally similar in magnitude across thermal environments.
388
389 *Functional Enrichment Analysis*

390 Functional enrichment of GO terms in the within-family member temperature contrasts
391 revealed both shared and distinct enriched functional characteristics across females, males, and
392 larvae. The most significantly enriched GO terms in parents were related to chitin and
393 carbohydrate metabolism (Supplementary Table 12–13), whereas larvae showed the most
394 enrichment for cellular housekeeping functions (e.g. ribosome biogenesis and rRNA processing;
395 Supplementary Table 14). Compared to the 20°C group, females, males, and larvae in the 24°C
396 group predominantly showed downregulation of functional pathways.
397 Actively parenting females showed significant positive enrichment in a range of
398 metabolic, catabolic, and biosynthetic functions compared to their respective non-interacting
399 controls (Supplementary Table 15). Forty-five of the 64 GO terms that were enriched in the
400 female parenting gene set were also significantly enriched in the overlap between female
401 parenting genes and larval parented genes, particularly terms related to the metabolism of
402 organic, carboxylic, and fatty acids (Supplementary Table 16). Conversely, analysis of GO terms
403 in males revealed significant downregulation of non-overlapping GO terms (e.g. larval cuticle
404 patterning; Supplementary Table 17). Larval functional pathways were predominantly
405 upregulated in response to interactions with parents and spanned many categories, from cellular
406 organization and differentiation to whole organism development (Supplementary Table 18).
407 Finally, we examined Gene Ontology term enrichment under the interaction of parenting
408 and thermal environment. Buffering genes of males (n = 4) showed enrichment in three pathways
409 related to lipid transport and localization (Supplementary Table 20) while those in larvae (n =
410 26) showed enrichment in five pathways (Supplementary Table 21), the most significant of
411 which (synaptic target inhibition and negative chemotaxis) were upregulated in parented larvae
412 responding to thermal stress. In females interacting with larvae, we found 45 significantly

413 enriched GO term annotations for genes that were differentially expressed between 20°C and
414 24°C, including 26 GO terms that were also enriched in the female parenting gene set but in the
415 opposite direction (Supplementary Tables 12, 19; Fig 3D). Top GO terms related to the oxidation
416 and catabolism of fatty and organic acids and comprised genes that were significantly
417 downregulated, on average (Supplementary Table 19).
418
419
420 **DISCUSSION**
421 Parenting is expected to evolve to buffer environmental stress, but parenting involves an
422 interaction between the parent and the offspring. For parenting to be effective, the family
423 interaction needs to be predictable and the mechanisms responsible for stabilizing these
424 interactions across variable environments are not well understood. Plasticity of gene expression
425 could allow families to optimize dynamics for their current environment. If true, then parenting-
426 related responses to an ecologically relevant stressor should have a gene expression signature.
427 We test this prediction in a subsocial insect with biparental care. We found that thermal and
428 social conditions both shape patterns of gene expression in *N. orbicollis* females, males, and
429 larvae, and that many responses are distinct to specific family members. Further, we found
430 evidence for some plasticity of gene expression in response to the combination of social and
431 thermal stressors. Specifically, parenting males and females, but not parented larvae, showed
432 weaker gene expression responses to interactions with larvae under stressful temperatures. In
433 females 79 genes responded to parenting in an entirely temperature-dependent manner, showing
434 significant changes in expression at the benign temperature but not at the stressful temperature.
435 Taken together with our previous findings of limited behavioral plasticity of *N. orbicollis* parents

436 exposed to stressful temperatures (Moss & Moore, 2021), this implies that behavioral stability
437 under stress is associated primarily with maintenance of existing genetic programs, rather than
438 switching to an alternative or supplemented genetic program for the same complex behavior.
439 Parental investment and offspring development are sensitive to environmental quality as
440 poor conditions can impose competing physiological demands between parenting and thermal
441 response. To capture and compare the gene expression and hence the molecular pathways
442 involved in responses to thermal stress, we sampled families from a benign (20°C) and a
443 stressful (24°C) thermal environment across the breeding cycle. While our previous work
444 showed that *N. orbicollis* do not modify their behavior in response to temperature (Moss and
445 Moore 2021), constitutive changes of gene expression of adults and larvae exposed to high
446 temperatures across stages of care suggest that any phenotypic stabilization over this thermal
447 range is at least partly due to the buffering action of background physiological processes. In
448 parents, the predominant gene expression response to heat stress was downregulation,
449 particularly of pathways involved in cuticle formation and carbohydrate metabolism
450 (Supplementary Tables 12–13). Similarly, larvae showed downregulation of many pathways
451 involved in cellular housekeeping functions (Supplementary Table 14). These findings are
452 consistent with several long-term acclimation experiments reporting genome-wide
453 downregulation in response to extreme heat stress, which could either point to an inability to
454 mount systemic responses (i.e., due to insufficent evolutionary history of such extremes; Levine,
455 Eckert, & Begun 2011; Becker et al., 2018) or an adaptive molecular mechanism for metabolic
456 compensation (Yampolsky et al. 2014). The high temperature applied here was at the upper limit
457 but still within the range experienced in the habitat our beetles were collected (Moss and Moore,
458 2021), suggesting the latter as a more likely explanation. In sum, thermal stress appears to be

