

¹ Corticosterone exposure is associated with long-term
² changes in DNA methylation, physiology, and breeding
³ decisions in a wild bird.

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¹⁰ **ABSTRACT**

¹¹ When facing challenges, vertebrates activate an evolutionarily conserved hormonal stress
¹² response that can dramatically alter behavior and physiology. Although this response can be
¹³ costly, conceptual models suggest that it can also recalibrate the stress response system,
¹⁴ priming more effective responses to future challenges. Little is known about whether this
¹⁵ process occurs in wild animals, particularly in adulthood, and if so, how information about
¹⁶ prior experience with stressors is encoded. One potential mechanism is hormonally-mediated
¹⁷ changes in DNA methylation. We simulated the spikes in corticosterone that accompany an
¹⁸ acute stress response using non-invasive dosing in female tree swallows (*Tachycineta bicolor*)
¹⁹ and monitored the phenotypic effects one year later, and DNA methylation both shortly
²⁰ after treatment and a full year later. The year after treatment, experimental females had
²¹ stronger negative feedback and initiated breeding earlier – traits that are associated with
²² stress resilience and reproductive performance in our population – and higher baseline
²³ corticosterone. We also found that natural variation in corticosterone predicted patterns of
²⁴ DNA methylation. Finally, corticosterone treatment influenced methylation on short (1-2
²⁵ weeks) and long (1 year) time scales; however, these changes did not have clear links to
²⁶ functional regulation of the stress response. Taken together, our results are consistent with
²⁷ corticosterone-induced priming of future stress resilience and support DNA methylation as a
²⁸ potential mechanism, but more work will be needed to demonstrate functional consequences.
²⁹ Uncovering the mechanisms linking experience with the response to future challenges has
³⁰ implications for understanding the drivers of stress resilience.

³¹ *Keywords:* reduced representation bisulfite sequencing, stress, endocrinology, carryover effects,
³² tree swallow

33 INTRODUCTION

34 Wild organisms regularly encounter challenging conditions that require rapid behavioral and
35 physiological responses. In vertebrates, the glucocorticoid mediated stress response plays an
36 essential role in allowing animals to successfully avoid or tolerate stressors (Sapolsky,
37 Romero, & Munck, 2000; Wingfield et al., 1998). While an appropriate response is beneficial
38 (Wingfield et al., 1998), an inappropriate or prolonged elevation of glucocorticoids can result
39 in a variety of negative consequences for health and fitness (Korte, Koolhaas, Wingfield, &
40 McEwen, 2005). Accordingly, the dominant paradigm in behavioral ecology and
41 endocrinology is that the immediate benefits of the stress response are balanced by long-term
42 costs.

43 But are the long-term effects of a physiological stress response always costly? Some
44 conceptual models propose that activating the stress response system—even in
45 adulthood—primes more effective responses to future challenges (Del Giudice et al., 2018;
46 Hilker et al., 2016). These models predict that initiating a response calibrates the stress
47 response system, increasing organismal ability to return to normal functioning after a
48 disturbance (resilience) or the ability to maintain normal performance despite a disturbance
49 (robustness) (Crespi et al., 2021). Because physiological priming results from activating the
50 stress response system rather than from learning, it could occur even in the absence of
51 exposure to an identifiable external threat. Physiological studies have provided some
52 evidence that stressor priming can occur, including outside of critical developmental periods
53 (Andrade-Linares, Lehmann, & Rillig, 2016; Marasco, Boner, Heidinger, Griffiths, &
54 Monaghan, 2015). However, we know little about the degree to which stressor priming
55 operates in natural populations, affecting later life behavior, physiology, and fitness.
56 Similarly, the mechanism(s) that link activation of the stress response to physiological
57 regulation of subsequent responses are not well understood.

58 One mechanism that could play a role in the calibration of stress response systems is changes

59 in DNA methylation. Epigenetic modification by DNA methylation can alter phenotypes by
60 making genes or promoters more or less accessible for transcription (Anastasiadi,
61 Esteve-Codina, & Piferrer, 2018; Lea et al., 2018; Sepers et al., 2019). While the majority of
62 methylation differences may be genetically determined (Sepers, Chen, Memelink, Verhoeven,
63 & Oers, 2023), early life experiences can also have profound programming effects on DNA
64 methylation patterns that often persist throughout the individuals' lifetime (McGowan et al.,
65 2009). For example, classic work in lab rodents demonstrates that early life experiences
66 regulate methylation of the gene producing the glucocorticoid receptor, which results in
67 lifelong changes to glucocorticoid secretion in response to challenges (Liu et al., 1997; Weaver
68 et al., 2004). A growing number of studies in wild animals also report patterns consistent
69 with early life programming of DNA methylation in wild populations associated with
70 dominance hierarchies (Laubach et al., 2019), brood size (Sheldon, Schrey, Ragsdale, &
71 Griffith, 2018), temperature and weather (Metzger & Schulte, 2017; Rubenstein et al., 2016),
72 or urbanization (Holdt, Kartzin, Oers, Verhoeven, & Ouyang, 2023).

73 While they are less well documented, experiences during adulthood can also result in changes
74 in DNA methylation and these adjustments can occur rapidly (Bentz et al., 2021; Metzger &
75 Schulte, 2017). For example, brief periods of experimental competition and aggression in tree
76 swallows (*Tachycineta bicolor*) resulted in altered DNA methylation of brain regions
77 associated with hormone signaling, suggesting a priming effect in preparation for future
78 aggression (Bentz et al., 2021). Conceptual models of the stress response have long
79 recognized that the sequence, frequency, duration, and intensity of stressors should change
80 the optimal behavioral and physiological response (Romero, Dickens, & Cyr, 2009; Zimmer,
81 Woods, & Martin, 2022). Yet it is often unclear how the experience of challenges during
82 adulthood would be biologically encoded to alter responses to future challenges. Altered
83 DNA methylation is a promising mechanism because i) it can change rapidly even during
84 adulthood (Bentz et al., 2021; Hu, Pérez-Jvostov, Blondel, & Barrett, 2018), ii) it might
85 persist over moderate to long time scales (Lea, Altmann, Alberts, & Tung, 2016;

⁸⁶ **zanas2015?**), iii) it has been shown to change with challenging experiences (Hu et al., 2018;
⁸⁷ Metzger & Schulte, 2017), and iv) it can directly alter an individual's phenotype (Angers,
⁸⁸ Castonguay, & Massicotte, 2010; Sagonas et al., 2020).

⁸⁹ We experimentally simulated a series of acute corticosterone spikes using a non-invasive
⁹⁰ dosing procedure (Vitousek et al., 2018) and monitored both long-term phenotypic effects
⁹¹ and changes in DNA methylation. In this population we previously found that brief
⁹² increases in corticosterone have effects on behavior and performance that persist throughout
⁹³ at least the length of the breeding season (Taff, Zimmer, & Vitousek, 2018; Vitousek et al.,
⁹⁴ 2018) and that genome-wide methylation predicts resilience to experimental challenges (Taff,
⁹⁵ Campagna, & Vitousek, 2019). Here, we extended those results to ask whether brief
⁹⁶ increases in corticosterone altered regulation of the stress response and breeding decisions a
⁹⁷ full year later. We coupled this approach with reduced representation bisulfite sequencing
⁹⁸ (RRBS) and a newly improved reference genome assembled for this study to examine
⁹⁹ genome-wide patterns of DNA methylation at high resolution. We used RRBS to first assess
¹⁰⁰ covariation between methylation and natural variation in corticosterone regulation during an
¹⁰¹ acute stress response. Next, we compared DNA methylation in corticosterone treated
¹⁰² females to controls to determine whether brief increases in corticosterone resulted in altered
¹⁰³ DNA methylation at either short (1-2 weeks) or long (1 year) timescales.

¹⁰⁴ If activation of the stress response machinery primes future coping ability (Del Giudice et al.,
¹⁰⁵ 2018; Hilker et al., 2016), experimentally treated females should exhibit long-term
¹⁰⁶ (across-year) differences in key phenotypic traits. Specifically, we predicted that in the year
¹⁰⁷ after treatment females would have a robust stress-induced increase in corticosterone and
¹⁰⁸ strong negative feedback, measured as the ability to quickly down regulate corticosterone
¹⁰⁹ after handling stress. These traits that have been previously shown to predict stress
¹¹⁰ resilience in this population (Zimmer et al., 2019). We also predicted that these females
¹¹¹ would initiate breeding earlier in the subsequent year, which is associated with higher

reproductive performance in tree swallows (Winkler et al., 2020). Finally, given previous work demonstrating a correlation between coping ability and both genome-wide methylation (Taff et al., 2019) and natural variation in rapid corticosterone regulation (Vitousek, Taff, Hallinger, Zimmer, & Winkler, 2018), we predicted that natural variation in corticosterone (baseline, stress-induced increase, and efficacy of negative feedback) would be associated with DNA methylation. However, a correlation here could arise through early life programming, prior activation of the acute corticosterone response, or any conditions that impact the regulation of both methylation and corticosterone (e.g., body condition). In contrast, for the experimental manipulation we predicted that differences in DNA methylation between control and treatment groups would only be present if brief increases in corticosterone have an effect on altering methylation patterns. We assessed the time course and persistence of any such changes using comparisons 1-2 weeks after treatments and 1 year after treatments. If methylation changes play a role in altering future corticosterone secretion then we expected to find more differences near genes and promoters associated with endocrine regulation.

