

1 **Sex-biased transcriptomic response of the reproductive axis to stress**

2 Rebecca Calisi^{1*}, Suzanne H. Austin¹, Andrew S. Lang², Matthew D. MacManes²

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4 ¹Department of Neurobiology, Physiology and Behavior, University of California, Davis

5 ²Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire

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8 **ABSTRACT**

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10 Stress is a well-known cause of reproductive dysfunction in many species, including birds,
11 rodents, and humans. However, little is known of the genomic basis for this dysfunction and how
12 it may differ between the sexes. Using the classic reproductive model of the rock dove (*Columba*
13 *livia*), we conducted the most in-depth investigation to date of how stress affects all gene
14 transcription of a biological system essential for facilitating reproduction - the hypothalamic-
15 pituitary-gonadal (HPG) axis. The HPG transcriptome responded to stress in both sexes, but
16 females exhibited more differential expression than males, and these stress responsive genes
17 were mostly unique to females. This result may be due to 1) fluctuations in the female endocrine
18 environment to facilitate ovulation and follicle maturation, and 2) their evolutionary history. We
19 offer a vital genomic foundation on which sex-specific reproductive dysfunction can be studied,
20 as well as novel gene targets for genetic intervention and therapy investigations.

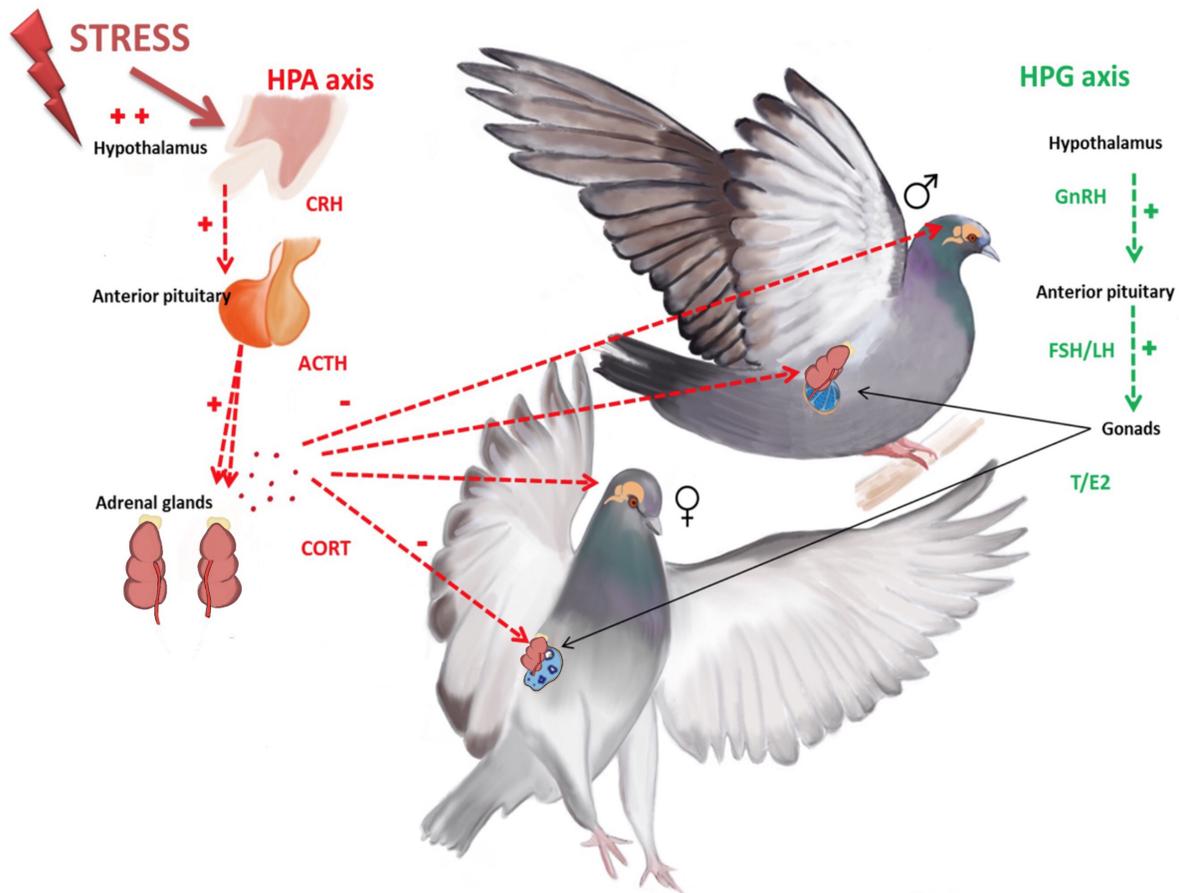
31 INTRODUCTION

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33 Stress can disrupt reproduction in multiple, complex ways [1–3]. The perception of a stressor
34 activates the hypothalamic-pituitary-adrenal (HPA) axis, which results in a synthesis of stress
35 hormones (glucocorticoids) [4]. Glucocorticoid hormones (cortisol in humans, corticosterone in
36 birds and rodents) are synthesized by the adrenal cortex and exert both rapid and gradual actions
37 on vertebrate physiology [5]. This activation of the HPA system can cause suppression of the
38 reproductive system, i.e., the hypothalamic-pituitary-gonadal (HPG) axis, at multiple levels (Fig.
39 1), including inhibiting gonadotropin-releasing hormone (GnRH) secretion from the
40 hypothalamus, suppressing luteinizing hormone (LH) and follicle stimulating hormone (FSH)
41 release from the pituitary, sex steroid hormone release from the gonads, and ultimately reducing
42 or eliminating sexual behavior and reproduction [6–9]. However, questions remain as to 1) how
43 stress affects all gene activity of the HPG axis, and 2) if these effects are sex-specific. Evidence
44 suggests regulatory mechanisms of the HPG system under stress can be sex-specific (eg. human:
45 [10]; rodent: [11]; birds: [12,13]), but the full extent of sex-biased changes is still largely
46 unknown. In general, males have historically dominated animal studies [14–16], obscuring
47 discovery of potential sex differences that could inform and guide further research and clinical
48 studies [17].

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50 Here, we tested the effects of stress on the genomic activity of the male versus female HPG axis
51 using the model of the rock dove (*Columba livia*). Doves have been historically used to study
52 reproductive behavior [18–20] and now are proving to be a valuable model for genomics
53 research [21–24]. We exposed sexually mature males and females to 30 min of restraint stress,
54 which successfully activates the stress response as measured through significantly increased
55 circulating plasma glucocorticoids. We compared the genomic expression of the HPG axis of
56 stressed females and males to each other and in comparison with unstressed controls. We report
57 patterns of tissue-specific and sexually dimorphic gene expression, with females showing a
58 greater stress response at the level of the transcriptome in all three tissues - the hypothalamus,
59 pituitary, and gonads - as compared to males. These data offer a valuable resource to advance
60 stress and reproductive research with the potential for devising future therapeutic strategies to
61 ameliorate stress-induced HPG axis dysfunction.



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64 Figure 1. Depiction of the hypothalamic-pituitary-adrenal (HPA), or “stress”, axis and its
65 intersection with the hypothalamic-pituitary-gonadal, or “reproductive”, axis (HPG). Illustration
66 by Natalia Duque.

