Title: Parent and offspring genotypes influence gene expression in early life

Running Title: Parental effects on offspring gene expression

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Abstract

Parents can have profound effects on offspring fitness. Little, however, is known about the mechanisms through which parental care variation influences offspring physiology in natural systems. White-throated sparrows *Zonotrichia albicollis* (WTSPs) exist in two genetic morphs, tan and white, controlled by a large polymorphic supergene. Morphs mate disassortatively, resulting in two pair types: tan male x white female (TxW) pairs, which provide biparental care and white male x tan female (WxT) pairs, which provide female-biased care. To investigate the effects of parental care variation, we performed RNA-seq on WTSP nestlings sampled from nests of both pair types. Pair type had the largest effect on nestling gene expression, with 881 genes differentially expressed (DE) and seven correlated gene co-expression modules. The DE genes and modules up-regulated in nests with female-biased parental care primarily function in metabolism and stress-related pathways resulting from the overrepresentation of stress response and proteolysis genes. These results show that parental genotypes, a proxy for parental care in this system, alter nestling physiology and highlight avenues of further research investigating the ultimate implications of alternative parental care strategies. Nestlings also exhibited morph-specific gene expression, driven by innate immunity genes and co-expression of genes located in the supergene. Remarkably, we identified the same regulatory hub genes in these blood-derived expression networks as were previously identified in WTSP brains (EPM2A, BPNT1, TAF5L). These hub genes were located within the supergene, highlighting the importance of this gene complex in structuring regulatory networks across diverse tissues.

Keywords

Transcriptome, parental care, early life stress, nestling, RNAseq, ornithology

Introduction

Parents can have profound impacts on offspring development and fitness. Parental effects can manifest throughout the developmental period, both pre- and postnatally (reviewed in Meaney 2001, Lupien et al. 2009). Postnatal effects can be particularly critical in altricial species, where offspring rely entirely on their parents for proper development and growth. Parental effects in these species can be mediated through genetic and physiological aspects of the parent or result from parental behaviors during early development (Trivers 1972).

A major component of these parental effects is mediated by the mother, termed maternal effects. In altricial species, maternal effects are crucial in the prenatal stage, where the mother's genotype and/or phenotype directly influence the offspring phenotype (reviewed in Gluckman et al. 2008). In this case, offspring develop in a maternally created environment and are exposed and influenced by maternal hormones (Wolf & Wade 2009). Maternal hormones can accumulate in the prenatal environment (e.g. uterus, egg) and influence offspring physiology (reviewed in Cottrell & Secki 2009).

Beyond hormone mediated maternal effects, parental behaviors (both maternal and paternal) can also impact offspring. These parental effects can appear prenatally during nest building. Nest location will determine susceptibility to impacts from temperature or sun exposure, humidity, food availability, pollution, and predation (e.g. Lloyd & Martin 2004, Sofaer et al. 2012, Mulholland et al. 2018). However, the magnitude of parental effects is likely largest during the postnatal period, where offspring rely entirely on the parents for provisioning and must compete with siblings during feeding bouts. Thus, this postnatal environment, mediated

56 through parental care, can be a potential source of early life stress (ELS) in offspring, which may
57 result in life-long fitness effects.

58 A prominent area of ELS research focuses on neural development and neuroendocrine
59 signaling (McEwen et al. 2007). The HPA axis is among the most well studied physiological
60 mechanisms of the stress response. ELS is associated with impaired neuroendocrine function and
61 corresponding impaired HPA response, which leads to lifetime consequences in behavior and
62 fitness (e.g. Heim et al. 2008, Crespi et al. 2012, Spencer 2017). Additionally, increased levels of
63 ELS can result in altered behaviors as organisms develop and mature including symptoms of
64 anxiety and depression in the postnatal environment (Noguera et al. 2017) and result in impaired
65 behavior as reproductive adults, with ultimate consequences in fitness. For example, ELS can
66 slow brain development in songbirds, leading to altered song learning and production, which
67 directly impacts the ability to produce and recognize songs as adults, a crucial aspect of songbird
68 reproduction (Spencer et al. 2003, MacDougall-Shackleton & Spencer 2012, Sewall et al. 2018)

69 Epigenetic impacts of ELS have also been extensively studied (Szyf et al. 2007, Szyf
70 2009). In particular, the quality of parental care can have profound impacts on offspring health.
71 Classic studies assessing maternal licking behavior in mice revealed strong epigenetic effects and
72 corresponding physiological consequences in offspring receiving poor parental care (Liu et al.
73 1997, Meaney et al. 2001, Weaver et al. 2004). While there are a variety of epigenetic marks,
74 including histone modification and chromatin accessibility, changes in DNA methylation are
75 often used as an indicator of ELS. Specifically, methylation of the exon 1_F promoter for the
76 glucocorticoid receptor, NR3C1, has received considerable attention. NR3C1 is often
77 hypermethylated in offspring experiencing ELS, which has been linked to physiological disorders
78 later in life (McGowan et al. 2009, Romens et al. 2014, Turecki & Meaney 2016). In general,

these epigenetic modifications are thought to alter transcriptional activity of genes in the modified genomic region. However, very few studies assess genome-wide transcription under ELS, particularly in the context of parental effects (but see: Weaver et al. 2006).

In this study, we studied the white-throated sparrow (WTSP, *Zonotrichia albicollis*) in order to assess the role of parents and ELS on offspring gene expression. WTSPs exist in two plumage morphs, tan (T) and white (W), that are found in both sexes and in roughly equal frequencies (Lowther 1961). These morphs are genetically determined by alternative alleles of a supergene (Schwander et al. 2014), resulting from a complex chromosomal rearrangement comprising multiple inversions (hereafter referred to as "inversion" or "inverted"). This inverted region contains ~1,100 genes on chromosome two, termed $ZAL2^m$ (Throneycroft 1975, Thomas et al. 2008, Romanov et al. 2009, Tuttle et al. 2016). W morphs are nearly always heterozygous for the inversion ($ZAL2/ZAL2^m$) and T morphs are always homozygous (ZAL2/ZAL2) and do not contain the inversion (Thorneycroft 1966, 1975). The morphs of both sexes differ dramatically in behavior, where W morphs are highly territorial, sing frequently, and maintain higher levels of hormones at different times of the breeding season. T morphs are far less territorial and aggressive and maintain lower levels of circulating hormones (Lowther 1962, Kopachna & Falls 1993, Tuttle 2003, Spinney et al. 2006, Swett & Breuner 2009, Horton & Holbertson 2010, Horton et al. 2014). Importantly, males of each morph differ in paternal investment (Knapton & Falls 1983). W morph males are promiscuous and provision nestlings very little. T morph males defend their within-pair paternity through mate guarding and are highly paternal. Additionally, morphs nearly always mate disassortatively, resulting in two stable pair types: T male x W female (TxW) and W male x T female (WxT) (Lowther 1961, Tuttle 2003, Tuttle et al. 2016). Because males differ in paternal investment, this results in two distinct

parental care strategies. TxW pairs provide biparental care and WxT pairs provide female-biased parental care.