MATERIALS AND METHODS

We studied tree swallows breeding at field sites in and around Ithaca, New York, U.S.A. from April to July 2014 to 2017. This population of tree swallows has been continuously studied since 1986 and we followed well-established monitoring protocols (for details see Winkler et al., 2020). Adult females were captured on day 6 to 7 after the beginning of incubation and again on day 3 to 7 after eggs had hatched. In the year after treatment, any returning females were captured on day 6 to 7 of incubation. At each capture we collected blood samples ($< 70\mu\text{l}$ each) to measure baseline (< 3 minutes) and stress-induced (30 minutes) corticosterone (Vitousek et al., 2018). Immediately after the second blood sample was taken, females were injected with $4.5 \mu\text{l/g}$ of dexamethasone in the pectoralis muscle (Mylan 4mg ml⁻¹ dexamethasone sodium phosphate; previously validated in Zimmer et al., 2019). This

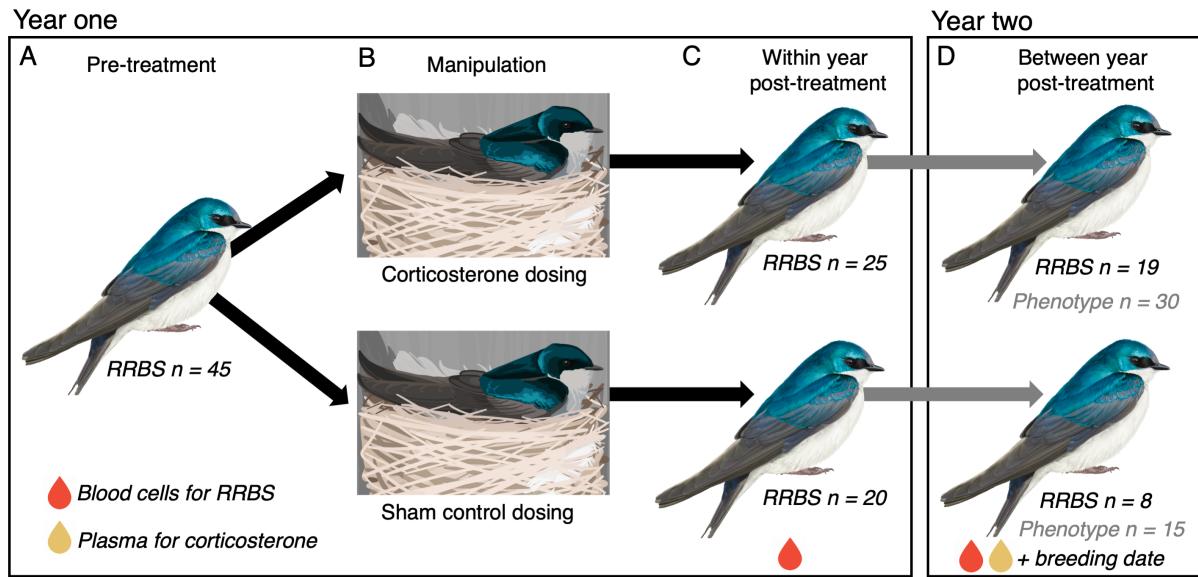


Figure 1: Schematic illustration of the experimental treatment and samples collected for RRBS. Models comparing natural corticosterone to DNA methylation used pre-treatment samples (A). After treatments were applied (B), models testing for within-year effects of treatment used post-treatment samples (C), while controlling for initial methylation (A). Models testing for between-year effects used post-treatment samples (D), while controlling for initial methylation (A). Models testing for between-year phenotypic effects on corticosterone adn breeding timing used a larger set of females (D). See text and table S1 for description of birds included in each group. Note that sample sizes indicate number of samples used in sequencing and analysis, but are not indicative of different between year return rates in each treatment because there were more corticosterone treatments applied.

¹³⁷ injection stimulates strong negative feedback, which is the process resulting in
¹³⁸ downregulation of circulating corticosterone after an acute increase. A final blood sample
¹³⁹ was collected 30 minutes after injection to measure the efficacy of negative feedback. We also
¹⁴⁰ collected a set of standardized morphological measurements and monitored reproductive
¹⁴¹ success (Vitousek et al., 2018). All birds received a unique USGS aluminum band and
¹⁴² passive integrated transponder (PIT) tag if they were not previously banded.

¹⁴³ Between the first and second capture in year one, females were randomly assigned to either a
¹⁴⁴ control or experimental treatment group (experiment schematic and sample sizes at each
¹⁴⁵ stage are shown in Figure 1). We later confirmed that females in these groups did not differ
¹⁴⁶ significantly in any pre-treatment corticosterone measures. In the experimental group, we
¹⁴⁷ simulated a brief spike in corticosterone once per day on five consecutive days between the
¹⁴⁸ two captures. To accomplish this, we applied two 60 μ l doses of corticosterone dissolved in
¹⁴⁹ DMSO gel one hour apart to a fake egg anchored in the nest cup at a randomly chosen time
¹⁵⁰ during the day when females were absent from the nest. Upon returning, females incubated
¹⁵¹ the clutch and absorbed the corticosterone across the skin on their brood patch. For the
¹⁵² purposes of this study, we considered females as part of the corticosterone treatment group if
¹⁵³ they received any of the three dose concentrations described in Vitousek et al. 2018 (high = 4
¹⁵⁴ mg ml⁻¹ corticosterone once per day followed by sham once per day; low = 2 mg ml⁻¹ once
¹⁵⁵ per day followed by sham once per day; long = 2 mg ml⁻¹ twice per day one hour apart).

¹⁵⁶ We previously validated that this dosing method results in a brief (< 180 minutes) increase
¹⁵⁷ in corticosterone within the range of natural acute corticosterone responses (Vitousek et al.,
¹⁵⁸ 2018). The elevation and time course of clearance from a single dose using this method is
¹⁵⁹ within the normal parameters for a natural acute corticosterone response triggered by a
¹⁶⁰ standard handling protocol. Control nests received either no manipulation or a sham control
¹⁶¹ in which they were dosed as described above but with DMSO gel only with no corticosterone
¹⁶² added. We previously found no difference at any time point in physiology, behavior,

163 reproductive success, or survival between control and sham control birds receiving this
164 treatment (Taff et al., 2018; Vitousek et al., 2018) and we combined both control groups in
165 the analyses described here.

166 For methylation analyses, we focused on the set of females that were manipulated in 2015
167 and—if they returned—recaptured in 2016. In analyses focused on between year effects of
168 treatments on later corticosterone regulation and breeding decisions, we also included a
169 smaller number of females observed from 2014 to 2015 and from 2016 to 2017. These
170 additional samples included slight variants on corticosterone dosing that we considered part
171 of the corticosterone treatment group (six doses or three doses of 4 mg ml⁻¹ corticosterone
172 once per day during incubation, Taff et al., 2018). In the year after exposure, we only
173 considered potential carryover effects of treatment on corticosterone regulation when females
174 were captured on day 6 of incubation and on the timing of clutch initiation because most
175 females were subsequently entered into unrelated experiments that could have influenced
176 later season measures. The slight variations on corticosterone exposure all result in
177 categorically different regulation of corticosterone when compared to control treatments
178 (Figure S4) and given our sample size, we only considered comparisons between control and
179 combined corticosterone exposure treatments. The details sample sizes for each set of
180 analyses are included in Table S2).

181 *Tree swallow reference genome assembly*

182 For this study, we improved upon a previously published reference genome sequenced from a
183 female belonging to this study population (Taff et al., 2019) by first extracting high
184 molecular weight DNA from this same individual. We performed a phenol-chloroform
185 extraction followed by an ethanol precipitation and finally a bead cleanup. The Duke Center
186 for Genomic and Computational Biology core facility used the DNA to produce a large insert
187 library (15 to 20 kb), which was subsequently sequenced on 3 cells of a Pacific Biosciences
188 RSII instrument. This produced a total of 9.6 Gbp of data with an average read length of

12,053 bp and an N50 subread length of 15,643 bp. We used bamtools version 2.5.1 (Barnett, Garrison, Quinlan, Strömberg, & Marth, 2011) to merge the reads from the difference cells and retain only those that were longer than 4,500 bp (47.6% of the original raw reads). We improved our first assembly with the PBJelly2 module of PBSuite version 15.8.24 (English et al., 2012), which uses long reads to fill or reduce gaps. This pipeline produced an assembly which was moderately improved from the previous version (Taff et al., 2019). The total length of the assembly was 1.22 Gb (previously 1.14 Gb) and was contained in 49,278 scaffolds (previously 92,148), with an N50 of 82.9 kb (originally 34 kb) and 1.9% Ns (vs. 5.8%). We assessed the completeness of our assembly by running BUSCO version 5.2.2 (Simão, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015), using the passeriformes dataset of 10,844 conserved genes. We found 80.5% of these genes in a single and complete copy, 3.9% were fragmented, 1.8% were duplicated, and 13.8% were missing. Finally, we annotated the genome following the pipeline described in Taff et al. (2019). The assembly generated for this project is deposited on GenBank (BioProject ID PRJNA553513).

203 *Sample processing*

Blood samples collected in the field were immediately stored on ice in a cooler and processed in the lab within 3 hours of capture. Red blood cells were separated from plasma by centrifugation and added to 1 mL of ice cold cryopreservation buffer (90% newborn calf serum, 10% DMSO, Haussmann & Mauck, 2008). Samples were then frozen at a constant cooling rate with isopropyl alcohol and stored at -80° C until further processing. Cryopreserved blood samples were thawed and DNA was extracted using the DNeasy Blood & Tissue spin column extraction kits according to the manufacturer's protocol (Qiagen Sciences Incorporated). Plasma was used to measure corticosterone after a triple ethyl acetate extraction with commercially available ELISA kits following the manufacturer's protocol (DetectX Corticosterone K014-H5, Arbor Assays, Ann Arbor, MI). The protocol has been previously validated for tree swallows from this population in our lab (Vitousek et

²¹⁵ al., 2018).