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70 RESULTS

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72 *Corticosterone Assay*

73 Plasma corticosterone was assayed from 48 birds (stress treatment group: 12 male, 12 female;
74 control group: 12 male, 12 female) using a rodent Corticosterone RIA kit and a 1:20 dilution
75 (MP Biomedicals, Orangeburg, NY). Plasma corticosterone concentrations were significantly

76 higher in both stressed birds as compared to controls (treatment: $F_{1,46}=73.5$, $P<0.001$). Neither
77 sex nor an interaction effect of treatment*sex were statistically significant (sex: $F_{1,46}=0.5$,
78 $P=0.505$; treatment*sex= $F_{1,46}=1.0$, $P=0.333$).

79

80 ***Sequence Read Data & Code Availability***

81 In total, 24 hypothalami (12 male, 12 female), 24 pituitary glands (12 male, 12 female), 12 testes,
82 and 12 ovaries from 24 birds were sequenced. Each sample was sequenced with between 2.3
83 million and 24.5 million read pairs. Read data corresponding to the control birds are available
84 using the European Nucleotide Archive project ID PRJEB16136; read data corresponding to the
85 stressed birds are available at PRJEB21082. Code used for the analyses of these data are
86 available at <https://git.io/vPA09>.

87

88 ***Transcriptome assembly characterization***

89 The Rock Dove version 1.1.0 transcriptome (available <https://goo.gl/S8goSM>, and at Dryad *post*
90 *acceptance*) contains 92,805 transcripts, of which 4,794 were added as part of this study to the
91 previous version 1.0.4 transcriptome. This newly compiled transcriptome data improves genic
92 contiguity, increasing the number of complete BUSCOs 0.2% to achieve 86.1% relative to the
93 version 1.0.4 assembly.

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95 ***Sequence Read Mapping and Estimation of Gene Expression.***

96 Raw sequencing reads corresponding to individual samples of hypothalami, pituitary glands, and
97 gonads were mapped to the Rock Dove reference HPG axis transcriptome version 1.1.0 using
98 Salmon, resulting in 80% to 90% read mapping. These data were imported into R and
99 summarized into gene-level counts using tximport, after which, edgeR was used to generate
100 normalized estimates of gene expression. 17,263 genes were expressed in the HPG.

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102 ***Evaluation of Candidate Gene Expression.***

103 Using the assembled transcriptome, HPG-specific and sexually dimorphic gene expression
104 patterns were characterized in stressed versus control animals. *A priori*, target genes were
105 identified for investigation that are well known to play a role in reproduction and the stress
106 response (Table 1). Using a generalized linear model and least-squares means for post-hoc tests

107 of significance ($P < 0.05$), we found sex and tissue-specific differences in HPG transcriptomic
 108 activity in response to the stress treatment.

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Entrez ID	Gene	Abbreviation	Hypothalamus		Pituitary		Ovary	Testes
			Female	Male	Female	Male	Female	Male
422165	Androgen receptor	AR						
414854	Aromatase CYP19A1	CYP19A1						
771773	Arginine vasotocin-like receptor 1A	AVPR-like 1A						
428263	Arginine vasotocin-like receptor 1B	AVPR-like 1B						
423433	Corticosteroid Binding Globulin	CBG						
374218	Corticotropin Releasing Hormone Receptor 1	CRHR1						
395940	Deiodinase iodothyronine type I	DIO1						
373903	Deiodinase iodothyronine type II	DIO2						
395939	Deiodinase iodothyronine type III	DIO3						
427633	Dopamine receptor D1	DRD1						
428252	Dopamine receptor D2	DRD2						
770757	Dopamine receptor D3	DRD3						
423016	Dopamine receptor D4	DRD4						
427552	Dopamine receptor D5	DRD5						
396099	ER alpha	Er α						
395575	ER beta	Er β						
395962	FSH Receptor	FSH-R						
374108	Follicle stimulating hormone beta subunit	FSH β						
396288	GABRG gamma-aminobutyric acid A receptor	GABA α R						
408185	Ghrelin/obestatin prepropeptide	GHRL						
423117	Galanin	GAL						
416343	Glucocorticoid receptor	GR						
378785	Gonadotropin inhibitory hormone	GnIH						
378784	Gonadotropin inhibitory hormone receptor	GnIH-R						
770134	Gonadotropin releasing hormone I	GnRH-I						
427517	Gonadotropin releasing hormone I receptor	GnRH I-R						
374223	Leptin receptor	LEPR						
395776	Luteinizing hormone/choriogonadotropin receptor	LHCGR						
374131	Mineralocorticoid receptor	MR						
429211	Oxytocin-like receptor	OT-like-R						
396198	Progesterone receptor	PGR						
422011	Proopiomelanocortin receptor	POMC-R						
396453	Prolactin	PRL						
395660	Prolactin receptor	PRL-R						
396251	Thyroid hormone receptor alpha	THR α						
396431	Thyroid hormone receptor beta	THR β						
428900	Thyroid stimulating hormone receptor	TSHR						
427860	Vasotocin-like receptor	VT-like-R						
396323	Vasoactive Intestinal Peptide	VIP						
395329	Vasoactive Intestinal Peptide Receptor 1	VIP-R-1						

As compared to same-sex controls:

 Down-regulates
 Up-regulates

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112 **Table 1.** Results of candidate gene expression analysis. Target genes identified *a priori* due to
 113 their known role in reproduction and/or the stress response. Expression of these genes was
 114 present in all HPG axis tissues. Genes that significantly ($P < 0.05$) upregulated in expression in
 115 response to stress are indicated with a lighter shade; genes that significantly downregulated in
 116 response are indicated with a darker shade.

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120 ***Global Evaluation of Gene Expression***

121 Global patterns of gene expression were analyzed using edgeR to observe how the entire
122 transcriptome of the HPG axis responds to stress. After controlling for over 17,000 multiple
123 comparisons, the count data was normalized using the TMM method [25], which, in brief, uses a
124 set of scaling factors for library sizes to minimize inter-sample log-fold changes for most genes.
125 This analysis revealed a significant transcriptomic response of the HPG axis to stress,
126 particularly in the female, especially in the pituitary and the ovary (Table 2, Fig. 2, 3). A
127 complete list of differentially expressed genes in female and male HPG tissue in response to
128 stress can be found at <https://goo.gl/mypuFv>. A brief description of each gene's reported
129 functionality in vertebrates is given in forthcoming text to offer insight into potential systems
130 affected by stress. However, it is important to note that the gene functionality given may not
131 have yet been confirmed in an avian model.

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Tissue	Sex	In response to stress:		
		Higher DE	Lower DE	Total DE
Hypothalamus	Female	105	131	136
	Male	3	21	24
Pituitary	Female	541	55	596
	Male	46	119	165
Ovary	Female	345	793	1,138
Testes	Male	5	3	8

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143 **Table 2.** The number of differentially expressed (DE) genes in each tissue in response to stress
144 as compared to controls.