459 mitigated through the regulation of different functional pathways in parents vs larvae but follows
460 the same general pattern – tempering all gene expression responses at high temperatures – which
461 could impose carry-over effects on gene expression underlying parent-offspring and offspring-
462 parent interactions

463 With our study, we provide a glimpse into these mechanisms by comparing the gene
464 expression profiles of family members before and 24 hours after offspring colonize the brood
465 ball. Thousands of differentially expressed genes were detected in larvae spanning many
466 functional categories, some due to differences of sampling ages dictated by experimental design.
467 While we cannot disentangle the direct effects being parented *per se* on gene expression
468 (Capodeanu-Nägler et al., 2018; Mashoodh et al., 2021) from indirect effects associated with the
469 rapid development and growth occurring at this early stage (Won et al., 2018), our sampling
470 design was biologically realistic given that orphaned *N. orbicollis* larvae usually succumb to
471 starvation within a day (Capodeanu-Nägler et al. 2018). In comparison, changes of parental gene
472 expression associated with having larvae to parent were subtle (~100–200 differentially
473 expressed genes), in line with the hypothesis that the modification of behavior within a state
474 involves a much smaller subset of the genes required to transition between states (i.e., *N.*
475 *vespilloides* transitioning into parenting from a non-reproductive state differentially express up to
476 650 genes*;* Parker et al. 2015, Cunningham et al. 2017, Bell, Bukhari, & Sanogo, 2016;
477 Benowitz, McKinney, Cunningham, et al., 2017; Cunningham et al., 2019).

478 As in previous work on the related *N. vespilloides* (Parker et al. 2015), males and females
479 parenting together showed strong concordance in overall gene expression patterns even though,
480 as has been found for every study of parenting in male burying beetles, we detected fewer
481 significantly differentially expressed genes for males. More so than males, however, actively

482 parenting females showed intriguing patterns of overlap (Fig 3B) and concordance (Fig 3G) with
483 parented larvae in terms of differentially expressed genes and pathways (i.e., males showed anti-
484 concordance with larvae; Fig 3H). Given that mothers in biparental pairs appear to perform a
485 disproportionate share of larval provisioning (Moss & Moore, 2021), we propose that these
486 overlapping pathways, which include lipid and carboxylic acid metabolism, could play a role in
487 self-feeding (i.e., from a shared resource) and/or nutrient exchange (i.e., mouth-to-mouth
488 regurgitation). In either case, genes with shared or reciprocal functional roles between family
489 members could serve as important targets of selection during adaptation to stressful
490 environments, which may demand more efficient performance of caregivers, offspring, or both.
491 Finally, we characterized patterns of gene expression in response to the combined
492 stressors of parenting/being parented and thermal stress. If plasticity of gene expression is
493 important for maintaining behavioral stability of families across environments, then genes
494 underlying parent-offspring interactions should be expressed differently or include
495 more/different genes dependent on environment. The first way we tested this was by comparing
496 the magnitude of differential expression of parenting/being parented genes between 24°C and
497 20°C. We predicted that if plasticity of gene expression underlies the ability of parents to buffer
498 environmental stress or for offspring to compensate independently, then responses would be
499 more pronounced at the higher temperature. Instead, we found that genes for receiving care (i.e.,
500 in larvae) were differentially expressed to the same degree regardless of temperature and that
501 genes for providing care (i.e., in males and females) showed less robust expression changes at
502 24°C than at 20°C (Fig 3A,B). Weaker differential expression at 24°C is nevertheless a form of
503 plasticity, and like global gene expression responses to thermal stress, could reflect adaptive
504 metabolic compensation by parents.