Thus, WxT pairs may be a source of ELS (via reduced provisioning) for nestlings. We expect nestlings in WxT nests to elicit a distinct transcriptional stress response relative to nestlings in TxW nests. Alternatively, W morph females may produce higher levels of oestradiol resulting from singing and territorial behavior (e.g. Horton et al. 2014), and this may act as a source of hormone mediated maternal effects in TxW nests. In this case, we may expect to see the opposite pattern, with a transcriptional stress response in nestlings from TxW nests as they cope with the immunosuppressive effects of estrogens (e.g. al-Alfaleq & Homeida 1998). Furthermore, each nest will produce nestlings of both morphs, offering the unique opportunity to investigate the interaction between offspring and parental morphs. As adults, W morphs must cope with high levels of energy expenditure via singing and territorial behavior relative to T morphs. Thus, we predicted W morph nestlings may be better suited to handle ELS mediated through female-biased parental care in WxT nests, or maternal effects in TxW nests, and we would see an elevated stress response in T morph nestlings in these nests. Lastly, we investigate morph-specific gene expression, independent of the pair type of the nestling, to assess the role of nestling morph (i.e. presence or absence of $ZAL2^{m}$ inversion) on nestling gene expression.

Methods

Field based sample collection

All nestling samples in this study came from a breeding population of WTSPs at the Cranberry Lake Biological Station in northern New York, USA (SUNY-ESF, 44.15ºN, 74.78ºW) and were collected during the 2015 breeding season. We only utilized samples collected during

125 the first clutch (June 6 - June 14, 2015), as WTSP males may increase paternal investment in
126 replacement broods (Horton et al. 2014). Nestlings were measured daily (tarsus length, mass)
127 from hatch date or upon locating a nest with nestlings. Nestlings were banded on days 5-7 post-
128 hatch and ~80µL blood was collected in capillary tubes via brachial venipuncture.
129 Approximately 60µL blood was preserved in Longmire's lysis buffer (Longmire et al. 1992) for
130 genotyping and ~20µL was immediately placed in RNAlater. Within six hours of collection,
131 samples were placed temporarily into liquid nitrogen, before being shipped overnight on dry ice
132 to -80ºC storage until RNA extraction. All animal sampling protocols were approved by the
133 Indiana State University Institutional Animal Care and Use Committee (IACUC 562158-
134 1:ET/RG, 562192-1:ET/RG).
135
136 *Molecular sexing & genotyping*
137 Nestling DNA was extracted from erythrocytes using the DNA IQ® magnetic extraction
138 system (Promega Corp, Madison, WI USA). To determine sex and morph, we used PCR to
139 fluorescently label and amplify a region of the chromo-helicase-DNA-binding gene, and a region
140 of the vasoactive intestinal peptide following Griffiths (1998) and Michopolous et al. (2007). The
141 PCR products were run and analyzed on an ABI PRISM™ 310 genetic analyzer.
142
143 *RNA extraction, library preparation, & sequencing*
144 We sampled a total of 32 nestlings for RNA extraction and sequencing. These samples
145 represent 23 nestlings from eight TxW pairs and nine nestlings from three WxT pairs.
146 Additionally, these data represent 18 females, 14 males, 15 T morph, and 17 W morph
147 individuals.

148 We removed RNAlater and homogenized whole blood tissue samples with Tri-Reagent
149 (Molecular Research Company). Total RNA was purified with a Qiagen RNeasy mini kit
150 (Valencia, CA, USA), followed by DNase treatment and further purification. We quality assessed
151 RNA with an Agilent Bioanalyzer (Wilmington, DE, USA). Both library preparation and
152 sequencing were performed at the University of Illinois Roy J. Carver Biotechnology Center. A
153 library was prepared for each RNA sample using the Illumina HT TruSeq (San Diego, CA, USA)
154 stranded RNA sample prep kit. Libraries were distributed into four pools with equimolar
155 concentrations and quantitated via qPCR. Each of the pools was sequenced on an individual lane
156 of an Illumina HiSeq 2500 using the Illumina TruSeq SBS sequencing kit v4 producing 100
157 nucleotide single-end reads.
158
159 *Creation of masked reference genome*
160 The WTSP reference genome was generated from a male T morph individual (Tuttle et al.
161 2016). Thus, the reference genome does not contain any sequence data from the ZAL2^{m}
162 inversion. To avoid any potential bias in mapping reads derived from W morph individuals onto
163 a T morph genome, we generated a masked reference genome for this study. To do so, we used
164 previously published whole genome sequences from three W morph adults (Tuttle et al. 2016).
165 Reads were adapter trimmed with *Trim Galore!* v0.3.8
166 (https://github.com/FelixKrueger/TrimGalore) and aligned to the WTSP reference genome with
167 *bwa mem* v 0.7.10-r789. We converted and sorted the resulting SAM alignment to BAM format
168 with *samtools view* and *samtools sort*, respectively (*samtools* v1.2, Li et al. 2009). We then
169 merged all genomic scaffolds corresponding to the ZAL2^{m} inversion with *samtools merge*. We
170 called SNPs within the inversion using *samtools mpileup* and *bcftools call* v 1.2 (Li 2009). We

171 only kept SNPs that were heterozygous in each of the three individuals with *SnpSift* v 4.3p
172 (Cingolani et al. 2012) and used these SNPs to mask the reference genome with *bedtools*
173 *maskfasta* v 2.21.0 (Quinlan & Hall 2010).
174
175 *Quality control, read mapping, differential expression, & gene ontology*
176 We trimmed Illumina sequencing adapters from each of the 32 libraries with *Trim*
177 *Galore!* v 0.3.8 which uses *Cutadapt* v1.7.1 (Martin 2011). Trimmed reads were then mapped to
178 the masked reference genome with *STAR* v2.5.3a (Dobin et al. 2013). The mapping results were
179 then quantified and assigned gene IDs with *htseq-count* v0.6.0 (Anders et al. 2015) specifying '-s
180 reverse' and '-i gene'. Genes with an average read count of ≥ 5 were used for downstream
181 analyses.
182 All statistical analyses were performed with R v3.5.0 (R Core Team 2013). We identified
183 outlier samples as having a normalized connectivity below -2.5 (Horvath 2011). Two samples,
184 one T female and one T male representing an entire TxW nest, were identified as outliers and
185 removed from all future analyses (Figure S1). We normalized reads accounting for sequencing
186 depth and assessed differential expression (DE) with *DEseq2* (Love et al. 2014). We performed
187 variance stabilizing transformation of reads in *DEseq2* and performed PCA and hierarchical
188 clustering of gene expression profiles with *pcaExplorer* v2.6.0 (Marini 2018). DE analyses
189 utilized pairwise comparisons between nestling morph and pair type (i.e. parental morphs). In
190 each case, we controlled for sex, morph, and/or nest ID. We did not include nestling age in
191 analyses, as most samples were 6 days old (n=21), limiting comparisons with nestlings aged Day
192 5 (n=3) or Day 7 (n=6). Network analysis (see below) did not reveal any effect of age on
193 variables of interest (morph, pair type; data not shown). We also tested for an interaction between