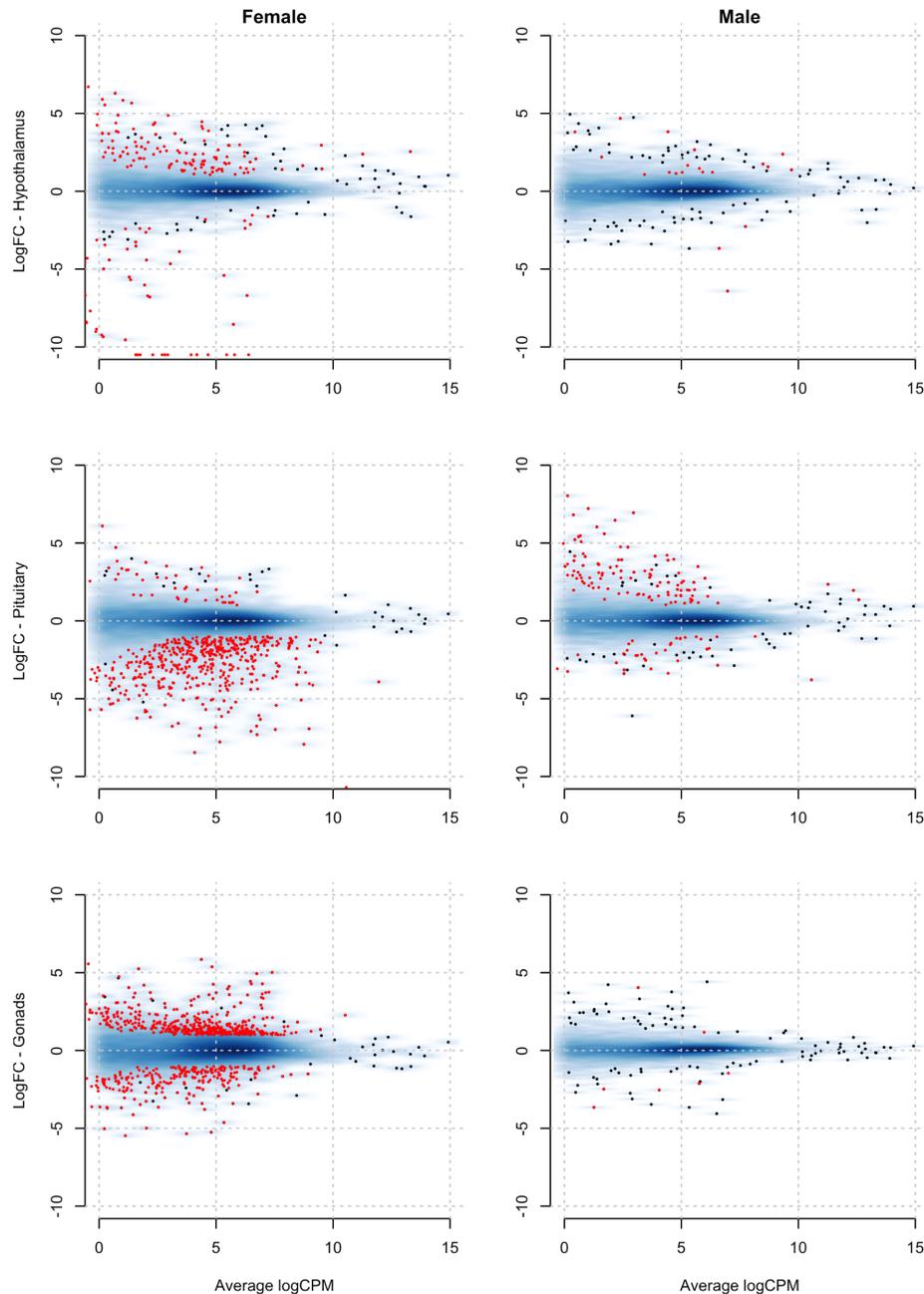
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173 Figure 2. MA plots depicting the amount of genes differentially expressed throughout the HPG
174 axis of females (left column) as compared to males (right column). Red dots indicate statistically
175 differentially expressed genes. The X axis represents the average value for gene expression (units
176 = average counts per million). The Y axis indicates the log fold change (LogFC) of genes
177 expressed in the hypothalamus (top), pituitary (middle) and gonads (bottom). Red dots above
178 zero indicate higher expression in the control animals; red dots below zero indicate higher
179 expression in stressed animals.

180

181 *Hypothalamus*

182 A global transcriptome analysis yielded 236 differentially expressed genes in the hypothalami of
183 stressed females (106 upregulated and 131 downregulated) as compared to female controls,
184 while 24 genes were significantly differentially expressed in the hypothalami of stressed males
185 (3 upregulated and 21 downregulated; Table 2, Fig. 3) as compared to male controls. Genes
186 more highly expressed in the hypothalamic tissue of stressed females include *UNC45B*, which is
187 a progesterone regulator and co-chaperone for heat shock protein of 90 kDa (*HSP90*) [26]
188 (logFC -8.6, FDR 9.0e-12). Additionally, *interferon induced transmembrane protein 5*
189 (*IFITM5*), which was more highly expressed, can interact with serotonin receptor [27] as well as
190 several solute carriers [28] responsible for transporting newly synthesized prostaglandins PGD2,
191 PGE1, PGE2, leukotriene C4, and thromboxane B2 (logFC -11.1, FDR 3.0e-10). Several long
192 non-coding RNAs, *LOC107050862* and *LOC101747554* were some of the most differentially
193 expressed genes between stress and control groups (logFC -12.6, FDR 1.0e-13 and logFC -11.3,
194 FDR 4.5e-11 respectively), yet their function is currently unknown. In addition to these, a strong
195 signal of differential expression of the Myosins was uncovered (*e.g.*, *MYH1E*, *MYH1F*, *MYOZ2*).
196 Gene ontology terms enriched for this treatment group to describe groups of genes of similar
197 function responsive to stress include terms related to muscle development and function (gene
198 ontology term myofibril assembly, GO:0030239). This pattern is driven in part by Myosin
199 proteins, which can play a role in secretory function [29,30].

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201 Genes more lowly expressed in the hypothalamic tissue of stressed females as compared to
202 controls include *LUC7L2* (logFC 1.9, FDR 1.1e-07), a gene previously found to be differentially
203 expressed in response to stress [31], and *LOC107051530* (logFC 4.9, FDR 6.4e-5), a non-coding
204 RNA whose function is unknown. *Prolactin* is more lowly expressed in response to stress (logFC
205 2.5, FDR .0002), as is *GABA type A receptor-associated protein* (logFC 1.2, FDR .007), the
206 latter which has been shown to be regulated by *Leptin* in guinea pigs [32]. No significantly
207 enriched gene ontology terms were revealed for genes more lowly expressed in the female
208 hypothalamus in response to stress. The full differential dataset for the female hypothalamus is
209 available at <https://git.io/vD4LU>.

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211 As compared to the females, fewer genes were differentially expressed in the male hypothalamus
212 in response to stress. One gene more highly expressed in stressed males as compared to controls
213 is *PH domain leucine-rich repeat-containing protein phosphatase 2 (PHLPP2)*, which can play a
214 role in the apoptotic process (logFC -3.6, FDR 2.4e-09). A gene more lowly expressed in
215 response to stress, *Pro melanin concentrating hormone (PMCH)* (logFC 3.8, FDR 2.532022e-
216 05), can inhibit stress-induced ACTH release, stimulate anxiety and sexual behavior, and
217 antagonize the inhibitory effect of alpha melanotropin on exploration behavior [33–35]. *C-*
218 *RFamide protein*, also known as *prolactin releasing hormone 2* [36] was also more lowly
219 expressed in response to stress and can play a role in the release of prolactin as well as several
220 gonadotropes [37] (logFC 4.7, FDR 2.52e-05). No significantly enriched gene ontology terms
221 were revealed for genes differentially expressed in the male hypothalamus in response to stress
222 The full differential dataset for the stress response of the male hypothalamus is available at
223 <https://git.io/vD40k>.