²¹⁶ *Corticosterone and breeding timing data analysis*

²¹⁷ We used general linear models to compare corticosterone and the timing of breeding between
²¹⁸ control and experimental females one year after corticosterone manipulations. We fit four
²¹⁹ models in total with either the date of clutch initiation or circulating corticosterone levels
²²⁰ (baseline, stress-induced, or post-dexamethasone injection) as the response variable.

²²¹ Predictors included treatment and year as a categorical fixed effect. The model for
²²² stress-induced corticosterone also included baseline corticosterone as a predictor because
²²³ baseline levels can influence the amount of stress induced increase. The model for
²²⁴ post-dexamethasone corticosterone also included stress-induced corticosterone as a predictor
²²⁵ because the stress-induced level directly influences post-dexamethasone levels.

²²⁶ *Reduced representation bisulfite sequencing*

²²⁷ We prepared our samples for reduced representation bisulfite sequencing (RRBS) in house
²²⁸ using the Diagenode Premium RRBS Kit and closely following the manufacturer's protocol
²²⁹ (Veillard, Datlinger, Laczik, Squazzo, & Bock, 2016). Briefly, samples were diluted to 3.85
²³⁰ ng/ μ l and 26 μ l of diluted sample was used for library preparation. The process included
²³¹ enzymatic digestion with Mspl and size selection to increase coverage of CpG-rich regions,
²³² such as CpG islands and enhancers. Individual samples received one of twenty-four unique
²³³ barcodes and were pooled in randomized groups of 8 before bisulfite conversion. We also
²³⁴ included a methylated and unmethylated spike in control with each sample to confirm the
²³⁵ efficiency of bisulfite conversion.

²³⁶ From the available samples, we selected 120 samples to process from 61 unique birds (three
²³⁷ samples per bird n = 14, two samples n = 31, one sample n = 16, Figure 1). We prioritized
²³⁸ selection of individuals that had data from both years and that had complete data for the
²³⁹ planned analyses. For birds with the same available samples, we selected which individuals
²⁴⁰ to include randomly. Prior to RRBS processing, these 120 samples were randomly sorted to

241 account for any batch effects. Libraries were prepared with the Diagenode kit in two batches
242 (one set of 24 and one of 96). Prepared libraries were checked for the expected size
243 distribution by digital PCR prior to sequencing. Sequencing was performed at the Cornell
244 BRC using NextSeq sing-end 1x75 with 20% PhiX and 85% of the normal cluster density. In
245 total, we ran our samples on five sequencing lanes with 24 samples per lane.

246 Raw sequence data were first processed with **Trim Galore!** version 0.6.6 using the default
247 RRBS settings. Visual inspection of FastQC files confirmed high quality reads for all
248 samples. Next, we used **Bismark** version 0.23.0 to align each sequence in end-to-end mode to
249 the prepared genome and extract the methylation status for each CpG, CpH, or CHH site
250 (Krueger & Andrews, 2011). As expected, global methylation at CpH and CHH sites was
251 extremely low (1.0% and 0.6%, respectively, Figure S1) and we only considered methylation
252 at CpG sites in our subsequent analyses. We also used **Bismark** to determine the
253 methylation conversion efficiency for each sample based on methylated and unmethylated
254 spike in controls and following the instructions in the Diagenode RRBS kit (Krueger &
255 Andrews, 2011; Veillard et al., 2016). Finally, we used **Bismark** to visually inspect m-bias
256 plots and determined that no further trimming was necessary.

257 *General methylation patterns*

258 Our process resulted in 9.8 ± 4.3 million (SD) total reads per sample (Figure S1). Across all
259 samples, we were able to align 51.1% of the total reads produced, which is comparable to
260 several recent studies in wild birds (Mäkinen et al., 2019; Watson, Powell, Salmón, Jacobs, &
261 Isaksson, 2021). Spiked controls in each sample indicated that our bisulfite conversion
262 worked efficiently and within the recommended kit parameters (conversion of methylated
263 control sites = $1.9\% \pm 1.4$; conversion of unmethylated control sites = $99.5\% \pm 0.6$).

264 Among 45 pre-treatment samples, we had sufficient coverage to estimate methylation at
265 148,167 CpGs (minimum of 10 reads from a minimum of 10 individuals). In total, the
266 average percentage methylation across all sites was $35.5\% \pm 34.0$ with a wide distribution

267 (Figure S2A). After assigning CpGs hierarchically to promoter (within 2kb upstream of a
268 TSS) > exon > intron, we found that 12.1% of sites were in promoters, 7.9% in exons, 11.8%
269 in introns, and 68.1% in intergenic regions. At the level of genomic features, promoters had
270 the lowest methylation (median = 5.3%, mean \pm SEM = 20.5% \pm 0.5), introns had
271 intermediate methylation (median = 43.5%, mean \pm SEM = 41.0% \pm 0.5), and exons had
272 the highest methylation (median = 54.3%, mean \pm SEM = 46.7% \pm 0.7). However, each of
273 these features had a wide distribution of methylation percentages across different genes
274 (Figure S2B).

275 *RRBS Data Analysis*

276 Output data from the sequence processing described above was analyzed in R version 4.1.1
277 (R Core Team, 2020). We processed the aligned sequence data with **MethylKit** version
278 1.14.2 (Akalin et al., 2012). Using **MethylKit**, we extracted the number of total aligned
279 reads and number of methylated or unmethylated reads for each CpG site. As recommended
280 by the package authors, we filtered out CpGs above the 99.5th percentile to account for
281 potential PCR bias.

282 For analyses of corticosterone and treatment associations, we filtered these CpGs to include
283 only those that met the following criteria. First, we required a minimum coverage of 10 reads
284 per sample to retain data for that sample at a given CpG. We further filtered the dataset to
285 remove any CpGs that were mostly invariant (i.e., more than half of samples had
286 methylation percentage of 0 or 100%) as well as CpGs that had extremely low variation (SD
287 less than 5% across all samples, Husby, 2022; Lundregan et al., 2022). For models comparing
288 treatment effects, we required that females have data at a given CpG from both pre- and
289 post-treatment sampling points to be included. We also required a minimum of six
290 individuals per treatment group for a CpG to be included in the analysis. For basic
291 descriptions of methylation patterns, we used all CpGs that had 10 reads or more in the
292 pre-treatment samples.

293 The built-in differential methylation techniques in **MethylKit** are designed for two group
294 comparisons with limited flexibility in modeling options. Because we had repeated measures
295 before and after treatments for both groups, we could not specify the necessary models
296 within **MethylKit** itself. Therefore, we exported and combined the filtered CpG records for
297 all groups so that we could fit generalized linear mixed models (GLMMs) for each CpG site
298 (as in Lindner et al., 2021) using the **glmer** function in R package **lme4** version 1.1-29
299 (Bates, Mächler, Bolker, & Walker, 2014). We fit a separate set of models for natural
300 corticosterone variation before treatments occurred (baseline, stress-induced, or
301 post-dexamethasone), for the within-year treatment effect, and for the between-year
302 treatment effect. Each of these datasets were constructed separately since they included
303 different subsets of both individual birds and of CpGs that met the criteria described above.

304 For natural variation in corticosterone, we included only the pre-treatment samples. Using
305 these samples, we fit a GLMM for each CpG with the number of methylated and
306 unmethylated reads as the binomial response variable. We fit this set of models separately
307 with baseline ($n = 78,143$ CpGs), stress-induced ($n = 78,027$ CpGs), or post-dexamethasone
308 ($n = 69,189$ CpGs) corticosterone measured in ng/ml as the single continuous predictor
309 variable. The models included a random effect for female identity to account for repeated
310 sequencing of the same CpG sites within each female. We excluded the results for any
311 models that failed to converge because we could not reliably estimate effects in those cases.

312 For within-year ($n = 48,070$ CpGs) and between-year ($n = 6,787$ CpGs) comparisons after
313 treatments, we fit a single GLMM for each included CpG with the number of methylated
314 and unmethylated reads as the binomial response variable. Predictors included
315 pre-treatment methylation percentage at the CpG being modeled, a fixed effect of treatment
316 (control vs. corticosterone), and a random effect for female identity. In each model,
317 significance of the comparison between control and corticosterone treated birds was assessed
318 using the **emmeans** version 1.5.3 package in R (Lenth, 2019), which employs the

³¹⁹ Satterthwaite approximation method for binomial mixed models. We also evaluated the
³²⁰ stability of methylation within individuals in these models by summarizing the regression
³²¹ coefficient of pre-treatment methylation on post-treatment methylation.

³²² We accounted for multiple comparisons in each of these analyses by adjusting all p-values
³²³ using the q-value approach implemented by the `qvalue` package version 2.20.0 in R with the
³²⁴ false discovery rate (FDR) set at 0.05 (Storey, Bass, Dabney, & Robinson, 2019). The
³²⁵ q-value approach accounts for multiple comparisons by considering both the number of
³²⁶ comparisons made and the distribution of accumulated p-values. We only report and
³²⁷ interpret estimates with q-values < 0.05.