505 Another possibility is that plastic responses to parenting/being parented mediated by
506 thermal stress involve completely different genes to those captured by the main effects of
507 parenting. If true, then many differentially expressed genes should be captured by the interaction
508 between parenting and temperature, and changes in expression of these genes before and during
509 parenting should be more pronounced and/or change their sign at the higher temperature. Overall
510 few genes satisfied this condition in any family member. While not entirely surprising given that
511 interaction terms have lower statistical power than main effects, this suggests that individuals in
512 the same behavioral state but at a different temperature still rely on more-or-less the same
513 genetic program. The largest gene expression response occurred in heat stressed actively
514 parenting mothers, and closer inspection of this 'buffering' gene set (n = 79) revealed the nature
515 of this plasticity. Genes that were differentially expressed at 20°C (i.e., significantly increased in
516 expression during parenting) did not change expression or a temporal shift followed by sustained
517 expression (i.e., increased before parenting but then showed no further change during parenting;
518 Fig 3C) at 24°C. Thus, while temperature may independently influence the expression of some
519 parenting genes the effects are not synergistic, and rather than amplify differences between
520 behavioral states, thermal stress appears to have a tempering effect.
521 Our inferences based on differential expression analysis were further corroborated by
522 functional enrichment analysis of maternal buffering genes, which revealed significant
523 downregulation of pathways involved in fatty acid oxidation and lipid processing
524 (Supplementary Table 18). Lipids are the principal macronutrient used for fueling energetically
525 intensive behavior in insects (e.g. flight: Canavoso et al. 2003), yet previous efforts to link post-
526 hatching care (i.e., presumed to be the costliest form of care) to increased lipid metabolism in
527 burying beetles found no relationship (Benowitz, McKinney, Roy-Zokan, Cunningham, &