224

225 *Pituitary Gland*

226 A global transcriptome analysis of the HPG stress response yielded 596 differentially expressed
227 genes in the pituitary of stressed females (541 upregulated and 55 downregulated) as compared
228 to female controls, while 165 genes were differentially expressed in the pituitary of stressed
229 males (46 upregulated and 119 downregulated; Table 2, Fig. 3) as compared to male controls.
230 For example, in females, *Proteolipid Protein 1 (PLP1)* [38], a gene implicated in the stress
231 response, as well as *Myelin basic protein (MBP)* [39], which can interact with PLP1 to regulate
232 apoptosis, are more highly expressed in response to stress (logFC -10.7, FDR 4.4e-12 and logFC
233 -3.9, FDR 4.4e-12). Genes like *Cytokine inducible SH2 containing protein (CISH)* and
234 *glutamate metabotropic receptor 3 (GRM3)* are also more highly expressed in females in
235 response to stress (logFC -1.8, FDR 1.9e-10; logFC -3.8, FDR 4.2e-10, respectively). *CISH* is
236 involved in the immune response via negatively regulation of cytokine signalling [40], and
237 *GRM3* has been reported as a candidate gene for schizophrenia [41]. Gene ontology terms
238 enriched for genes more highly expressed in the female pituitary in response to stress are related
239 to oligodendrocyte differentiation, positive regulation of gliogenesis, G-protein coupled receptor
240 signaling pathway, and behavior.

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242 Genes more lowly expressed in the pituitary of stressed females as compared to controls include
243 *Angiopoietin like 7 (ANGPTL7)* (logFC 2.6, FDR 5.9e-5) whose function is currently unknown
244 but may be related to the regulation of growth and metabolism (Hato et al. 2008). *Chemokine (C-*
245 *C motif) ligand 4 (CCL4)*, a gene previously implicated in the heat stress response in chickens
246 (Tu et al. 2016), is also more lowly expressed in response to stress. No significantly enriched
247 gene ontology terms were revealed for genes differentially expressed in the female pituitary in
248 response to stress. The full differential dataset for the stress response of the female pituitary is
249 available at <https://git.io/vD49n>.

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251 Much like the pattern observed in the hypothalami of males as compared to females, the male
252 pituitary also has less differential gene expression than the female pituitary. For example, genes
253 that were more highly expressed in the male pituitary in response to stress include *Nuclear*
254 *receptor subfamily 4 group A member 3 (NR4A3)* (logFC -2.9, FDR 5.3e-10), which has been
255 implicated in mechanisms related to feeding behavior and energy balance [42,43], and 5-
256 *hydroxytryptamine receptor 3A (HTR3A)* (logFC -1.9, 4.42e-05), a receptor whose ligand
257 (serotonin) is widely accepted to be involved in mood disorder and anxiety [43–46]. A signal of
258 cell-cycle arrest may be evident, as the gene *CCAAT/enhancer binding protein (C/EBP), delta*,
259 known to be involved in preventing the cell cycle from continuing through the G1 phase [47] is
260 more highly expressed in response to stress (logFC -2.6, FDR 3.1e-09). *Activating transcription*
261 *factor 3 (ATF3)*, which has been previously reported to respond to stress [48,49], is highly
262 expressed in the male pituitary in response to stress. Gene ontology terms enriched for in genes
263 more highly expressed in the stressed male pituitary in response to stress are related to the term
264 for “muscle development”. Again, this signal is descendent from actin and myosin genes
265 involved in secretion [29] as opposed to actual skeletal or smooth muscles.

266

267 Genes more lowly expressed in the male pituitary in response to stress include the *class II major*
268 *histocompatibility complex (MHC) antigen and DM beta chain type 1 (DMB1)* (logFC 2.11 FDR
269 0.0009), which has important implications for stress given their well known roles in immune
270 function [50,51]. Other genes include the Heat shock 27kDa protein 1 (HSPB1) (logFC 2.8.,
271 FDR 0.008) and Synaptotagmin 2 (logFC 1.21, FDR 0.009), the latter which is known to be
272 involved in the stress response. [52,53]. The full table of differential expression results for the

273 male pituitary in response to stress is available at <https://git.io/vD4xL>.

274

275 *Ovary*

276 The ovary was the site of the most differential expression in response to stress as compared to
277 the hypothalamus, pituitary, and testes. In sum, 1138 genes were differentially expressed (345
278 higher in stress, 793 lower; Table 2, Fig. 3) in the ovary of stressed females as compared to
279 controls. Genes that were more highly expressed include Heat Shock Protein 25 (HSP25) (logFC
280 -2.9, FDR 5e-07) and *Heat shock 70kDa protein 8 (HSPA8)*, logFC -1.5, FDR 2.5e-08), the latter
281 has been reported to prevent protein aggregation under stress conditions [54]. Additionally,
282 *Thyroid hormone responsive protein (THRSP)* was more highly expressed in the ovary in
283 response to stress (logFC -5.1, FDR 1.1e-05). *THRSP* has been reported to play a role in the
284 regulation of lipogenesis, especially in lactating mammary gland [55]. Gene ontology terms
285 enriched for genes more highly expressed in the ovary in response to stress are related to
286 inflammatory response, defense response, and response to stress.

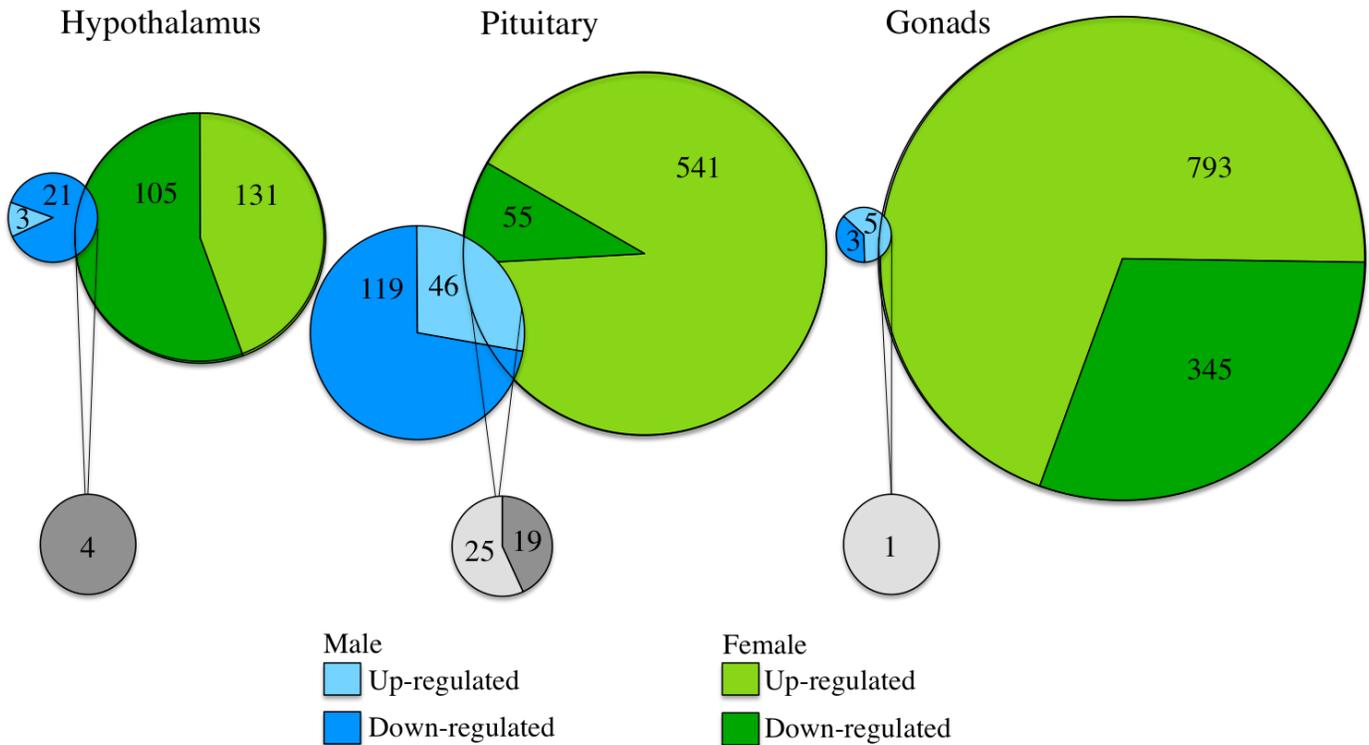
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288 *Testes*