194 nestling morph and pair type utilizing a grouping variable as outlined in the *DEseq2* manual.
195 *DEseq2* determines DE with a Wald test followed by Benjamini & Hochberg (1995) FDR
196 correction. Genes were considered DE if the FDR corrected p-value was < 0.10. Details for each
197 model run, including the R code used, are in this project's GitHub repository.
198 We next grouped DE genes into gene ontology (GO) categories with *GOrilla* (Eden et al.
199 2007, 2009). For each *DEseq2* comparison, we ordered the list of genes based on ascending FDR
200 values, excluding any genes in which *DEseq2* did not assign a FDR value. The WTSP genome is
201 not completely annotated, so any loci without a gene symbol were excluded from GO analyses
202 (n=1,926). *GOrilla* places greater weight on genes located at the top of the list (i.e. DE genes),
203 while accounting for the contribution of each gene in the given comparison. GO categories were
204 considered significantly enriched if the FDR corrected p-value <0.05. *GOrilla* does not support
205 WTSP annotation; so, all analyses were based on homology to human gene symbols.
206
207 *Weighted gene co-expression network analysis (WGCNA)*
208 We used the *WGCNA* package in R (Zhang & Horvath 2005, Langfelder & Horvath
209 2008) to identify modules of co-expressed genes in our dataset. We first exported variance
210 stabilizing transformed (vst) read counts from *DEseq2*, removed genes with an average vst < 5
211 across all 30 samples, and imported the subsequent list of 8,982 genes into *WGCNA*. To build the
212 co-expression matrix, we chose a soft thresholding power (β) value of 12, at which the network
213 reaches scale-free topology (Figure S2). We generated a signed network with minimum module
214 size of 30 genes and merged highly correlated modules (dissimilarity threshold = 0.20, which
215 corresponds to $R^2 = 0.80$). We then correlated the eigengene, which is the first principal

216 component of a module, of these merged modules with external traits (pair type, morph, sex, nest
217 ID). Modules with $p < 0.05$ were considered significantly correlated with a given trait.
218 To visualize the interaction of genes within a module, we generated the intramodular
219 connectivity (IM) score for each gene, which represents the interconnection of module genes. We
220 exported all IM scores for modules of interest and imported into *VisAnt* v5.51 (Hu et al. 2013) for
221 visualization. To maximize network clarity, we only plotted the top 300 interactions based on IM
222 scores. Thus, we only visualized the most connected genes. To identify hub genes, we visualized
223 the Degree Distribution (DD) for the network and selected the most connected genes above a
224 natural break in the distribution. This resulted in one to nine hub genes per module.
225 Lastly, to understand the biological function of modules correlated with traits of interest,
226 we performed a target vs background GO analysis in *GOrilla*. For each module, we tested the
227 assigned genes for each module against the entire list of 8,982 genes used for the *WGCNA*
228 analysis. GO categories were significant with a FDR corrected p-value < 0.05.
229
230 **Results**
231 *Sequencing results*
232 We sequenced each sample to an average depth of 29.4 million reads (range = 16.2-58.5
233 million reads). The 32 libraries were distributed into four pools in equimolar concentration. One
234 pool contained only four samples, which corresponded to the four samples with lowest RNA
235 concentrations. This pool was sequenced to an average depth of 56.17 million reads per library.
236 The remaining three pools were sequenced to an average depth of 25.62 million reads per library.
237 Samples mapped to our masked genome at an average rate of 91.08% (range = 88.19%-92.87%)

238 (Table S1). A total of 8,982 genes had count values ≥ 5 across all samples. Samples do not
239 segregate by pair type or morph in clustering analyses (Figures S3, S4).
240
241 *Differential Expression – Morph*
242 Testing for morph-specific expression resulted in 92 genes DE. Sixty-five of these genes
243 were in the supergene (Table S2). Additionally, many of these 92 genes were up-regulated in W
244 morph nestlings and function in innate immunity (e.g. IFIT5, IL20RA, EIF2AK2, RSAD2).
245 There was GO enrichment of four categories, two of which are immunity related: "immune
246 response" ($p = 0.019$) and "defense response to virus" ($p = 0.049$) (Table S3).
247
248 *Differential Expression – Pair Type*
249 Pair type had the largest effect on gene expression, with 881 genes differentially
250 expressed (DE) between offspring from the two different pair types (FDR < 0.10, Table S2).
251 Many genes associated with stress responses were up-regulated in nestlings in WxT nests,
252 including the glucocorticoid receptor (NR3C1), superoxide dismutase (SOD)1 & SOD2, DEP
253 domain-containing mTOR-interacting protein (DEPTOR), and several ubiquitin-mediated
254 proteolysis pathway genes (e.g. UBE2D3, PSMD3, PSMD6). Additionally, several immune
255 system related genes were also up-regulated in WxT nests, including cytokines (e.g. IL2RA,
256 IL7R), suppressor of cytokine signaling 1 (SOCS1), and five putative major histocompatibility
257 complex (MHC) class I loci. No GO categories were significantly enriched, however.
258 We next tested for a morph-specific response to pair type. Within WxT nests, 40 genes
259 were DE ($p <0.10$) between T and W morph nestlings, 12 of which are in the supergene.
260 Additionally, 34/40 genes are uniquely DE between morphs in WxT nests and do not overlap

261 with the overall list of 92 genes DE between morphs described above, suggesting a prominent
262 role of the WxT pair type on nestling morph-specific gene expression. Only two genes (THSD7B
263 & CFAP44) were DE between morphs within TxW nests, both of which are uniquely DE
264 between morphs in TxW nests. No GO categories were enriched in either comparison.
265
266 *WGCNA – Morph*
267 WGCNA revealed 26 modules, five of which were correlated with morph (Table 1,
268 Figure 1). The light cyan module (183 genes, R^2=0.67, p=5x10^{-5}) and white module (72 genes,
269 R^2=-0.66, p=9x10^{-5}) contained genes up-regulated and down-regulated respectively in W morph
270 nestlings. These genes are primarily located in the chromosomal inversion (light cyan = 70/183
271 genes, white = 40/72) (Figure S5). The hubs of each of these modules are also located in the
272 chromosomal inversion (Table 1, Figure S5). Additionally, the sky blue module (58 genes,
273 R^2=0.53, p=0.003) and dark red module (102 genes, R^2=0.47, p=0.009) (Figure S6) contained
274 genes up-regulated in W morph nestlings and many of these genes overlap with the immune
275 related genes described in the morph DE tests above. Additionally, the hubs of these networks
276 (e.g. sky blue: EIF2AK2, IFIT5, OASL; dark red: TRAF5) (Table 1) reflect a conserved innate
277 immunity network structure in avian blood (Kernbach et al., in review) (Figure S6).
278
279 *WGCNA – Pair Type*
280 We found seven modules correlated with pair type (Table 2, Figure 1). The blue module
281 represented genes that are up-regulated in nestlings from WxT nests (1,142 genes, R^2 = -0.45,
282 p=0.01). This module contained both the largest number of genes and correspondingly strongest
283 functional enrichment. Many of these GO enrichments were related to protein function, resulting

284 from the presence of ribosomal genes. Interestingly, several GO categories for metabolism,
285 catabolism, and proteolysis were also enriched, driven by genes encoding ubiquitin-conjugating
286 enzymes and proteasome subunits (e.g. "proteasomal protein catabolic process", $p=2.34x10^{-4}$;
287 "proteasome-mediated ubiquitin-dependent protein catabolic process", $p=5.32x10^{-4}$) (Table S4).
288 Many of these (e.g. PSMF1, PSMD3, PSMD6, UBE2D2, UBE2D3, UBE3C) were also DE
289 between offspring of the two pair types (Figure 2). Lastly, the blue module contains one hub
290 gene, NDUFB3 (DD=42) (Figure 2), which is involved in the mitochondrial electron transport
291 chain.