528 Moore, 2017). We suggest that females restrict the use of stored energy even more in an
529 energetically demanding environment, implying that the combined stressors of temperature and
530 parenting could directly mediate trade-offs between current and future reproduction. In line with
531 this, actively parenting cichlids exposed to stressful levels of noise show pronounced gene
532 expression changes in brain regions responsible for homeostatic functioning, suggesting that
533 stressful environments may trigger a neural switch from offspring-promoting to self-promoting
534 behaviors (Butler & Maruska 2021). Offspring of stressed mothers in our study in turn
535 upregulated genes involved in forming the larval serum protein complex – a structure that
536 sequesters amino acids early in development for the synthesis of adult proteins (Chrysanthis,
537 Kaliafas, & Mintzas, 1994). Whether this gene expression response by larvae may help
538 compensate for constrained parental investment or could simply be an indirect effect of plasticity
539 in mothers remains to be seen. What is clear is that parental gene expression responses to larvae
540 are tempered at higher temperatures but generally involve the same genes, meaning that the
541 adaptive function most likely fulfilled by plasticity of gene expression is metabolic
542 compensation. Thus, the scope for temperature adaptation to proceed *via* selection on alternative
543 genetic programs underlying care would appear limited.
544
545
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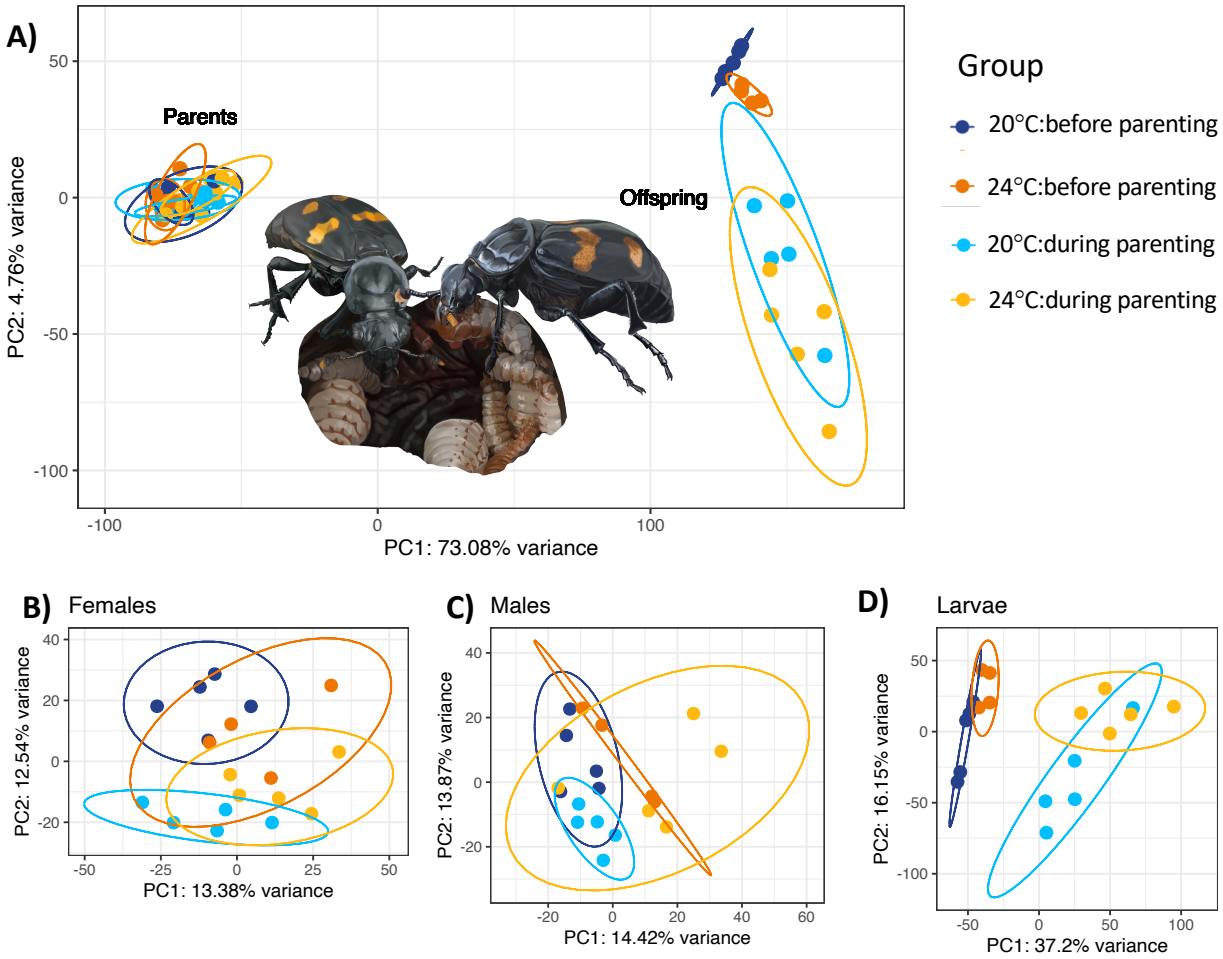
744 **DATA ACCESSIBILITY:** All sequencing data is deposited in NCBI GenBank under the
745 BioProject ID PRJA71530. Raw sequence reads are deposited in the Sequence Read Archive
746 (SRA) under submission SUB9220525 and Accession numbers SAMN18349415–
747 SAMN18349472 and the assembled transcriptome is deposited in the Transcriptome Shotgun
748 Assembly (TSA) Sequence Database under submission SUB9312680. Supporting data and R
749 scripts are deposited as an Open Science Framework project: osf.io/ztyqa.
750
751 **BENEFITS GENERATED:** Benefits from this research accrue from the sharing of our data and
752 results on public databases as described above.
753
754 **AUTHOR CONTRIBUTIONS:** JBM and AJM conceived and designed the study. JBM, ECM,
755 CBC, and AJM collected and analyzed the data. JBM, CBC, and AJM wrote the paper with input
756 from ECM. All authors gave final approval for publication.

757 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
Parenting	**27.238**	<0.0001	**88.311**	<0.0001
Family Member x Temperature	0.499	0.611	**11.406**	<0.0001
Family Member x Parenting	**4.852**	0.012	**92.516**	<0.0001
Temperature x Parenting	2.139	0.151	0.925	0.341
Family Member x Temperature x Parenting	1.653	0.203	1.287	0.286

758



759

760 Figure 1: Principal components analysis (PCA) of normalized transcriptomic profiles across the
761 three family members (females, males, and larvae) and four treatment groups (20°C:before
762 parenting, 20°C:during parenting, 24°C:before parenting, and 24°C:during parenting). (A)
763 Sample clustering along the first two principal components of the full model PCA (~Family
764 Member*Temperature*Parenting). Eclipses are drawn for 95% confidence intervals. (B–D)
765 Clustering of female, male, and larval samples, respectively, when analysis is repeated using
766 only those samples. Illustration of *N. orbicollis* family in Fig 1A by K.E. Kollars.