289 Analysis of differential expression in the testes in response to stress resulted in only 8
290 differentially expressed genes (Table 2, Fig. 3). These genes include *Heat shock 27kDa protein 1*
291 (*HSPB1*) (logFC -2.5, FDR 0.005) and *Thyroid hormone receptor interactor 11 (TRIP11)* (logFC
292 -1.55, FDR 0.01), both of which increase in expression in response to stress as compared to
293 controls. *TRIP11* has been previously implicated in the stress response [56], yet its function in
294 the testes is currently unknown. The full table of differential expression results for the male
295 testes in response to stress is available at <https://git.io/vDBTw>.

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300 **Fig. 3.** A weighted Venn diagram depicting the overlap of the number of differentially expressed
301 genes between the sexes in the hypothalamus, pituitary, and gonads in response to stress as
302 compared to controls. Genes that upregulated in expression in response to stress are shown in a
303 lighter color; genes that downregulated in response exhibit a darker color. Numbers within shaded
304 areas indicate the number of stress-responsive genes. Pie charts of shared, stress-responsive
305 genes have been magnified and are not to scale.

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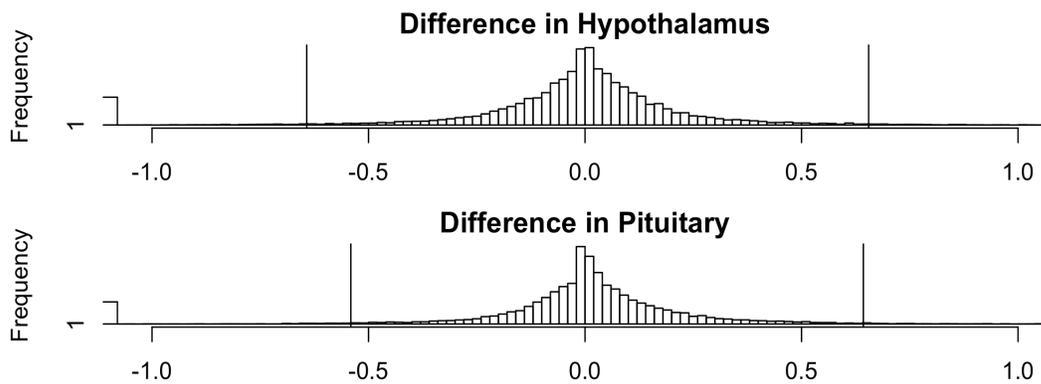
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309 *Sex-biased Response to Stress*

310 In the previous analyses, changes in gene expression in response to restraint stress were
311 identified in each sex as compared to same-sex controls. Here, changes in gene expression in
312 response to restraint stress are compared between the sexes, specifically in hypothalamic and
313 pituitary tissue. Male and female gonads are structurally and functionally different and thus were
314 not directly compared. A distributional analysis was used to identify genes whose expression
315 patterns varied by sex. Specifically, the sex difference in gene expression in response to stress

316 (defined as $(\text{Median Expression}_{\text{Male Tissue Stress}} - \text{Median Expression}_{\text{Male Tissue Control}}) - (\text{Median}$
317 $\text{Expression}_{\text{Female Tissue Stress}} - \text{Median Expression}_{\text{Female Tissue Control}})$ was calculated, with genes whose
318 response was different (defined as being more responsive than 98% of all other genes, $n=173$ in
319 each tail of the distribution, Fig. 4), deemed to be expressed in a sex-biased manner.
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325 **Figure 4.** The transcriptomic stress response distribution histogram in males versus females in
326 the hypothalamus (top) and pituitary (bottom). Vertical lines in the tails of the distributions
327 indicate genes in the upper and lower 1% of the distribution, beyond which genes were deemed
328 to be expressed in a sex-biased manner. The X axis represents the difference in expression
329 response, and the Y axis represents the frequency. To the right of 0.0 point are genes more highly
330 expressed in males, while genes to the left of 0.0 point are more highly expressed in females.

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334 *Hypothalamus*

335 Genes that were more responsive to stress in the female hypothalamus relative to the male
336 hypothalamus (defined as $\geq 99\%$ differences; examples in Figure 5a) included *Cholecystokinin*
337 (*CCK*; $\Delta\text{Median} = 0.75$), which has been previously implicated in opiate antagonism [57],
338 appetite [58], and the stress response [59,60]. Another gene of interest discovered to be more
339 responsive in females was *Calcitonin Gene-Related Peptide I* (*CALCA*, also known as CGRP,

340 Δ Median = 1.02), known to be an important regulator of stress-induced reproductive suppression
341 [61]. Actin beta-like 2 (ACTBL2), a gene whose function may be linked to secretory function
342 [29] as well as to leptin and insulin mediated signalling [62], were more responsive to stress in
343 the female hypothalamus as compared to the male hypothalamus (Δ Median = 1.39), as was
344 *Growth Hormone* (GH) (Δ Median = .77), which can stimulate growth and cell reproduction as
345 well as respond to stress by increasing glucose and fatty acids. The full table of genes, more
346 responsive to stress in the female hypothalamus as compared to the male hypothalamus is
347 available at <https://git.io/vDzKT>.

348

349 Genes that were more responsive to stress in the male hypothalamus relative to the female
350 hypothalamus (examples in Figure 5b) included Toll-like Receptor 15 (TLR15) and MHC DM-
351 beta2 (DMB2), which play a role in immune function (Δ Median = 0.74 and 0.89,
352 respectively)[63,64] which can affect the stress response [65]. Hypothalamic genes more highly
353 responsive to stress in males versus females appear to be related to Mitogen-Activated Protein
354 Kinase (MAPK) activity, including nerve growth factor (NGF), Filamin C (FLNC), and ECSIT
355 Signalling Integrator (ECSIT). The MAPK cascade is thought to coordinate the response to a
356 wide variety of stressors, given it receives stimuli from a diverse group of signalling pathways
357 including growth factors, G protein-coupled receptors, pathogen-associated molecular patterns
358 (PAMPs) and danger-associated molecular patterns (DAMPs), reviewed in [66]. The full table
359 of differential expression results of genes more responsive to stress in the male hypothalamus as
360 compared to the female hypothalamus is available at <https://git.io/vDz9C>.