292 The remaining modules were not enriched for any GO categories, but the tan and light
293 green modules represented candidate stress response networks. These modules showed
294 contrasting expression patterns in nestlings from WxT nests. Within the tan module (335 genes,
295 $R^2=-0.61$, $p=3x10^{-4}$), genes were up-regulated and DEPTOR was the single hub (DD=39), which
296 functions as an inhibitor of the mTOR pathway in response to stress (e.g. Desantis et al. 2015)
297 (Figure 3). The tan module also contained NR3C1, which is activated in response to increased
298 glucocorticoid secretion. Lastly, the light green module (116 genes, $R^2=0.60$, $p=4x10^{-4}$)
299 contained genes down-regulated in TxW nests. There were three hub genes (DD > 28), CDK19,
300 CHD4, and EPG5, each with previously described roles in the stress response (Figure 4). We did
301 not observe modules correlated with pair type that were also correlated with nestling morph or
302 sex, suggesting there is no morph or sex-specific response to a given pair type at the network
303 level.

304

305 **Discussion**

306 By assessing genome-wide transcription in nestlings raised by different WTSP pair types,
307 a proxy for parental investment, we have identified distinct transcriptomic signatures that suggest
308 WxT pairs (female-biased parental care) induce a stress response in developing nestlings relative
309 to TxW pairs. This is reflected both by differential expression of several genes involved in
310 protein degradation as well as networks of co-expressed genes with stress response hubs.
311 Additionally, we identified morph-specific gene expression driven by innate immunity genes and
312 genes located in the chromosome 2 supergene. As adults, the genes within the supergene strongly
313 influence the WTSP neural transcriptome (Balakrishnan et al. 2014, Zinzow-Kramer et al. 2015).
314 Our results here suggest that as nestlings, parental genotypes and associated behaviors, rather
315 than nestling genotype, have the strongest influence on the nestling transcriptome.
316
317 *Gene expression differences resulting from pair type, a proxy for parental care*
318 WTSP morphs differ in the amount of paternal provisioning; with W morph males
319 providing less than their T male counterparts. Therefore, we expected nestlings in WxT nests to
320 be stressed nutritionally or to suffer increased sibling competition due to uniparental feeding
321 visits (e.g. Yosef et al. 2012). Indeed, we find 881 genes DE between nestlings raised under the
322 two pair types. Many of these genes function in the proteasome or ubiquitin-mediated
323 proteolysis. Cells naturally use the proteasome for degradation of proteins targeted by the
324 ubiquitination process, but genes involved in proteasome formation (e.g. PSMD6, PSMD11) and
325 ubiquitination (e.g. UBE2B) are up-regulated in cells experiencing mild oxidative stress (Aiken
326 et al. 2011, Shang & Taylor 2011, Livneh et al. 2016) or organisms experiencing abiotic stress
327 (Dhanasiri et al. 2013, Tomalty et al. 2015). Thus, up-regulation of these genes in nestlings from
328 WxT nests suggests they are responding to oxidative stress. As a result, there is a physiological

329 cost to having a W morph father and T morph mother at the nestling stage, since W morph males
330 invest more in reproduction and territory defense than provisioning.

331 To complement our differential expression approach, we also constructed co-expression
332 networks with *WGCNA*. *WGCNA* identifies modules of co-regulated genes blind to the
333 experimental design. These modules are then correlated with external traits, offering a systems
334 level view into how conditions impact transcriptional networks. Within these networks, we can
335 then perform GO analyses as described above and identify network hubs, which are the most
336 highly connected genes within that network. Using this approach, we identified 26 modules of
337 co-regulated genes in this dataset (Figure 1). Seven of these modules were significantly
338 correlated with parental pair type. The blue module contains genes that are up-regulated in
339 nestlings in WxT nests. The blue module hub gene was NDUFB3 (Module Membership
340 [MM]=0.938, DD=42) (Figure 2), which encodes a subunit of the mitochondrial membrane
341 respiratory chain. Interestingly, many of the same proteolysis-related genes highlighted in the
342 differential expression results are also present in this module, resulting in the enrichment of
343 several metabolism and stress-related GO categories (Table S4).

344 Two modules, light green and tan, contained stress responsive hub genes. The light green
345 module contains genes that are down-regulated in nestlings in WxT nests, with three hub genes:
346 CDK19, CHD4, and EPG5 (Figure 4). The absence of EPG5 expression (via knockout) and
347 reduction in CHD4 expression (via knockdown) has been associated with increased DNA
348 damage (Zhao et al. 2013, Larsen et al. 2010). Similarly, down-regulation of CDK19 following
349 knockdown is associated with an increased stress response (Audetat et al. 2017). Down-
350 regulation of these genes in these nestlings could be indicative of increased cellular damage. The
351 tan module contains genes up-regulated in nestlings from WxT nests and contains one hub gene,

352 DEPTOR, which is an inhibitor of mTOR signaling (Figure 3). The exact role of DEPTOR
353 remains unclear, but up-regulation likely inhibits the mTORC1 pathway to reduce endoplasmic
354 reticulum stress, promote cell survival, and avoid apoptosis (Peterson et al. 2009, Desantis et al.
355 2015, Catena et al. 2016).

356 Up-regulation in these nestlings and the high connectivity of DEPTOR to other co-
357 expressed genes provides further support for a transcriptional stress response within WxT nests.
358 The tan module also contains two well-studied stress responsive genes, superoxide dismutase 2
359 (SOD2) and the glucocorticoid receptor (NR3C1). SOD2 mitigates the effects of exposure to
360 reactive oxygen species by scavenging free radicals (Zelko et al. 2002). NR3C1 binds
361 glucocorticoids and has primarily been studied in the context of ELS and methylation of an
362 upstream promoter. NRC3C1 methylation is often associated with down-regulation of NR3C1
363 (e.g. McGowan et al. 2009) and impairment of the HPA axis, but up-regulation following
364 methylation has also been observed as part of the stress response (Turner et al. 2006, Bockmühl
365 et al. 2015). Up-regulation observed here directly implicates the HPA axis and suggests these
366 nestlings may be activating SOD2 and NR3C1 to cope with elevated levels of reactive oxygen
367 species and corticosterone, respectively. However, further work is needed to investigate stress
368 physiology, corticosterone levels, and uncover the epigenetic state of NR3C1 in these nestlings
369 and how this may relate to ELS.

370 Importantly, we did not measure provisioning by the parents of these nestlings but instead
371 used pair type as a proxy for parental care. Reduced provisioning by W morph males appears to
372 be stable across populations resulting in female-biased parental care in WxT nests (Knapton &
373 Falls 1983, Horton et al. 2014). Therefore, reduced parental care is a likely a source of
374 behaviorally mediated maternal or paternal effect (see Crean & Bonduriansky 2014). We cannot,

375 however, ignore the possibility that provisioning rates did not differ between the nests we
376 sampled. Previous work revealed no effect of parental pair type on nestling mass (Knapton et al.
377 1984, Tuttle et al. 2017), and nestlings did not differ in mass at time of sampling between the
378 TxW and WxT nests used in this study (Smith et al. in review). Increased provisioning by
379 females to compensate for reduced care by males could explain this observation. In this scenario
380 reduced brooding and increased maternal separation; could also negatively impact nestling
381 physiology and act as a source of ELS (reviewed in Ledón-Rettig et al. 2013). Surprisingly,
382 given the gene expression findings described above, a recent study in the same study population
383 did not detect differences in reactive oxygen metabolites in plasma of offspring of the two
384 different pair types (Grunst et al. 2018b). Our finding of transcriptional differences in stress-
385 responsive genes in the absence of significant phenotypic differences highlights the utility of
386 RNA-seq to uncover subtle changes in physiology.