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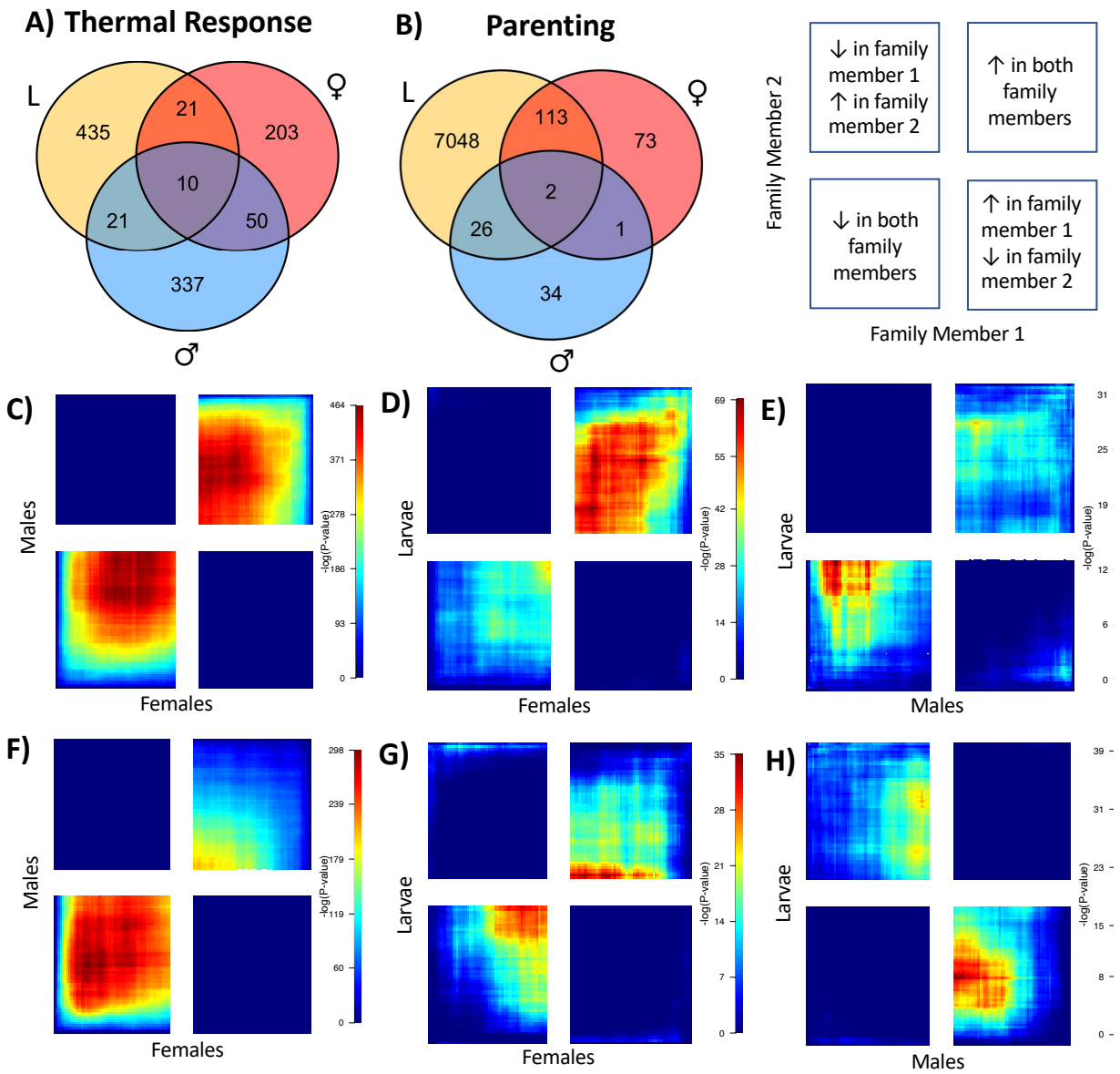

Figure 2: Concordance in differential gene expression between family members as estimated by direct overlap of differentially expressed genes (A–B) and rank-rank hypergeometric overlap (RRHO; C–H). Numbers of shared differentially expressed genes associated with (A) thermal response and (B) parenting are shown in Venn diagrams for females (red), males (blue), and larvae (yellow). The bottom panel shows the results of the RRHO analysis of all 12,406 genes for each of the respective contrasts (20°C vs 24°C: C–E; and with vs without parenting: F–H). The x (family member 1) and y (family member 2) axes of each plot correspond to the