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374 Figure 5. Violin plots depicting genes that were more responsive to stress in the a) female
375 hypothalamus relative to the male hypothalamus, and b) male hypothalamus relative to the
376 female hypothalamus. Plot titles are of the format Gene Name:Entrez ID:Tissue. The Y-axis
377 indicates levels of gene expression as presented by taking the log of transcripts per million
378 (logTPM). The shape of each half of the plot represents the kernel density estimation of the data,
379 with darker grey indicative of data from control animals and lighter grey from stressed animals.
380 The diamond symbol represents the median value of the control group while the plus sign
381 represents the median value of the stressed group. Arrows represent the direction and amount of
382 change in gene expression in the stressed group as compared to controls (up for upregulated,
383 down for downregulated).

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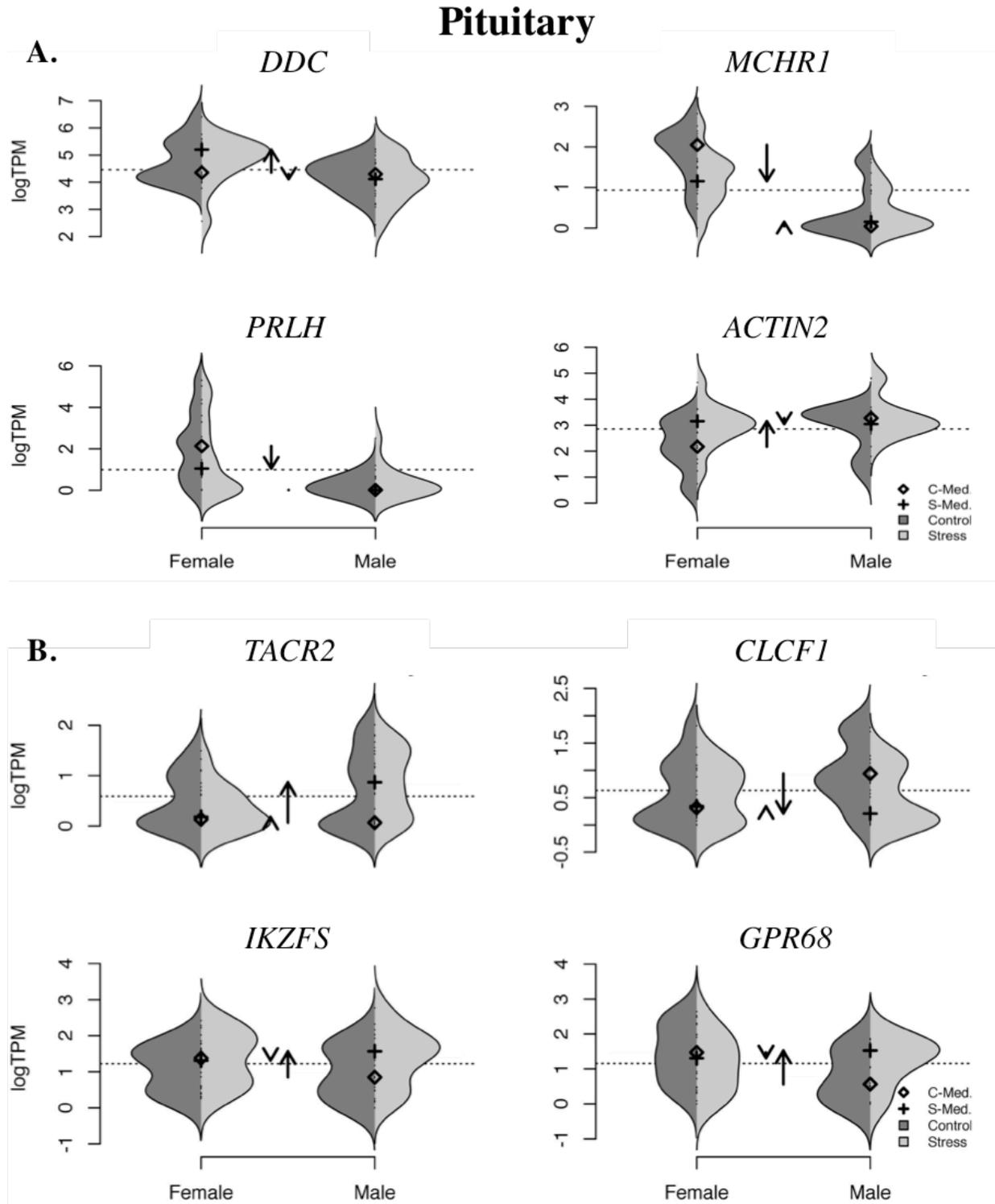
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386 *Pituitary*

387 Genes more responsive to stress in the female pituitary relative to the male pituitary include
388 (examples in Fig. 6a) melanin concentrating hormone receptor 1 (MCHR1), Δ Median = 1.32), a
389 gene whose function can impact hunger and feeding [67], and anxiety [68], as well as reduce the
390 impacts of stress [69]. Dopa Decarboxylase (DDC), a gene which encodes the the enzyme that
391 synthesizes norepinephrine (NE) from dopamine [70], is similarly more responsive in the female
392 pituitary ($FC_{\text{difference}} = 4.76$), as is Prolactin releasing hormone (PRLH) (Δ Median = 1.082668).
393 Actin gamma 2 (ACTG2) (Δ Median = .75). The full table of differential expression results of
394 genes more responsive to stress in the female pituitary as compared to the male pituitary is
395 available at <https://git.io/vDzQ1>.

396

397 Genes more responsive to stress in the male pituitary relative to the female pituitary (examples in
398 Fig. 6b) include tachykinin receptor 2 (TACR2, Δ Median = -1.37), a gene known to have
399 important reproductive correlates, specifically in loss of sexual cyclicity in females
400 [71,72]. [71,72] Genes that play key roles in the immune response, cardiotrophin-like cytokine
401 factor 1 (CLCF1) [73,74]) and IKAROS family zinc finger 3 (IKZF3) [75,76] G protein coupled
402 receptor (GPR68)(Δ Median = 0.79). The full table of differential expression results of genes
403 more responsive to stress in the male pituitary as compared to the female pituitary is available at
404 <https://git.io/vDzA8>.



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409 Figure 6. Violin plots depicting genes that were more responsive to stress in the a) female
410 pituitary relative to the male pituitary, and b) male pituitary relative to the female pituitary. Plot
411 titles are of the format Gene Name:Entrez ID:Tissue. The Y-axis indicates levels of gene
412 expression as presented by taking the log of transcripts per million (logTPM). The shape of each
413 half of the plot represents the kernel density estimation of the data, with darker grey indicative of
414 data from control animals and lighter grey from stressed animals. The diamond symbol
415 represents the median value of the control group while the plus sign represents the median value
416 of the stressed group. Arrows represent the direction and amount of change in gene expression in
417 the stressed group as compared to controls (up for upregulated, down for downregulated).

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420

421 **DISCUSSION**

422

423 By leveraging a highly-replicated and sex-balanced experimental approach to understand the
424 transcriptomic effects of stress on the HPG axis, we provide an unparalleled glimpse into how
425 males and females respond to stress. We exceeded replication standards for RNAseq experiments
426 [77], while performing gene-level quantitation [78], which resulted in exceptionally robust
427 estimates of gene expression across tissues and treatments. Our results provide evidence of sex-
428 biased gene activity of a tissue system vital for vertebrate reproduction - the hypothalamus in the
429 brain, the pituitary gland, and the gonads (testes and ovaries).