³²⁸ *Annotation of differentially methylated CpGs*

³²⁹ After identifying CpGs that were significantly associated with either natural corticosterone
³³⁰ or experimental treatment with corticosterone, we identified genes associated with each CpG.
³³¹ We used the `bedtoolsr` package version 2.30.0-5 to select genes that had a significant CpG
³³² either within the gene body or within 2 kb upstream of the transcription start site
³³³ (Patwardhan, Wenger, Davis, & Phanstiel, 2019). We generated separate lists of genes
³³⁴ associated with CpGs for baseline corticosterone, stress-induced corticosterone,
³³⁵ post-dexamethasone corticosterone, within-year treatment effects, and between-year
³³⁶ treatment effects. For each of these comparisons we also generated a complete list of genes
³³⁷ associated with all of the CpGs that passed the filtering criteria described above to be used
³³⁸ as a null background list (see below).

³³⁹ Starting with the list of genes associated with each comparison set, we used the DAVID
³⁴⁰ functional annotation tool (Huang et al., 2007) to test whether our genes were enriched in
³⁴¹ any molecular functions or biological processes in the Gene Ontology knowledgebase
³⁴² (Ashburner et al., 2000; Consortium, 2021). For each comparison we used the custom
³⁴³ background list generated above. This background list is important for interpretation
³⁴⁴ because we were only able to test CpGs near a subset of genes in each comparison (number

³⁴⁵ of genes included in testing for baseline corticosterone = 4,143; stress-induced corticosterone
³⁴⁶ = 4,146; post-dexamethasone corticosterone = 3,863; within-year treatment = 2,913;
³⁴⁷ between-year treatment = 452).

³⁴⁸ Using DAVID we identified a set of GO terms associated with biological processes or
³⁴⁹ molecular functions that were over represented in the list of significant CpGs compared to
³⁵⁰ the background list for that comparison (Consortium, 2021). We filtered this list to include
³⁵¹ only GO terms with p-values < 0.05 after applying a false discovery rate correction. We
³⁵² initially visualized the GO terms for each comparison using REVIGO (Supek, Bošnjak,
³⁵³ Škunca, & Šmuc, 2011); however, our study identified a relatively small number of GO terms
³⁵⁴ and no clearly identifiable clusters of terms were identified in REVIGO. Therefore, we report
³⁵⁵ the complete list of genes and GO terms associated with CpGs in each comparison.

³⁵⁶ RESULTS

³⁵⁷ *Corticosterone and breeding timing*

³⁵⁸ Birds that were treated with corticosterone in year one had higher baseline corticosterone in
³⁵⁹ year two (corticosterone treatment $\beta = 3.34$; 95% confidence interval = 1.43 to 5.24; Figure
³⁶⁰ 2A; Table S2). Corticosterone treatment in year one was not related to stress-induced
³⁶¹ corticosterone in year two (Table S2), but females that were exposed to corticosterone in
³⁶² year one had lower post-dexamethasone corticosterone in year two, indicating more robust
³⁶³ negative feedback ($\beta = -4.86$; 95% confidence interval = -9.01 to -0.70; Figure 2B; Table S2).
³⁶⁴ Finally, corticosterone-exposed females initiated their nesting attempt earlier in the following
³⁶⁵ year ($\beta = -2.34$; 95% confidence interval = -4.49 to -0.20; Figure 2C; Table S2).

³⁶⁶ *Association between natural or experimental corticosterone and methylation*

³⁶⁷ Using pre-treatment samples, we found that methylation percentage at 116 CpGs out of
³⁶⁸ 78,143 tested was significantly associated with baseline corticosterone after FDR correction

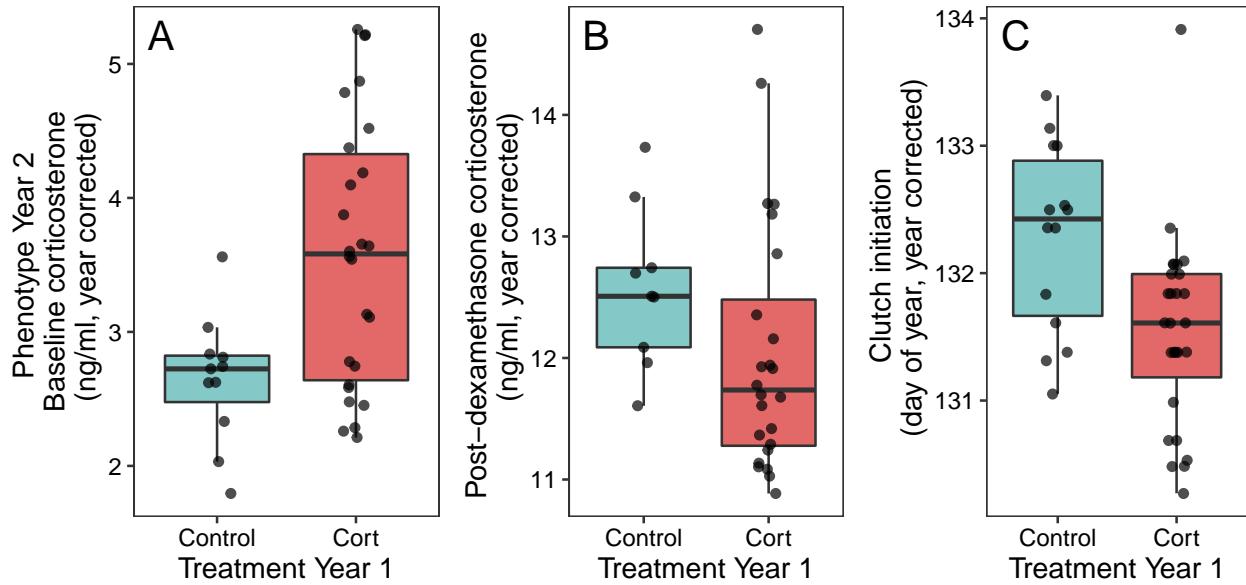


Figure 2: Effect of corticosterone treatment in one year on measures of baseline corticosterone (A), post-dexamethasone corticosterone (B), and clutch initiation date (C) in the following year. Points are partial residuals of raw data collected for average year effects. Boxes and whiskers show the median, interquartile range, and 1.5 times IQR.

369 (Figure 3A; Table S1). For stress-induced corticosterone, we found a similar significant
 370 association at 356 out of 78,027 CpGs that were tested (Figure 3B; Table S1). For
 371 post-dexamethasone injection samples, we found a significant association between
 372 corticosterone and methylation at 735 out of 69,189 CpGs tested (Figure 3C; Table S1).
 373 In models examining the effect of corticosterone treatment, we found that for samples
 374 collected within the same breeding season 1-2 weeks after treatment, 111 out of 48,070 CpGs
 375 tested showed significant evidence of differential methylation between treatment and control
 376 groups after FDR correction (Figure 3D; Table S1). We had fewer individuals and fewer
 377 CpGs that passed filtering for comparisons one year after treatment, but we found that 49
 378 out of 6,787 CpGs tested were significantly differentially methylated between treatment and
 379 control groups after one year (Figure 3E; Table S1). Although we were primarily interested
 380 in treatment effects, these models also showed that pre-treatment methylation at a given
 381 CpG site generally predicted post-treatment methylation both within a year (Figure S3A)
 382 and for samples collected one year later (Figure S3B).