387 Our study was carried out in the field as part of a long-term study and is limited by the
388 fact that was a non-experimental study. We aimed to mitigate potential environmental confounds
389 by restricting sampling of nestlings to a short time period of nine days. Certainly, the
390 environment may influence gene expression in our samples, but consistent changes among the
391 samples in the two pair types suggest the role of parents is a significant driver of nestling gene
392 expression, rather than temporal or spatial environmental variation.

393 Hormone-mediated maternal effects provide another potential driver of the observed
394 expression differences among pair types. In previous studies of WTSP, only oestradiol has been
395 shown to differ between adult female morphs during the breeding season and is higher in W
396 morph females during the pre-laying and laying stages (Horton et al. 2014). No baseline
397 differences in any other hormone measured to date (corticosterone, testosterone, DHEA, DHT)

398 have been described during the breeding season (Spinney et al. 2006, Swett & Breuner 2009,
399 Horton & Holberton et al. 2010, Horton et al. 2014), which would suggest that hormone
400 deposition into eggs does not dramatically differ between the morphs. Additional work is needed
401 to investigate the potential role of maternal effects in the WTSP, including measuring hormone
402 levels in both the egg and as nestlings. Although we cannot rule out hormone-mediated maternal
403 effects as a source of expression differences observed in offspring, given the current knowledge
404 of the system the differences we observed are likely driven by differences in parental care.
405
406 *Morph-specific gene expression*
407 We were also interested in morph-specific gene expression and how morphs may respond
408 to differences in pair type. We found 92 genes DE between morphs, including many innate
409 immune-related genes and genes located within the supergene (65/92 genes, Table S2). WGCNA
410 revealed five modules correlated with morph (Figure 1). These included two innate immunity-
411 related modules with up-regulation in W morphs (Dark Red & Sky Blue) and two modules
412 predominantly containing genes located in the supergene (White = 40/72, Light Cyan = 70/183)
413 (Figures S5, S6). The sky blue module contains nine hub genes and the dark red module contains
414 one hub gene, both of which include well-studied anti-viral genes (e.g. sky blue: OASL, RSAD2;
415 dark red: TRAF5). These genes also form a co-expression module in avian blood following West
416 Nile virus infection (Kernbach et al., in review). Adult WTSP morphs differ in their ability to
417 clear infection (Boyd et al. 2018), so the immune activation here may be indicative of an
418 increased parasite load in W morph nestlings, although further investigation is required. The light
419 cyan module contains genes up-regulated in W morph nestlings and contains eight hub genes,
420 each located in the supergene (Table 1). Three of these, EPM2A, BPNT1, and TAF5L, were also

421 identified as hub genes in neural tissues of adult W morph males (Zinzow-Kramer et al. 2015).
422 These nestlings thus exhibit transcriptional changes driven by the inversion prior to any
423 phenotypic or behavioral differences. Additionally, the conservation of network hub genes in a
424 different tissue and life stage highlights avenues for further investigation into WTSP
425 transcription.

426 Given adult W morphs are highly territorial, aggressive, sing frequently, and maintain
427 higher levels of stress hormones, we predicted W morph nestlings might be primed to handle
428 stress and fare better in WxT nests than their T morph siblings. Despite broad gene expression
429 differences between the morphs, within pair types morph-specific expression was limited. In part
430 due to small sample size, nestlings in TxW nests only have two genes DE between morphs. There
431 is a larger effect of morph within WxT nests, where the number of DE genes increased to 40.
432 These genes encompassed a wide range of gene functions without any obvious stress-related
433 candidate genes. Of these 40 genes, 34 are uniquely DE within WxT nests and do not overlap
434 with the overall list of 92 genes DE between morphs using all samples. Interestingly,
435 glucocorticoid-induced transcript 1 (GLCCI1) is up-regulated in W morph nestlings in WxT
436 nests. The function of GLCCI1 remains unclear (Kim et al. 2016), but expression differences
437 between morphs observed here implicates the role of glucocorticoids in response to pair type.
438 This suggests that nestling morphs may respond differently to the parental pair type though larger
439 sample sizes will be needed to explore this further.
440

441 **Conclusions**

442 Using the WTSP, a system with alternative parental care strategies, we show that
443 nestlings in WxT nests (female-biased parental care) have increased expression of stress-related

genes, and parental genotypes may act as a source of ELS in the species. Nestling morph also influences transcription, but pair type appears to have the greatest effect on their transcriptome. Combined, this supports the parental effects hypothesis (Wade 1998, Schrader et al. 2018), where offspring phenotypes are primarily a result of the nest environment and care received, rather than from offspring genotypes (i.e. T vs W). Nearly 54% of observed pairs have been WxT (Tuttle et al. 2016). Thus, roughly half of the nestlings in every population will experience reduced parental care. Our results suggest that these differences in parental care have at least short-term consequences on offspring physiology. While we have identified impacts at the level of transcription, an integrative approach assessing nestling WTSP physiology, for example by combining epigenetic and neuroendocrine approaches, will further elucidate the consequences of variation in parental pair type. Importantly, it remains unclear whether female-biased parental care or differences in maternal effects translate into long-term fitness consequences for offspring. WTSPs have been studied extensively as adults, but very rarely in other life stages. W morph males and T morph females exhibit earlier reproductive and actuarial senescence, potentially resulting from the high energy expenditure lifestyle of W morph males and biased parental care given by T morph females (Grunst et al. 2018a, Grunst et al. 2018c). There also appears to be seasonal variation in fitness between the morphs as adults. Following cold, wet winters, W morph males exhibit lower recruitment in the breeding grounds, leading to an overproduction of W morph male nestlings, potentially to stabilize morph frequencies in the population (Tuttle et al. 2017). Thus, there is a cost associated with parental genotype, as this less cooperative reproductive strategy accelerates senescence. We show here that this cost is also translated into nestlings within WxT nests via increased stress-related gene expression. This work sets the stage

466 to further explore morph-specific fitness consequences in nestlings experiencing alternative
467 parental care strategies.
468

469 **Acknowledgements**

470 We acknowledge Lindsay Forrette, Andrea Grunst, and Melissa Grunst for assistance in the field,
471 Sarah Ford for assistance with molecular work, Rachel Wright for WGCNA code, and Cranberry
472 Lake Biological Station. Funding provided by East Carolina University, Indiana State University,
473 The National Science Foundation (grant no. DUE-0934648) and the National Institutes of Health
474 (grant no. 1R01Gm084229 to E.M.T and R.A.G.) and a Sigma Xi Grants in Aid of Research
475 award to DJN. Birds were banded with color bands and a Fish and Wildlife band (Master
476 Banding Permit 22296 to EMT and permit 24105 to RAG). Dr. Alvaro Hernandez and Chris
477 Wright provided guidance and oversight on sequencing carried out at the University of Illinois.
478 All methods were conducted in accordance with legal and ethical standards and were approved
479 by Indiana State University's Institutional Animal Care and Use Committee (protocols 562158-
480 1:ET/RG and 562192-1:ET/RG).
481

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710 **Data Accessibility**

711 The 32 RNAseq libraries used in this study will be submitted to the NCBI Sequence Read
712 Archive (SRA). All files needed to produce these results, including code and counts files, will be
713 uploaded to this project's GitHub page: https://github.com/danielnewhouse/wtsp

714

715 **Author Contributions**

716 DJN designed and performed research, analyzed the data, and wrote the paper. MBS
717 performed research, contributed samples, and reviewed drafts of the paper. EMT designed and
718 performed research and contributed samples. RAG designed and performed research, contributed
719 samples, and reviewed drafts of the paper. CNB designed and performed research, contributed
720 reagents, and reviewed drafts of the paper.