430

431 More genes in the female HPG axis were responsive to stress as compared to males. This
432 differential expression of genes in response to stress was also mostly unique to each sex. Various
433 factors could contribute to a sex-biased response to stress, though we were able to control for
434 many of them with the rock dove model. For example, an uneven parental care strategy (one sex
435 cares more than the other) and age have been known to influence the stress response [79–81].
436 However, both rock dove sexes offer significant offspring care, though they were not caring for
437 offspring at the time of collection, and all birds were of similar age (2 years old). Males and
438 females of this species are similar in other ways: they are physically monomorphic, socially and
439 genetically monogamous [82], and we report that both sexes exhibit a similar increase in

440 circulating glucocorticoids in response to restraint stress. This classic method of measuring the
441 effects of stress on circulating glucocorticoid concentration would have suggested that rock dove
442 males and females have a similar stress response. However, a deeper look at gene transcription in
443 response to stress suggests otherwise. This begs the question as to whether a gene(s) is regulated
444 in a sex-specific manner to produce a sex-specific physiological or behavioral result in the face
445 of stress, such as female control over reproductive timing? Or, does expression resulting from
446 stress lead to sexually monomorphic gene-mediated reproductive processes that converge to
447 similar physiological and behavioral endpoints [83], such as the similar corticosterone response
448 we observed? These data we provide can inform and promote multiple lines of further
449 investigations to answer these questions, including hypothesis driven tests and manipulations of
450 newly identified sex-biased and stress-responsive genes. Here, we propose three, non-mutually
451 exclusive hypotheses to explain why more genes are responsive to stress in the female HPG axis
452 as compared to their male counterparts: the Reproductive Cycle Hypothesis, the Reproductive
453 Investment Hypothesis, and the Environmental Preparation Hypothesis.

454
455 Sexually mature females, unlike sexually mature males who maintain a relatively consistently
456 functioning HPG axis, experience cycling of their reproductive hormones to facilitate ovulation
457 and follicle growth; because of this, females may present a more complicated picture for
458 understanding the effects of stress on the HPG axis, especially due to the potential for feedback
459 mechanisms to change with the reproductive cycle [3,84]. The ovary was the site of the most
460 differential expression in response to stress as compared to the hypothalamus, pituitary, and
461 testes. Although we controlled for reproductive stage, selecting sexually mature birds that were
462 not actively breeding, as well as specific ovary tissue type and amount sampled, we were unable
463 to control for the specific stage of ovulation and follicle maturation at the point of sampling.
464 Endocrine processes associated with these reproductive processes thus may influence how the
465 HPG axis, particularly the ovary in this case, responds to environmental perturbations such as
466 stress. This may be why we found a greater array of genes active in the female at both baseline
467 sampling [24] and in response to stress. Alternatively, potential endocrine variation experienced
468 by the female HPG axis might create “noise” and thus *decrease* our statistical ability to identify
469 differentially expressed genes. Because we observed a significant increase, not decrease, in
470 differentially expressed genes in the female HPG axis at baseline and in response to stress as

471 compared to males, this is either not the case, or there is even more differential expression in
472 female HPG tissue than we were able to statistically uncover. In either event, females are
473 experiencing heightened HPG gene activity in response to stress, and this may be due to the
474 physiological variation they experience over the course of their reproductive period.

475

476 Another potential hypothesis to explain why females are more responsive to stress than males at
477 the level of their HPG transcriptome is an evolutionary one related to their reproductive
478 investment. Though both males and females of this species are socially and genetically
479 monogamous and offer biparental care [82], females generally invest more time and resources in
480 making and maturing gametes and eggs [85,86]. Thus, because reproduction is arguably more
481 energetically expensive for females during gamete and egg production, this may have supported
482 the adaptation of a more stress-responsive reproductive axis to influence when and how females
483 breed in response to the environment to optimize their lifetime reproductive success [87]. For
484 example, prolactin is a hormone that is involved in a multitude of biological processes, but it has
485 most notably been studied for its influential role in lactation and parental care in both birds and
486 mammals. In this experiment, *Prolactin* in the hypothalamus and pituitary decreased in
487 expression in response to stress in females but not males. Both male and female doves use
488 prolactin to facilitate reproductive behaviors like nest building, lactation, and offspring care [88–
489 90]. While males and females are responsive to stress at every level of their reproductive axis, a
490 heightened responsiveness of reproductive substrates like prolactin by females could have
491 evolved to support their need for increased sensitivity to the environment due to their higher
492 level of reproductive investment. In this same vein, glucocorticoid receptor (GR), which binds
493 the stress response hormone corticosterone, increased in expression in the female but not male
494 hypothalamus in response to stress, suggesting a potential increase in sensitivity to the stress
495 response by the female reproductive axis.

496

497 In another example, the use of RNAseq helped to uncover a less well-studied gene that could
498 play a pivotal role in suppressing reproduction in the face of stress, *Calcitonin Gene-Related*
499 *Peptide (CALCA)*. *CALCA* was more responsive to stress in the female hypothalamus relative to
500 the male hypothalamus. In another study, central administration of *CALCA* into the lateral
501 cerebral ventricle of ovariectomized rats resulted in suppression of LH pulses, which was

502 reversed by a CALCA receptor antagonist [61]. Stress-induced suppression of LH pulses was
503 also blocked with a CALCA receptor antagonist [61]. These data suggest CALCA could be
504 involved in stress-induced suppression of the reproductive axis. Here, we report that expression
505 of the gene for the CALCA peptide is responsive to stress, more so in females than in males.
506 However, *CALCA* gene expression decreased in response to stress. A decrease in expression
507 might suggest a decrease in peptide production, which seems counterintuitive to the reported
508 actions of CALCA in rats. However, the species, its physiology (for example, our birds were not
509 ovariectomized), the time course of sampling, and the potential for physiological feedback must
510 all be considered to gain a better picture of *CALCA* regulation. For now, its responsiveness to
511 stress in females coupled with reports of its actions [61] suggest it may play an important role in
512 regulating the reproductive system, and this may be related to reproductive investment.

513

514 A third hypothesis to explain increased female HPG genomic responsivity to stress is that
515 information about the external environment experienced by the female could shape embryo, egg,
516 and chick development, potentially priming offspring in a such a way as to increase their fitness
517 in that stressful environment. For example, global warming is a type of stress that poses a threat
518 to the survival of many species. Zebra finch parents acoustically signal high ambient
519 temperatures to their egg-bound embryos, adaptively altering their behavior, growth,
520 reproductive success, and thermal preferences as adults [91]. Indeed, maternal exposure to
521 prenatal or postnatal stress can alter the stress response and behavior in offspring [92–94]. Thus,
522 herein lies the potential for the environment experienced by the female to influence the
523 development of young pre-egg lay. Female rock doves experienced a significant change of
524 expression in stress- and reproduction-related genes in response to stress (Table 1) as well as in
525 those related to immune function, growth, and other processes (eg. a gene ontology term
526 enriched for genes more highly expressed in the ovary in response to stress was related to the
527 inflammatory response). Resulting alterations to physiology and behavior could lead to maternal
528 effects of epigenetic and genomic activity, setting into motion biological events that could
529 prepare offspring for the environment in which they will soon face.