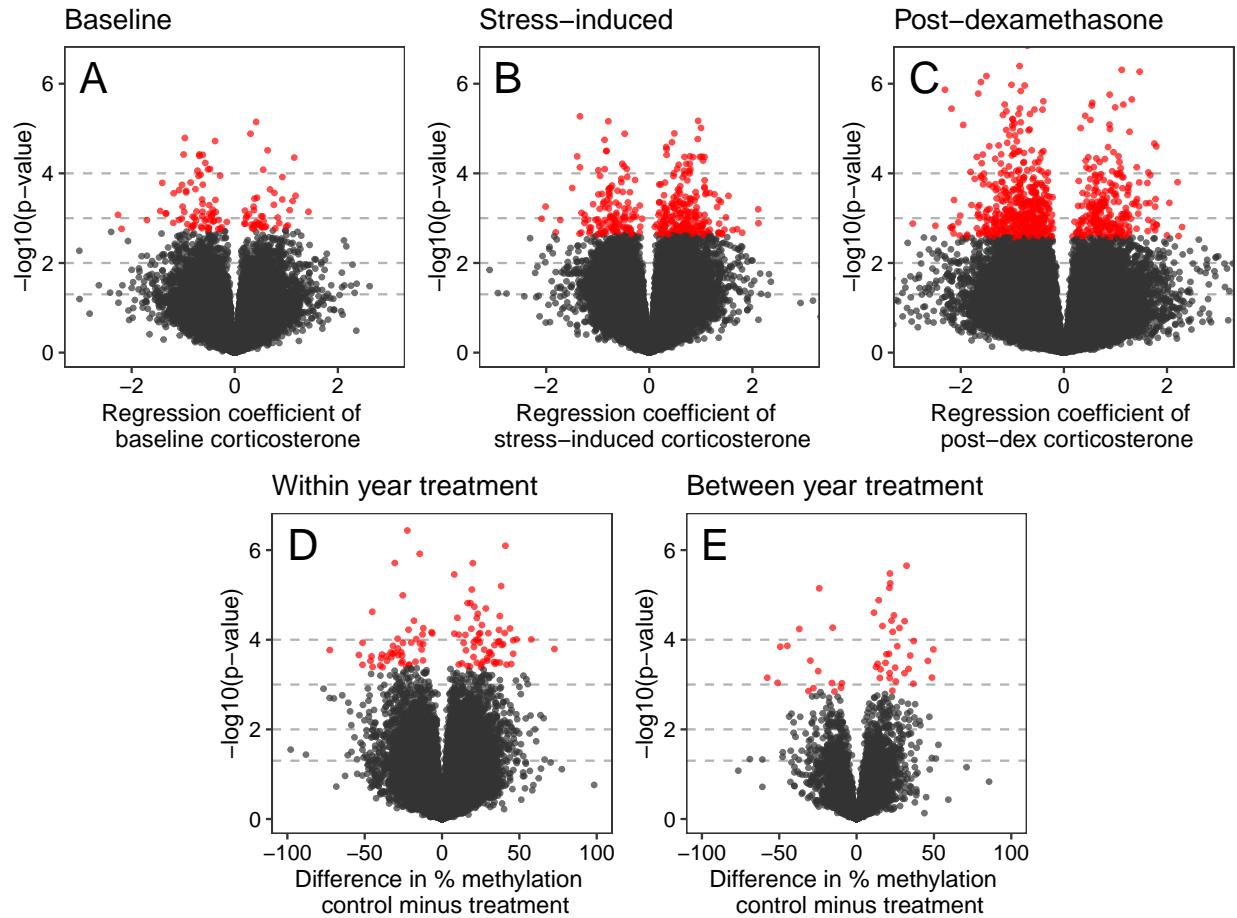


Figure 3: Association between DNA methylation and corticosterone from GLMMs based on observational and experimental study components. Panels A, B, and C show the pre-treatment regression coefficient for baseline corticosterone, stress-induced corticosterone, and post-dexamethasone corticosterone on methylation percentage, respectively. Panels D and E show the difference in methylation for control vs. treatment groups after accounting for pre-treatment methylation percentage for samples 1-2 weeks after treatment (D) and 1 year after treatment (E). In all plots, -log base 10 p-values are shown on the y axis with red points indicating CpGs that were significantly associated with corticosterone after applying the false discovery rate correction. Horizontal dashed lines indicate p-values of 0.05, 0.01, 0.001, and 0.0001 moving from the bottom to top of each plot to aid in interpretation.

³⁸³ Association between differentially methylated CpGs and genes

³⁸⁴ We found that CpGs that were significantly associated with baseline corticosterone,
³⁸⁵ stress-induced corticosterone, and post-dexamethasone corticosterone were located in or near
³⁸⁶ a total of 32, 176, and 236 identifiable genes, respectively (Table S3). When comparing
³⁸⁷ differentially methylated CpGs after treatment effects, within-year and between-year CpGs
³⁸⁸ were located in or near 52 and 16 genes, respectively (Table S3). A subset of these genes
³⁸⁹ were identified in two or three different comparisons (Figure 4). Because of our filtering
³⁹⁰ process many genes were not tested in each comparison (i.e., the background set of possible
³⁹¹ genes tested differed for each comparison).

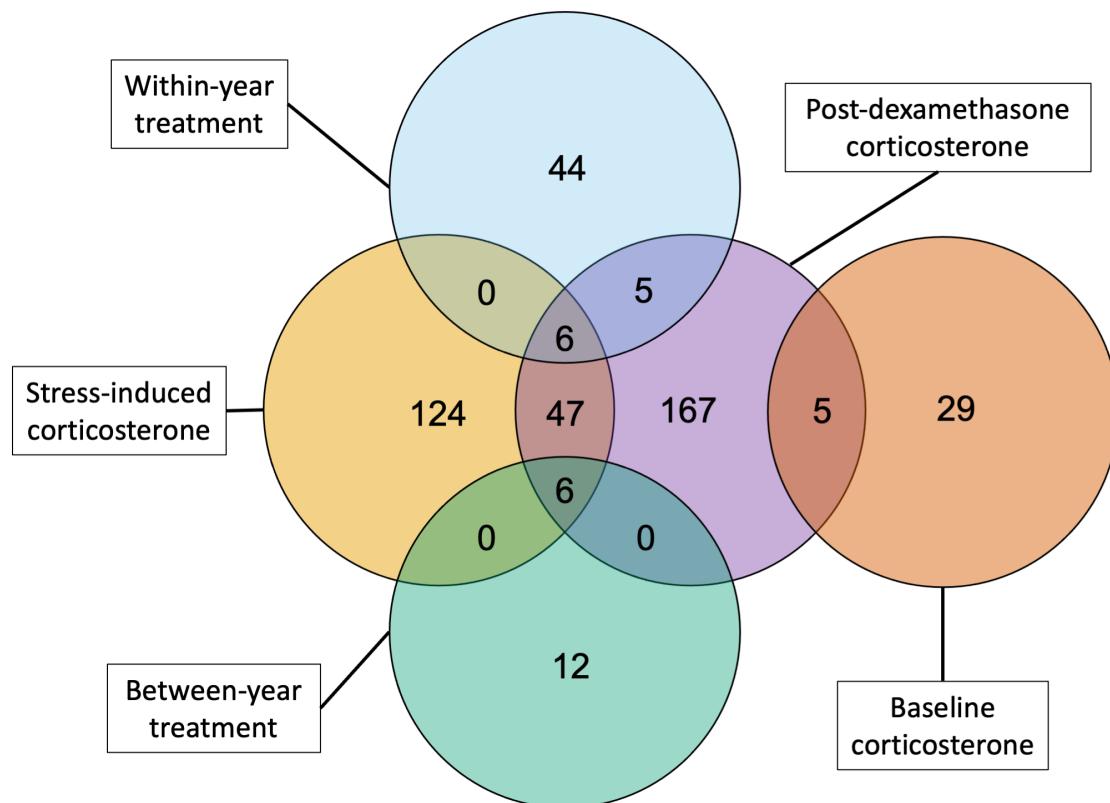


Figure 4: Number of genes near CpGs that were significantly associated with natural variation in corticosterone (baseline, stress-induced, and post-dexamethasone) or with experimental corticosterone elevation (within-year and between-year). No identified genes were shared between the comparisons with circles that do not overlap.

³⁹² In examining the function of genes identified in this process, we found no clear evidence for

393 direct links to genes known to be involved in regulation of the HPA axis. Stress-induced
394 corticosterone was linked to a single CpG associated with MC2R, which encodes the ACTH
395 receptor, but closer examination of the CpG in question suggested that this association
396 resulted from similar proteins included in our genome annotation and more likely gene was
397 MC3R, which is not directly involved in the HPA axis. Several genes known to be associated
398 with the HPA axis (e.g., CRH, CRHR1, FKBP5) did not have any CpGs near them in the
399 background set, so we could not test for differences associated with these genes.

400 *GO term analysis*

401 Using the gene lists from Table S3 as input, we identified GO terms that were significantly
402 associated with each comparison. With the false discovery rate set at 0.05, we identified 14
403 GO terms associated with baseline corticosterone, 22 terms associated with stress-induced
404 corticosterone, 10 terms associated with post-dexamethasone corticosterone, 2 terms for the
405 within-year treatment effect, and 27 terms for the between-year treatment effect (Table S4).
406 None of these lists resulted in any clear clustering of processes using the REVIGO
407 visualization tool and many terms were repetitive and attributable to the same few gene
408 associations.

409 Baseline corticosterone was associated with photoreceptor activity and response to light,
410 which was primarily driven by opsin and rhodopsin gene associations (OPN1SW, RHO,
411 LWS). Stress-induced corticosterone was associated with a wider range of processes connected
412 to a larger set of genes. These included a variety of cell signaling and receptor pathways
413 (e.g., MC2R, MC3R). Post-dexamethasone corticosterone was primarily associated with
414 signaling receptor activity driven by a relatively large number of associated genes (Table S4).

415 Differentially methylated CpGs for within-year corticosterone treatment were only related to
416 two GO terms associated with structural cell components and attributable to genes of
417 unknown function. Between-year corticosterone treatment was associated with a variety of
418 GO terms having to do primarily with transmembrane receptor signaling, but nearly all of

⁴¹⁹ these terms were selected from the same set of gene associations (BMPR1A and B, ACVR1,
⁴²⁰ and TGFBR1).

⁴²¹ **DISCUSSION**

⁴²² We found that experimental increases in corticosterone induced long-term phenotypic
⁴²³ changes. Females that experienced a few brief spikes in exogenous corticosterone had
⁴²⁴ stronger negative feedback in the HPA axis and bred earlier in the subsequent year; these
⁴²⁵ characteristics are typically associated with high stress resilience and reproductive success in
⁴²⁶ this population. Furthermore, natural variation in corticosterone was correlated with DNA
⁴²⁷ methylation, and experimental treatments altered DNA methylation patterns. Importantly,
⁴²⁸ regulation of DNA methylation in response to corticosterone occurred rapidly in adults
⁴²⁹ (within days) and resulted in detectable changes at least one year after treatment, paralleling
⁴³⁰ the changes in physiological and behavioral phenotypes. Taken together, these results
⁴³¹ support the idea that the activation of the stress response machinery changes traits
⁴³² associated with stress resilience, and thus may prime future responses to challenges. DNA
⁴³³ methylation could act as a key mechanism linking the prior experience of stressors—including
⁴³⁴ during adulthood—to subsequent coping ability. Rapid endocrine flexibility and adaptive
⁴³⁵ calibration of the stress response have emerged as key determinants of resilience to
⁴³⁶ challenges (Grindstaff, Beaty, Ambardar, & Luttbeg, 2022; Hau & Goymann, 2015; Taff &
⁴³⁷ Vitousek, 2016) and understanding the mechanistic basis of these patterns is an important
⁴³⁸ step in predicting when flexibility is sufficient for coping with changing conditions.