721 **Tables and Figures**

722 Table 1. WGCNA modules correlated with morph, strength of correlation (R^2), p-value, hub

723 gene(s) of module, and the degree distribution of hub gene(s).

Module	**R^2**	**p-value**	**Hub genes**	**DD of hub gene(s)**
Dark Red	0.47	0.009	TRAF5	32
Light Cyan	0.67	$5x10^{-5}$	BPNT1, EPM2A, LOC102066536 (GST-like), MAN1A1, MEI4, RNASET2, SLC18B1, TTC32	>27
Salmon	-0.5	0.005	NSL1	39
Sky Blue	0.53	0.003	DTX3L, EIF2AK2, IFIT5, LOC102064521 (OASL), LOC102065196 (IFI27L2), PARP9, PARP14, RSAD2, ZNFX1	>22
White	-0.66	$9x10^{-5}$	GOPC, HDAC2, HINT3, TAF5L, TRMT61B, MARC2	>29

724

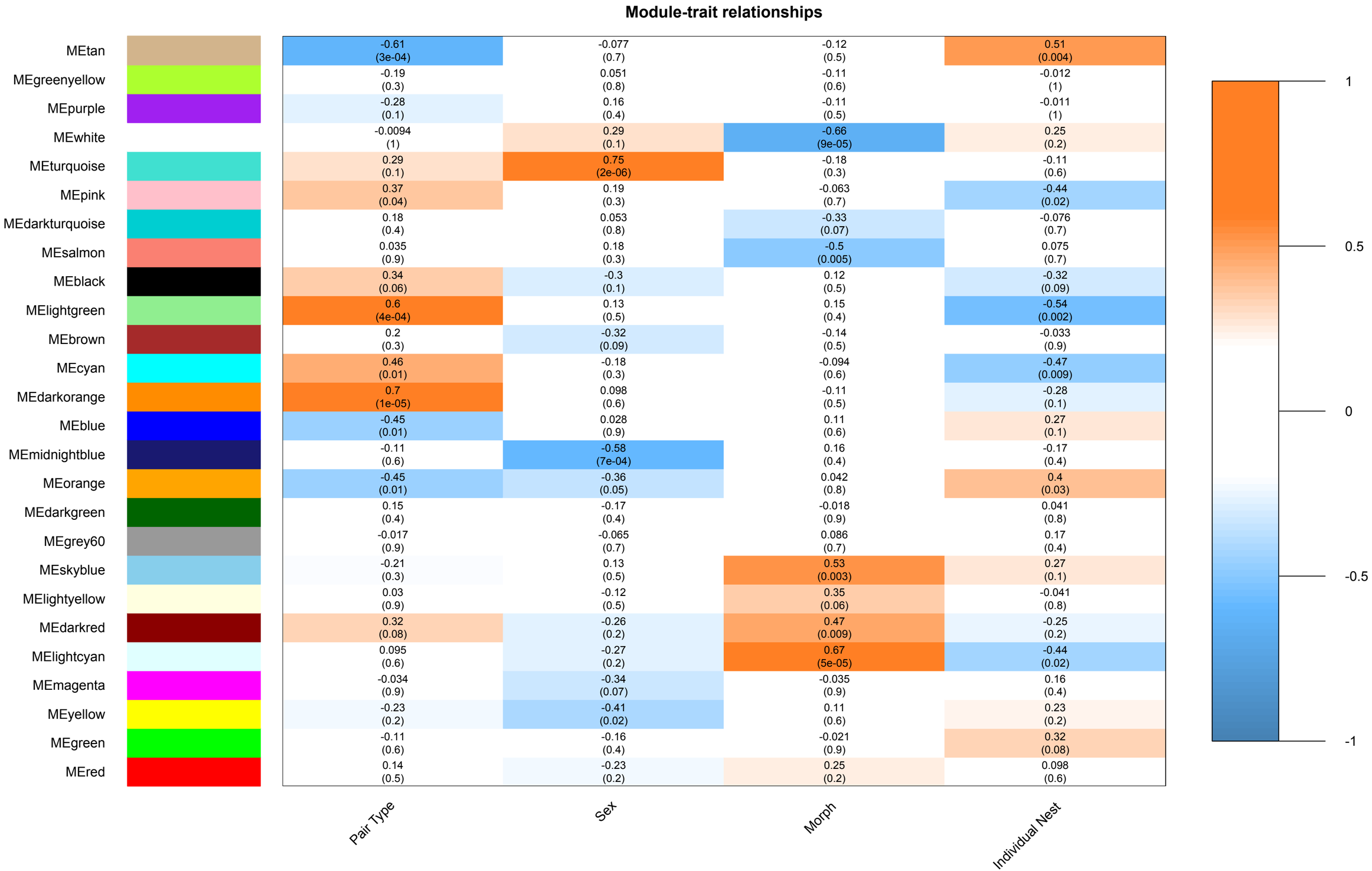
725 Table 2. WGCNA modules correlated with pair type, strength of correlation (R^2), p-value, hub

726 gene(s) of module, and the degree distribution of hub gene(s).

Module	**R^2**	**p-value**	**Hub genes**	**DD of hub gene(s)**
Blue	-0.45	0.01	NDUFB3	42
Cyan	0.46	0.01	HELZ	36