530

531 In summary, we report sex-specific changes in gene expression of the rock dove HPG axis in
532 response to stress, with females exhibiting increased genetic responsiveness at all levels of their

533 axis as compared to males. This phenomenon could be explained by the variation females
534 experience in their reproductive cycle as well as by their evolutionary history, including parental
535 investment and the potential for maternal effects to increase the reproductive success of
536 offspring. These hypotheses are not mutually exclusive and inspire future investigations of
537 genome to phenome causal effects. Presently, the data we report create a vital genomic
538 foundation on which sex-specific reproductive dysfunction and adaptation in the face of stress
539 can be further studied.

540

541

542

543 **ACKNOWLEDGEMENTS**

544 We thank the many undergraduate and graduate students of the Calisi Lab for their assistance on
545 this project, including their involvement in avian husbandry, conducting the experiment, and
546 tissue collection. We also thank the UC Davis Environmental Endocrinology Group, comprised
547 of the R. Calisi, T. Hahn, M. Ramenofsky, K. Ryan, and J. Wingfield Lab groups, for their
548 discussion of this project. This work was funded by NSF IOS 1455960 (to RMC and MM).

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550

551 **COMPETING INTERESTS**

552 The authors declare no competing interests.

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564 MATERIALS AND METHODS

565

566 *Animal Collection Methods*

567 Birds were housed at the University of California, Davis, in large aviaries (5'x4'x7'), with 8
568 sexually reproductive adult pairs per aviary. Food and water were provided *ad libitum*. To
569 control for reproductive stage and potential circadian rhythm confounds, we sampled males and
570 females that were paired but lacked eggs or chicks between 0900-1100 (PST) following animal
571 care and handling protocols (UC Davis IACUC permit #18895). Birds in the baseline, or
572 “unstressed,” group were sampled within 5min of entering their cage. Birds in the stress
573 treatment group were restrained in cloth bags for 30min prior to sampling. To sample tissue,
574 birds were first anesthetized using isoflurane until unresponsive (<2 min), at which point they
575 were decapitated. Trunk blood was collected to assay for plasma corticosterone concentrations.
576 Brains, pituitaries, and gonads were then immediately extracted and placed on dry ice, then
577 transferred to a -80 C freezer until further processing. Brains were sectioned coronally on a
578 cryostat (Leica CM 1860) at 100 μ M to best visualize and biopsy the hypothalamus. We used
579 Karten and Hodos' [95] stereotaxic atlas of the brain of the pigeon to locate the hypothalamus
580 and collect it in its entirety. In brief, we, collected hypothalamic tissue beginning at the point of
581 bifurcation of the tractus septomesencephalicus and ending after the cerebellum was well
582 apparent. Lateral septum tissue was included with the hypothalamus. We sequenced tissue from
583 whole homogenized testes and ovaries, the latter comprised of tissue from the oviduct and
584 ovarian follicles. Hypothalamic sections, pituitaries, and gonads were preserved in RNALater
585 and shipped from the UC Davis to the University of New Hampshire for further processing. This
586 technique to harvest hypothalamic, pituitary, and gonadal tissue from this species has been
587 previously validated by our research group [24].

588

589 *Hormone assay*

590 Fresh blood was centrifuged at 4200 rpms at 4C for 10min. Plasma was removed and stored at -
591 80C. We assayed plasma for corticosterone using radioimmunoassay (RIA), informed by a serial
592 dilution conducted prior to the assay. A dilution of 1:20 was used in a commercially available
593 Corticosterone RIA kit (MP Biomedicals, Orangeburg, NY) to determine corticosterone levels

594 (ng/ml). The assay was validated for cross-reactivity with *C. livia* corticosterone and the limit of
595 detection was estimated at 0.0385 ng/ml.

596

597 ***Illumina Library Preparation and Sequencing***

598 Tissues frozen in RNALater were thawed on ice in an RNase-free work environment. Total
599 RNA was extracted using a standard Trizol extraction protocol (Thermo Fisher Scientific,
600 Waltham, MA). The quality of the resultant extracted total RNA was characterized using the
601 Tapestation 2200 Instrument (Agilent, Santa Clara, CA), after which Illumina sequence libraries
602 were prepared using the TruSeq RNA Stranded LT Kit (Illumina). The Tapestation 2200
603 Instrument was, again, used to determine the quality and concentration of these libraries. Each
604 library was diluted to 2nM with sterile ddH₂O, and pooled in a multiplexed library sample. The
605 multiplexed library sample was then sent to the New York Genome Center for 125 base pair
606 paired-end sequencing on a HiSeq 2500 platform.

607

608 ***Transcriptome assembly evaluation and improvement***

609 The previously constructed Rock Dove transcriptome version 1.0.3 assembly [24] was evaluated
610 to ensure that transcripts expressed uniquely in the stress condition were included. To
611 accomplish this, reads from the pituitary, hypothalamus, and gonads from one male and one
612 female stressed individual were assembled following the Oyster River Protocol [96]. Unique
613 transcripts contained in this assembly relative to the previously described assembly were
614 identified via a BLAST procedure. Novel transcripts, presumably expressed uniquely in the
615 stress condition were added to the existing assembly, thereby creating the Rock Dove version
616 1.1.0 transcriptome. This new assembly was evaluated for genic content via comparison with the
617 BUSCO version 2.0 Aves database [97].

618

619 ***Mapping and Global Analysis of Differential Gene Expression***

620 After quality and adapter trimming to a Phred score =2, reads were quasimapped to the Rock
621 Dove version 1.1.0 transcriptome after an index was prepared using Salmon 0.7.2 [98]. Rock
622 dove transcript IDs were mapped to genes from *Gallus gallus* genome version 5, using BLAST
623 [99]. All data were then imported into the R statistical package (version 3.3.0) [100] using
624 tximport [101] for gene level evaluation of gene expression, which was calculated using edgeR

625 (version 3.1.4) [102] following TMM normalization and correction for multiple hypothesis tests
626 by setting the false discovery rate (FDR) to 1%. Gene ontology enrichment analysis was carried
627 out using the Kolmogorov-Smirnov test [103] for significance in the R package, topGO [104]. A
628 select set of genes found to be differentially expressed were plotted (*e.g.*, Figures 5 and 6) using
629 the ‘beanplot’ package available at <https://cran.r-project.org/package=beanplot>.

630

631 ***Candidate Gene Expression Evaluation***

632 To evaluate a set of candidate genes, we selected *a priori* genes of interest based on their known
633 involvement in stress and reproduction and associated behaviors (Table 1). Differences in gene
634 expression were evaluated between these genes in the hypothalamic, pituitary, and gonadal
635 tissues and between both sexes using a generalized linear model framework (expression ~ sex *
636 tissue * treatment) with significance for all pairwise combinations of factors tested using the
637 Bioconductor package lsmeans (<https://cran.r-project.org/package=lsmeans>) after correction
638 using a dunnettx adjustment.

639

640 ***Differential Response to Stress***

641 In addition to understanding patterns of differential gene expression between stressed and control
642 birds, genes were identified whose response to stress varied by sex. A distribution was generated
643 corresponding to the absolute difference in median gene expression between treatment and sex.
644 Genes located in the upper and lower 1% of this distribution were retained as significantly
645 different in the response to stress.

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