⁴³⁹ The changes in phenotype that we detected one year after experimentally elevating
⁴⁴⁰ corticosterone partially matched our predictions if exposure altered phenotype in ways that
⁴⁴¹ would increase future stress resilience. We found that, compared to controls, experimental
⁴⁴² females initiated breeding earlier and had stronger negative feedback in the subsequent year.
⁴⁴³ In tree swallows, clutch initiation date is a strong predictor of both seasonal and lifetime

444 reproductive success and is often considered a proxy for individual quality or condition
445 (Winkler et al., 2020). Similarly, the strength of negative feedback is consistently the best
446 physiological predictor of coping ability and reproductive success both under natural
447 conditions and after imposing experimental challenges (Taff et al., 2018; Zimmer et al., 2019).
448 However, contrary to our prediction, treatment had no effect on stress-induced corticosterone
449 the following year. We also found that compared to control females, corticosterone dosed
450 females had higher baseline corticosterone and no difference in stress-induced corticosterone
451 one year after treatment. Baseline corticosterone does not predict stress resilience in this
452 population (Zimmer et al., 2019). However, because baseline corticosterone often increases in
453 preparation for periods of high energetic demands, including the demands of reproduction
454 (the cort-adaptation hypothesis, Bonier, Moore, & Robertson, 2011; Casagrande et al., 2018;
455 Hau, Ricklefs, Wikelski, Lee, & Brawn, 2010), these results might reflect an increased
456 allocation to breeding in subsequent years in corticosterone treated females. For example,
457 female European starlings (*Sturnus vulgaris*) manipulated to increase parental investment
458 increased their baseline corticosterone during incubation (Love, Madliger, Bourgeon,
459 Semeniuk, & Williams, 2014). Similarly, tree swallows that increase baseline corticosterone
460 more over the reproductive period provision offspring at higher rates (Bonier et al., 2011).
461 Thus, our results might represent a combination of priming effects coupled with the
462 immediate energetic demands of breeding earlier. Alternatively, corticosterone treatment in
463 year one could have influenced measurements in year two indirectly, rather than by priming
464 responses to later challenges.

465 Our study also adds to the growing recognition of bidirectional links between coping ability
466 and DNA methylation. While this relationship has been demonstrated in laboratory-based
467 model systems (Liu et al., 1997; Weaver et al., 2004) the potential for environmental
468 stressors to trigger methylation, and affect subsequent coping ability, has only recently been
469 explored in wild animals. Early results in wild animals suggest patterns similar to those seen
470 in laboratory rodents. For example, early life maternal care and social connections in

⁴⁷¹ spotted hyenas (*Crocuta crocuta*) predict DNA methylation and glucocorticoid regulation as
⁴⁷² an adult (Laubach et al., 2019, 2021). Similar effects can play out in adulthood; for example,
⁴⁷³ in savannah baboons (*Papio cynocephalus*) high social status as an adult is associated with
⁴⁷⁴ more rapid changes in DNA methylation (epigenetic aging) as a consequence of the social
⁴⁷⁵ stress that accompanies high status (Anderson et al., 2021). Our results are consistent with
⁴⁷⁶ the results derived from lab rodents, wild mammals, and a few studies in wild birds (e.g.,
⁴⁷⁷ Rubenstein et al., 2016), suggesting that flexible adjustment of methylation may be a general
⁴⁷⁸ mechanism by which prior experiences of stressors are encoded in order to modulate future
⁴⁷⁹ responses to challenges.

⁴⁸⁰ While there has been a rapid increase in studies of methylation in wild birds in recent years
⁴⁸¹ Sheldon et al. (2018), relatively few studies have sampled the same adults multiple times.
⁴⁸² Our study design allowed us to assess the stability of genome-wide DNA methylation within
⁴⁸³ individuals. We found that many CpGs that we interrogated had large between individual
⁴⁸⁴ differences in methylation and that those differences were typically stable even in samples
⁴⁸⁵ collected one year apart. Compared to these individual differences, flexible changes in
⁴⁸⁶ methylation were relatively smaller and detectable at fewer CpGs. The stable individual
⁴⁸⁷ differences that we detected may be largely explained by genetic differences between
⁴⁸⁸ individuals (e.g., Sepers et al., 2023), but the consequences of early life conditions may also
⁴⁸⁹ play an important role in generating differences (Jimeno, Hau, Gómez-Díaz, & Verhulst,
⁴⁹⁰ 2019; Laubach et al., 2019; Sheldon et al., 2018). For example, early life climate conditions
⁴⁹¹ are related to lifelong methylation of the glucocorticoid receptor gene in superb starlings
⁴⁹² (*Lamprotornis superbus*, Rubenstein et al., 2016). We could not assess the possibility of a
⁴⁹³ similar pattern in our study because we did not have any information on early life conditions
⁴⁹⁴ for our birds. Regardless of the source of these initial differences, our results clearly
⁴⁹⁵ demonstrate that detecting subtle adjustments of methylation in adulthood to any treatment
⁴⁹⁶ of interest will often require accounting for pre-treatment methylation. Our results also
⁴⁹⁷ suggest that if changes in methylation are ecologically relevant for phenotypic flexibility

498 during adulthood, these effects are likely to occur through modest changes at specific
499 locations rather than through large scale modifications to methylation patterns.

500 At this point, any functional consequences of the specific methylation changes that we
501 detected are unclear. None of the genes or GO terms associated with natural variation in
502 corticosterone or with treatments had clear connections to HPA axis regulation. We
503 previously found that non-specific, genome-wide methylation predicts stress resilience to
504 experimental challenges in this population (Taff et al., 2019). Thus, the differences that we
505 detected might reflect large-scale regulation of methylation rather than targeted regulation of
506 sites with specific functional consequences. Alternatively, some of the changes that we
507 detected might have functional effects on stress response calibration that are not obvious
508 from the known effects of those genes. In support of this idea, we did find some overlap
509 between methylation in the genes associated with natural variation in corticosterone and
510 with the consequences of our experimental manipulation of corticosterone. In particular,
511 post-dexamethasone corticosterone, which is a strong predictor of stress resilience in tree
512 swallows (Taff et al., 2018; Zimmer et al., 2019), had the most extensive correlations between
513 methylation and identified genes and some of these genes were shared with the other
514 corticosterone measures and with treatment effects.

515 Another potential reason for our failure to find clear links between changes in DNA
516 methylation and genes associated with the stress response may result from limitations of our
517 approach. An advantage of RRBS is that it does not rely on pre-selecting candidate genes,
518 but a disadvantage is that not all relevant genes are necessarily tested. After filtering our
519 data, many of the genes with known roles in the HPA axis were not included in comparisons
520 or had coverage at only a few CpG sites. Thus we did not directly test for methylation
521 differences for many key genes. It is possible that deeper sequencing of our libraries would
522 have improved our ability to detect functional differences. Moreover, although we used the
523 most complete reference genome available for tree swallows, many CpGs mapped to

524 predicted genes whos function is unknown. Continued improvement of assembly and
525 annotation for reference genomes of non-model organisms is important for understanding the
526 functional importance of epigenetic changes. Studying DNA methylation in non-model
527 systems is a rapidly developing field and many recent papers outline the pros and cons of
528 various approaches (Beck, Ben Maamar, & Skinner, 2022; Laine et al., 2022; Sepers et al.,
529 2019). One particularly promising approach that may strike a balance between a focus on
530 candidate genes and the ability to detect genome-wide associations is to combine RRBS with
531 probes that enrich sequences at a potentially large number of target genes (target-enriched
532 enzymatic methyl sequencing, Rubenstein & Solomon, 2023).

533 Regardless of the functional consequences of the changes we detected, we found that brief
534 increases in corticosterone have effects on subsequent corticosterone regulation, breeding
535 decisions, and methylation a full year after dosing ended. At least some of the phenotypic
536 changes we detected support a hormone-mediated priming effect in which activation of the
537 stress response machinery improves the capacity to cope with future challenges, increasing
538 stress resilience. The fact that these changes in phenotype are coupled with changes in
539 methylation patterns implicates the regulation of DNA methylation as a potential
540 mechanism of flexibly adjusting the stress response system based on prior experiences,
541 although our study cannot determine whether changes in methylation directly cause adaptive
542 changes in the subsequent stress response and will need to be paired with analyses of gene
543 expression. Understanding the mechanisms that integrate experience with future stress
544 responsiveness has important consequences for predicting how and when individuals can cope
545 with repeated exposure to challenges. Conceptual models of the stress response suggest that
546 while repeated challenges can sometimes impose costs (McEwen & Wingfield, 2003; Romero
547 et al., 2009), calibration achieved through regular activation of the stress response may prime
548 more effective responses to future challenges (Del Giudice, Ellis, & Shirtcliff, 2011; Hilker et
549 al., 2016). Studying the mechanisms by which stress exposure is encoded biologically will
550 help to differentiate these possibilities and shed light on when and how individuals succeed

551 or fail through flexible regulation of the physiological response to challenges.

552 *Data and code availability*

553 The complete set of bioinformatic processing scripts, R code, and data associated with each
554 sample is available and permanently archived on Zenodo
555 (<https://doi.org/10.5281/zenodo.8125153>). Raw sequence data from RRBS is available on
556 GenBank (BioProject ID PRJNA953597).