776 significance of differential expression (ranked -log10(*p*-values)) multiplied by the signed
777 log2fold difference in gene expression for each family member-specific analysis. White
778 boundaries demarcate the switch from down- to up-regulation between behavioral groups for
779 each family member. Pixel color corresponds to the value of the -log10(*p*-value) from the
780 differential expression analysis, such that hot spots in the plot designate the strength and
781 directionality of concordance in gene expression between family members.
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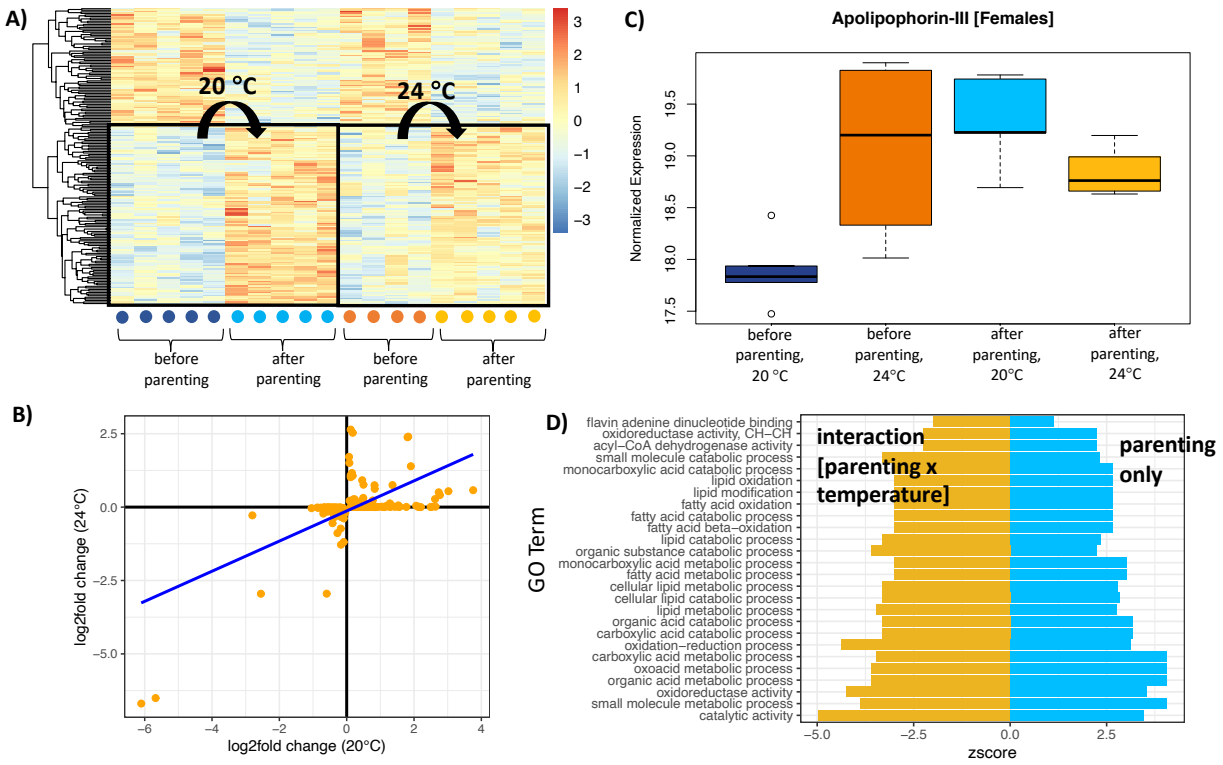

Figure 3: (A) Normalized expression profiles of the 189 genes that were differentially expressed in females before and during parenting depicted as heatmaps, with each column representing an individual sample (grouped by social and temperature treatment) and each row representing a gene (clustered in 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 between groups; (B) Regression of log2fold changes in expression of 189 genes that were differentially expressed in females before and during parenting between 24°C and 20°C environments; (C) Distribution of normalized expression values of Apolipophorin-III across four behavioral groups of females: before parenting at 20°C, before parenting at 24°C, during parenting at 20°C, and during parenting at 24°C; (D) Directionality of expression (calculated as a z-score) of 26 GO terms identified as significantly enriched in females in response to parenting only (blue bars) as well as in response

802 to the interaction of parenting and temperature (gold bars). Z-scores correspond to the

803 standardized number of genes annotated for a term that are upregulated relative to

804 downregulated, such that values less than zero correspond to overall downregulation.