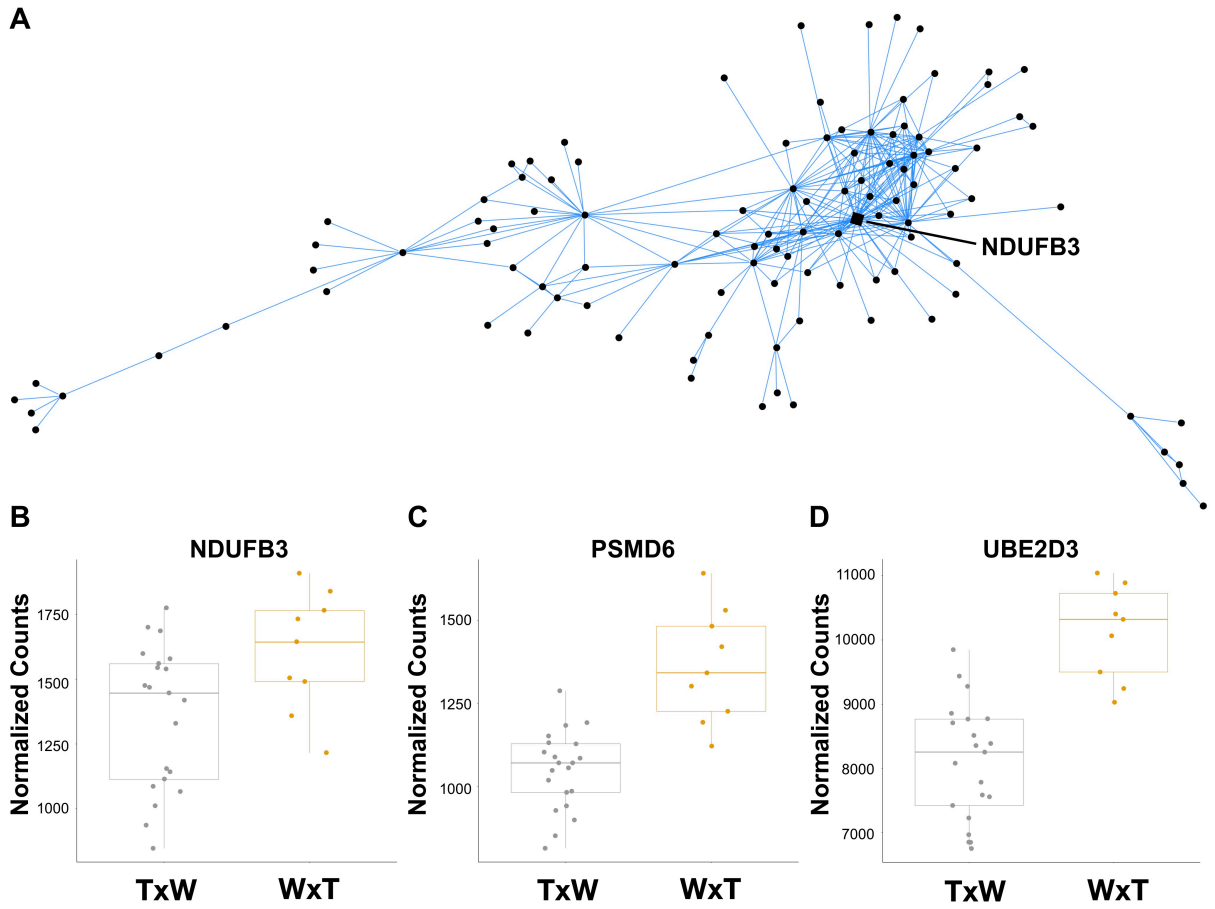
Dark Orange	0.7	$1x10^{-5}$	NCOA6	45
Light Green	0.6	$4x10^{-4}$	CDK19, CHD4, EPG5	>28
Orange	-0.45	0.01	ZFX	31
Pink	0.37	0.04	LOC102060916 (C12orf4)	19
Tan	-0.61	$3x10^{-4}$	DEPTOR	39

727



	Pair Type	Sex	Morph	Individual Nest
MEtan	-0.61 (3e-04)	-0.077 (0.7)	-0.12 (0.5)	0.51 (0.004)
MEgreenyellow	-0.19 (0.3)	0.051 (0.8)	-0.11 (0.6)	-0.012 (1)
MEpurple	-0.28 (0.1)	0.16 (0.4)	-0.11 (0.5)	-0.011 (1)
MEwhite	-0.0094 (1)	0.29 (0.1)	-0.66 (9e-05)	0.25 (0.2)
MEturquoise	0.29 (0.1)	0.75 (2e-06)	-0.18 (0.3)	-0.11 (0.6)
MEpink	0.37 (0.04)	0.19 (0.3)	-0.063 (0.7)	-0.44 (0.02)
MEdarkturquoise	0.18 (0.4)	0.053 (0.8)	-0.33 (0.07)	-0.076 (0.7)
MEsalmon	0.035 (0.9)	0.18 (0.3)	-0.5 (0.005)	0.075 (0.7)
MEblack	0.34 (0.06)	-0.3 (0.1)	0.12 (0.5)	-0.32 (0.09)
MElightgreen	0.6 (4e-04)	0.13 (0.5)	0.15 (0.4)	-0.54 (0.002)
MEbrown	0.2 (0.3)	-0.32 (0.09)	-0.14 (0.5)	-0.033 (0.9)
MEcyan	0.46 (0.01)	-0.18 (0.3)	-0.094 (0.6)	-0.47 (0.009)
MEdarkorange	0.7 (1e-05)	0.098 (0.6)	-0.11 (0.5)	-0.28 (0.1)
MEblue	-0.45 (0.01)	0.028 (0.9)	0.11 (0.6)	0.27 (0.1)
MEmidnightblue	-0.11 (0.6)	-0.58 (7e-04)	0.16 (0.4)	-0.17 (0.4)
MEorange	-0.45 (0.01)	-0.36 (0.05)	0.042 (0.8)	0.4 (0.03)
MEdarkgreen	0.15 (0.4)	-0.17 (0.4)	-0.018 (0.9)	0.041 (0.8)
MEgrey60	-0.017 (0.9)	-0.065 (0.7)	0.086 (0.7)	0.17 (0.4)
MEskyblue	-0.21 (0.3)	0.13 (0.5)	0.53 (0.003)	0.27 (0.1)
MElightyellow	0.03 (0.9)	-0.12 (0.5)	0.35 (0.06)	-0.041 (0.8)
MEdarkred	0.32 (0.08)	-0.26 (0.2)	0.47 (0.009)	-0.25 (0.2)
MElightcyan	0.095 (0.6)	-0.27 (0.2)	0.67 (5e-05)	-0.44 (0.02)
MEmagenta	-0.034 (0.9)	-0.34 (0.07)	-0.035 (0.9)	0.16 (0.4)
MEyellow	-0.23 (0.2)	-0.41 (0.02)	0.11 (0.6)	0.23 (0.2)
MEgreen	-0.11 (0.6)	-0.16 (0.4)	-0.021 (0.9)	0.32 (0.08)
MEred	0.14 (0.5)	-0.23 (0.2)	0.25 (0.2)	0.098 (0.6)

727 Figure 1. WGCNA module-trait correlation matrix. Each box contains the R^2 correlation value
728 followed by pvalue in parentheses of a given trait with the module. Correlation values range from
729 -1 to 1, with orange colors representing positive correlation and blue colors representing negative
730 correlation.



733

734 Figure 2. (A) Network of blue module, highlighting hub gene NDUFB3, along with normalized

735 expression plots of (B) NDUFB3, as well as ubiquitin-mediated proteolysis-related genes (C)

736 PSMD6 and (D) UBE2D3. TxW = samples from nests sired by a T male and a W female. WxT =

737 samples from nests sired by a white male and a tan female. Each circle represents a gene and

738 diamonds represent hub genes described in Table 2.