557 **ACKNOWLEDGMENTS**

558 We thank the members of the tree swallow research team for assistance with fieldwork
559 including, Joseph Byington, Joe Colcombe, Collin Dickerson, Garret Levesque, Jacob
560 Kolenda, Lyra Liu, Sophie Nicolich-Henkin, Teresa Pegan, Avram Pinals, Alyssa Rodriguez,
561 Vanesa Rodriguez-Arcila, Tom Ryan, David Scheck, Lauren Smith, Jocelyn Stedman, and
562 Cedric Zimmer. Bronwyn Butcher and the Lovette Lab provided input on lab methods and
563 members of the Vitousek Lab provided feedback on earlier versions of the manuscript.
564 Charlotte Holden produced the tree swallow illustrations.

565 **AUTHOR CONTRIBUTIONS**

566 CCT and MNV conducted the field based data collection. CCT and MNV conceived the
567 study. CCT and SMM conducted the lab work for RRBS. CCT and LC conducted the lab
568 work for creating the reference genome and LC carried out the bioinformatics for genome
569 assembly and annotation. CCT analyzed and visualized the data with assistance from SMM
570 and LC. CCT drafted the paper with input from all authors.

571 **ETHICAL NOTE**

572 All methods were approved by Cornell IACUC and sampling was conducted with
573 appropriate state and federal permits.

574 **FUNDING**

575 Research was supported by DARPA D17AP00033 and NSF-IOS grants 1457251 and 2128337.
576 The views, opinions and/or findings expressed are those of the authors and should not be
577 interpreted as representing the official views or policies of the Department of Defense or the
578 U.S. Government. CCT was supported by DARPA D17AP00033 and by the Rose
579 Postdoctoral Program at the Cornell Lab of Ornithology and SMM was supported by the
580 Rose Postdoctoral program at the Cornell Lab of Ornithology.

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777 SUPPLEMENTARY FIGURES AND TABLES

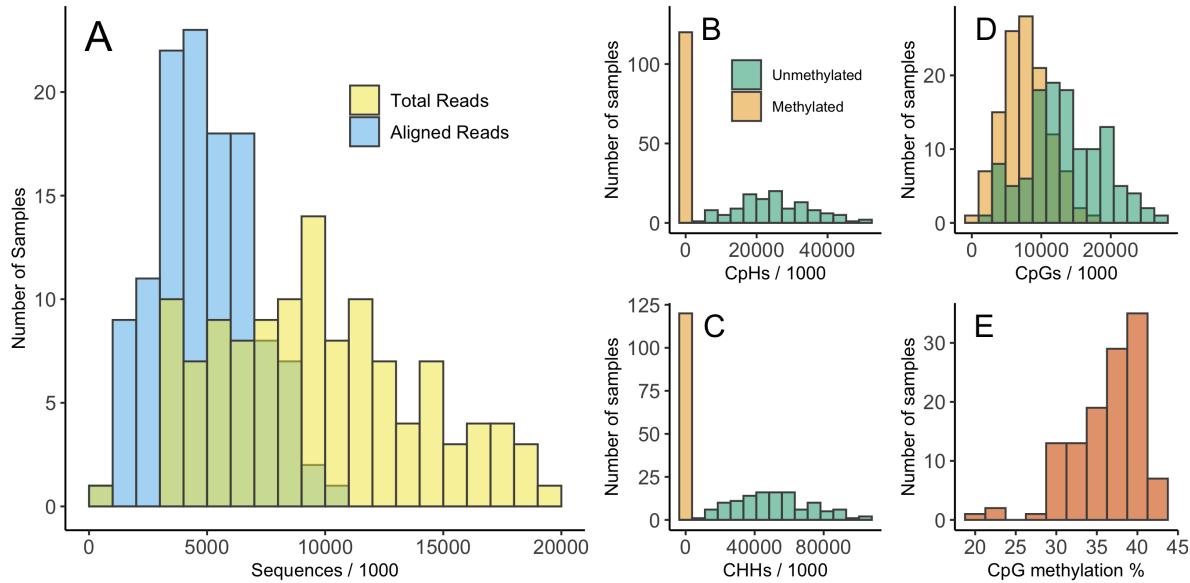


Figure S1: Summary of sequencing and methylation call results from raw sequence data. Panel A shows the distribution of the total number of sequences for each sample and number of sequences that aligned to the tree swallow genome. Panel B shows the number of CpH sites that were methylated or unmethylated for each sample. Panel C shows the number of CHH sites that were methylated or unmethylated for each sample. Panel D shows the number of CpG sites that were methylated or unmethylated for each sample. Panel E shows the percentage of total CpG reads that were methylated by sample. Note that these histograms are based on raw sequencing results that do not account for differential coverage between samples or locations in the genome and are included for illustration only.

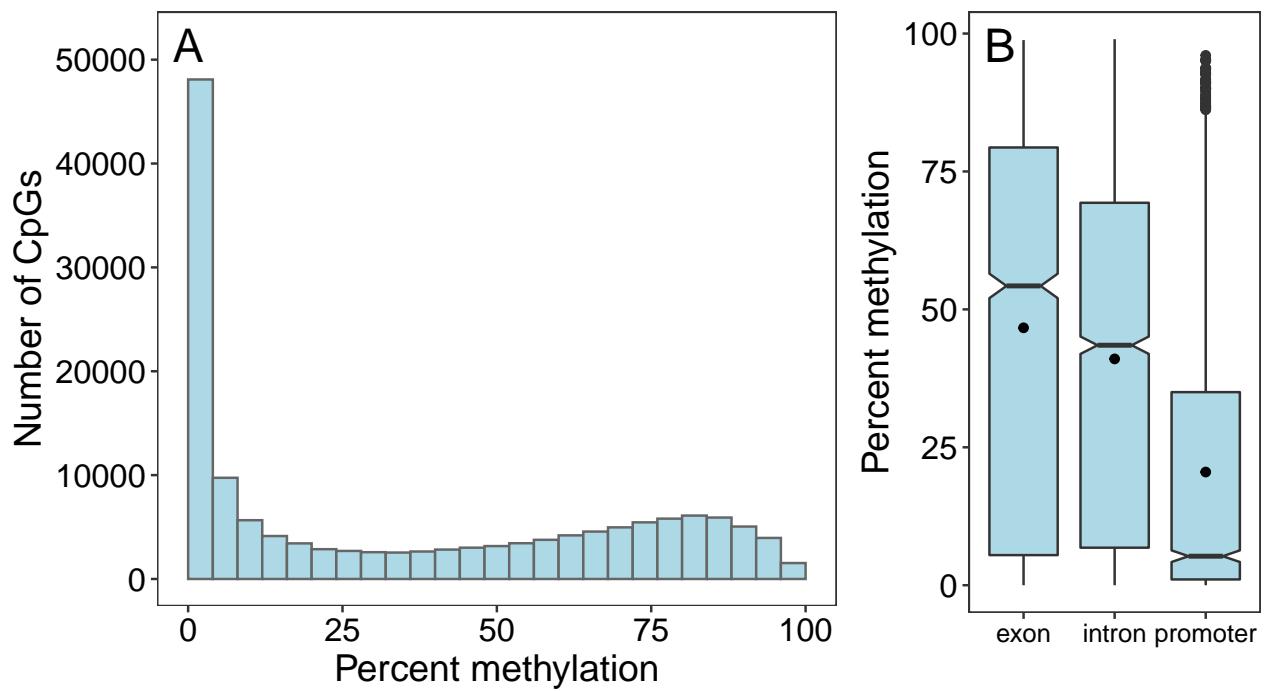


Figure S2: Distribution of methylation percentage across all CpGs and for different genomic features. Panel A shows entire distribution of methylation percentage for all 148,167 CpGs from pre-treatment samples before any filtering. Panel B shows the methylation percentage for exons, introns, and promoters that had CpGs identified within them. Horizontal lines, boxes, and whiskers show the median, interquartile range, and 1.5 times IQR respectively. The black circle within each box is the mean for that feature.

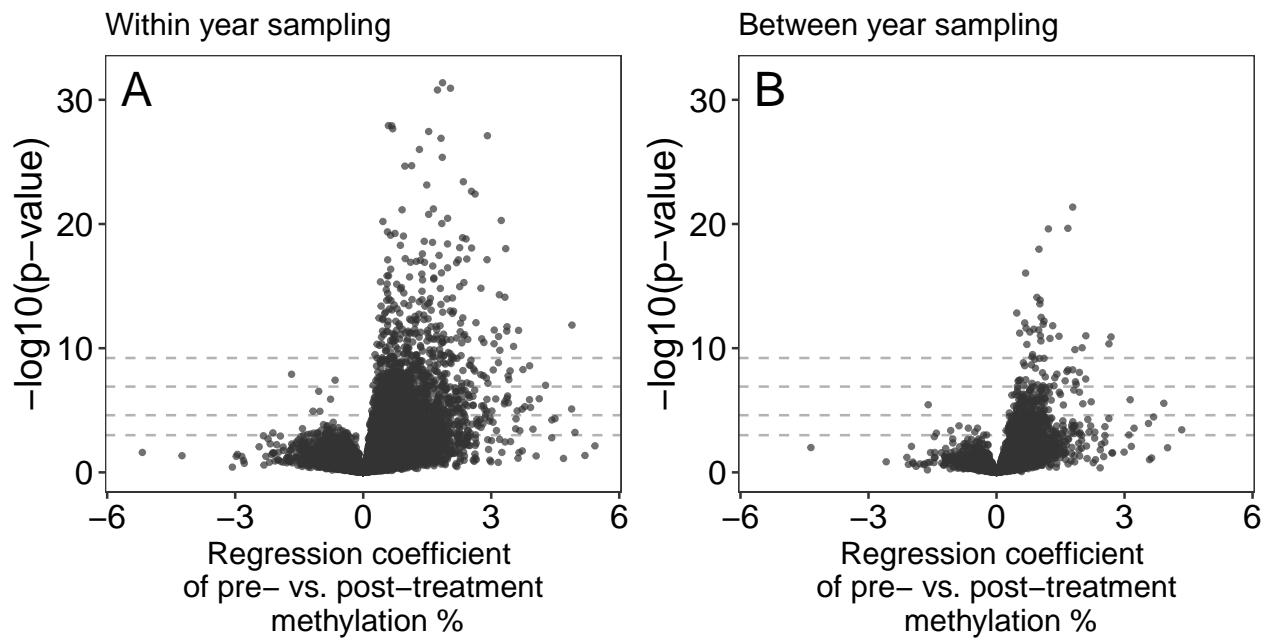


Figure S3: Association between pre-treatment methylation percentage and post-treatment methylation at each CpG for samples collected within a breeding season (panel A) and for samples collected one year apart (panel B). To help with interpretation, horizontal dashed lines indicate p-values of 0.05, 0.01, 0.001, and 0.0001 moving from the bottom to top of the plots.