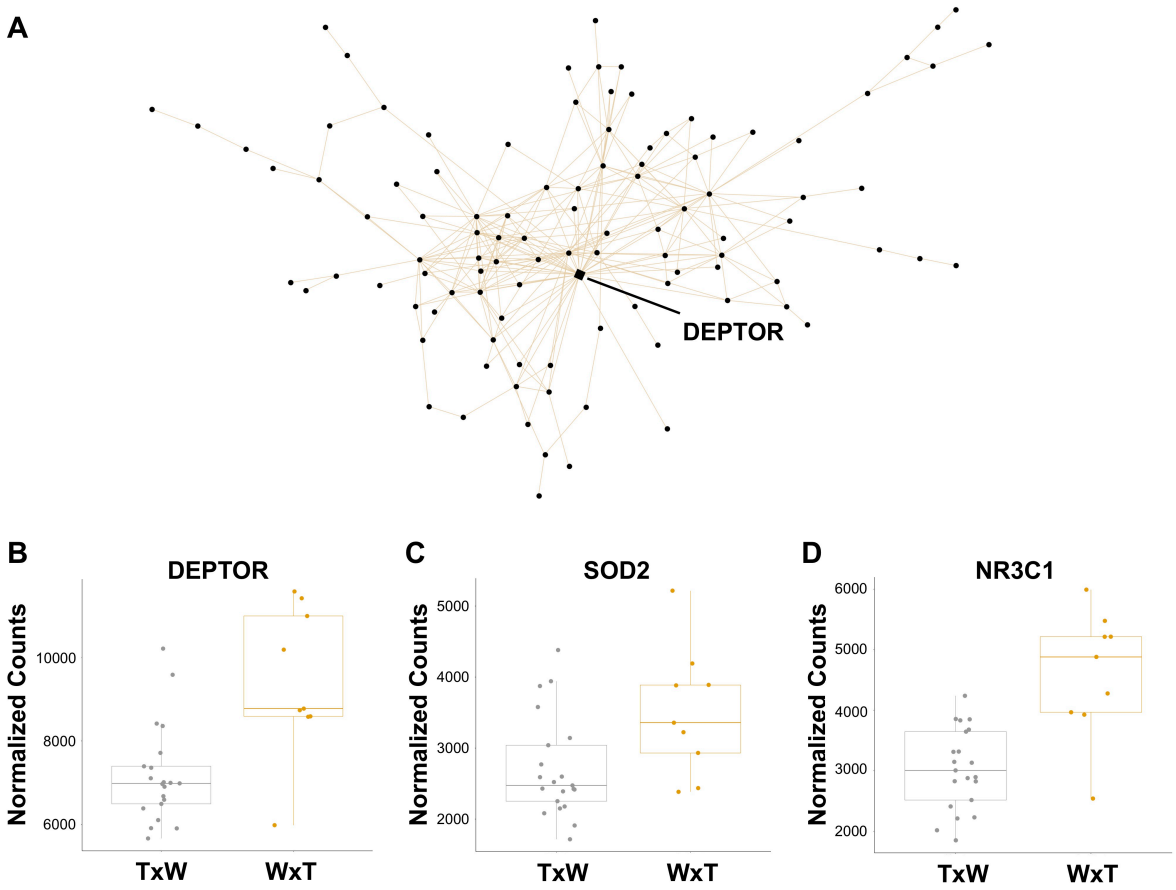


Figure 3. (A) Network of tan module, highlighting hub gene DEPTOR, along with normalized expression plots of hub gene (B) DEPTOR, as well as stress responsive genes (C) SOD2 and (D) NR3C1. TxW = samples from nests sired by a tan male and a white female. WxT = samples from nests sired by a white male and a tan female.

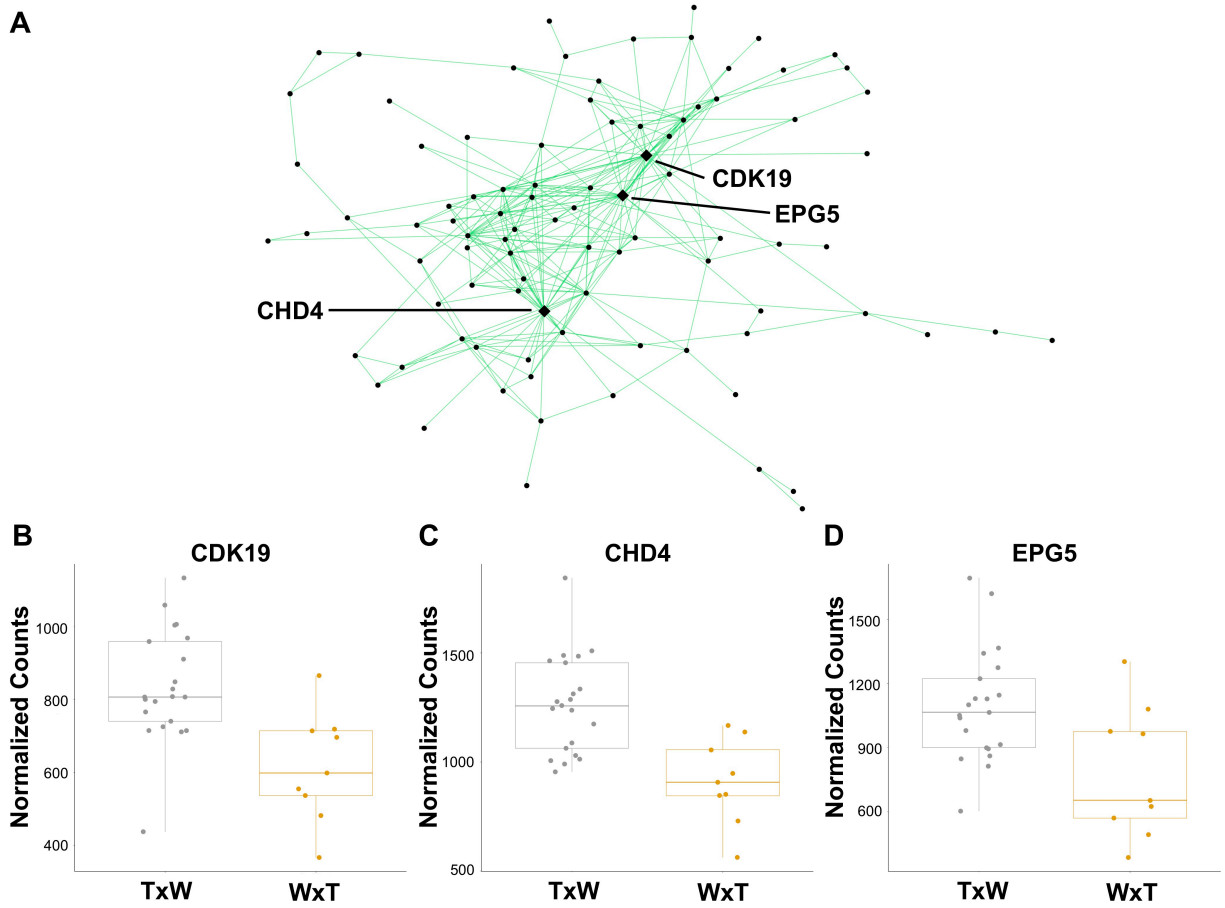


Figure 4. (A) Network of light green module and normalized expression plots of hub genes (B) CDK19, (C) CHD4, and (D) EPG5. TxW represents samples from nests sired by a T male and a W female. WxT represent samples from nests sired by a white male and a tan female. Each circle represents a gene and diamonds represent hub genes described in Table 2.