Table S1: Summary of GLMMs for each comparison with the number of CpGs significantly correlated with corticosterone or differentially methylated between treatment groups. One model was fit for each CpG site; see text for description of models.

Comparison	CpGs evaluated	Individuals per comparison	Significant CpGs	Associated Genes	Significant GO terms
Baseline corticosterone	78143	29.2 +/- 7.0	116	32	14
Stress-induced corticosterone	78027	30.6 +/- 7.3	356	176	22
Post-dexamethasone corticosterone	69189	14.2 +/- 3.0	735	236	10
Within year treatment	48070	24.5 +/- 8.8	111	52	2
Between year treatment	6787	9.8 +/- 1.5	49	16	27

Table S2: Results of GLMs for corticosterone and clutch initiation date in the year after experimental treatments were applied.

Predictor	Estimate	CI	P
Baseline corticosterone (n = 37)			
Intercept	2.18	0.25 - 4.12	0.028
Treatment (corticosterone)	3.34	1.43 - 5.24	0.001
Year (2015)	-2.69	-4.78 - -0.60	0.013
Year (2016)	-1.96	-0.51 - 4.43	0.116
Stress-induced corticosterone (n = 37)			
Intercept	26.04	10.36 - 41.71	0.002
Treatment (corticosterone)	7.21	-9.61 - 24.03	0.389
Year (2015)	1.98	-15.26 - 19.23	0.816
Year (2016)	0.27	-19.04 - 19.58	0.977
Baseline corticosterone	-0.88	-3.54 - 1.78	0.504
Post-dexamethasone corticosterone (n = 33)			
Intercept	21.56	16.21 - 26.91	<0.001
Treatment (corticosterone)	-4.86	-9.01 - -0.70	0.024
Year (2015)	-9.29	-14.76 - -3.81	0.002
Year (2016)	-12.18	-18.15 - -6.21	<0.001
Stress-induced corticosterone	0.10	0.00 - 0.20	0.047
Clutch initiation date (n = 41)			
Intercept	132.08	129.81 - 134.35	<0.001
Treatment (corticosterone)	-2.34	-4.49 - -0.20	0.033
Year (2015)	0.40	-2.00 - 2.81	0.735
Year (2016)	2.95	0.31 - 5.60	0.029

Table S3: List of genes with differentially methylated CpGs in exons, introns, or within 2kb upstream of the gene.

Comparison	Genes
Baseline corticosterone	ABHD8; CBX2; CCDC15; CLP1; HELZ; HMGA1; LIG3; LOC100220428; LOC100221958; LOC100223410; LOC100228079; LOC751972; LWS; MAST3; NFASC; NRCAM; OPN1SW; PCBP2; PCBP3; PCBP4; PCMT1; PUS3; RHO; SESN1; SESN3; STAMBPL1; STMN1; STMN2; STMN3; TAF6L; TMIE; ZFYVE19

Stress-induced corticosterone ACVR1; ACVR1B; ACVRL1; ADGRB1; ADGRB2; ADGRB3; AKT1; AKT3; ALPK1; ALX1; ARHGEF11; ARID3A; ARID3B; ARID3C; ASB3; AXIN1; AXIN2; BMPR1A; BMPR1B; CLDN1; CLDN14; CLDN5; COPA; DCLK1; DCX; ESRRG; FOSL2; FOXJ1; GALM; GATA4; GATA5; GATA6; GPR171; HID1; HNF4A; HNF4G; HOXA3; HOXB3; HSPA13; HSPA14; HSPA2; HSPA5; HSPA8; HSPA9; IER5L; IGHMBP2; IGSF9B; IKZF1; IKZF2; IKZF3; ITGA11; KIF1A; KIF1B; KLHL12; KLHL17; KLHL18; KLHL2; KLHL20; KLHL5; LAMB1; LAMB2; LAMB3; LDLRAP1; LECT2; LOC100217876; LOC100219031; LOC100220115; LOC100221543; LOC100222291; LOC100222941; LOC100223643; LOC100224585; LOC100224644; LOC100224843; LOC100225871; LOC100226582; LOC100226815; LOC100227201; LOC100227468; LOC100227703; LOC100229020; LOC100229354; LOC100229528; LOC100229609; LOC100229630; LOC100230278; LOC100230328; LOC100230362; LOC100231693; LOC100231893; LOC100232259; LOC101233849; LOC105758838; LOC105759110; LOC105759301; LOC105759399; LOC105759510; LOC105759937; LOC115491093; LOC115491353; LOC115491354; LOC115491358; LOC115491417; LOC115491512; LOC115494566; LOC115494567; LOC115496063; LOC115496268; LOC115497065; LOC115497066; LOC115497067; LOC115497068; LOC115497095; LOC115497281; LOC115498367; LOC116807418; LOC116807419; LOC121468018; MAPK1IP1L; MC1R; MC2R; MC3R; MC4R; MC5R; MEX3A; MEX3B; MEX3D; MITF; MNX1; MOB1B; MOB3C; MYO1D; NCOA5; NCOR2; NKX3-2; NR2E1; NR2E3; NR2F1; NR2F2; NR2F6; NR5A2; P2RY12; P2RY13; P2RY14; PCDH10; PCDH12; PCDH8; PIM1; PIM3; PKNOX1; PKNOX2; PLEKHA6; PLEKHO1; PTCH1; PTK7; RAX; RAX2; RXRA; SGK1; SGK2; SIX6; SLC25A10; SLC66A1; SRSF3; SYNPO⁴¹; TFEB; TFEC; TGFBR1; TIMM8A; TNRC18; UBALD1; UBALD2; UBXN7; WDR7; ZCCHC14; ZDHHC23

Post-dexamethasone corticosterone ACVR1; ACVR1B; ACVRL1; ADORA1; ADORA2A; ADORA2B; ADRA1B; AK8; ATOH7; BMPR1A; BMPR1B; C18H17orf58; CBX2; CCM2; CCNA1; CHGB; CHL1; CHST11; CHST12; CHST13; CLIP1; COPA; CORO2A; CORO2B; CRTC2; CRYBB1; CTRL; CTXN1; CTXN2; DENND4C; DNAJB13; DNMT3B; DOCK7; DOCK8; EMX2; ERICH3; ESRRG; EVA1B; FAM83A; FGF18; FGF8; FZD1; FZD10; FZD2; FZD3; FZD4; FZD5; FZD6; FZD7; FZD8; GAD1; GAD2; GATA2; GATA3; GNPTG; GPR171; GPR26; GPR78; GPR83; GRB2; HID1; HNF4A; HNF4G; ID=cds-NP_001232480.1; ID=cds-NP_001232635.1; ID=cds-NP_001243137.1; ID=cds-NP_001243138.1; ID=cds-XP_030112754.3; ID=cds-XP_030134736.2; ID=cds-XP_032603585.2; IGHMBP2; IKZF1; IKZF2; IKZF3; ILF3; IP6K1; IP6K2; IP6K3; ITGA11; KATNAL2; KCNA10; KCNA2; KCNA4; KCNA5; KDM5A; KHDRBS1; LOC100190025; LOC100218025; LOC100219450; LOC100220115; LOC100221543; LOC100221838; LOC100221958; LOC100223460; LOC100224073; LOC100225784; LOC100225871; LOC100226496; LOC100226582; LOC100226723; LOC100227201; LOC100227464; LOC100227703; LOC100229528; LOC100229609; LOC100229630; LOC100229947; LOC100230362; LOC100231785; LOC100232259; LOC101232904; LOC101232979; LOC101233511; LOC105758604; LOC105758698; LOC105758845; LOC105758859; LOC105759399; LOC105759510; LOC115490659; LOC115492615; LOC115494788; LOC115496018; LOC115496268; LOC115496876; LOC115497044; LOC115497045; LOC115497065; LOC115497066; LOC115497067; LOC115497068; LOC115497095; LOC115498277; LOC115498450; LOC115498504; LOC115498524; LOC116806919; LOC116807124; LOC116807173; LOC116807372; LOC116807374; LOC116807475; LOC116807566; LOC116807567; LOC116807569; LOC116807570; LOC116807571; LOC116807574; LOC116807590;

Treatment within year	ACTA1; ACTA2; ACTB; ACTC1; ACTG1; ACTG2; ACTR1A; ASS1; ESPN; GALNT3; GALNT6; GMPS; GPR27; GPR85; INHBB; IRF7; ITGA11; KCNG1; KCNG2; KCNG4; LOC100190135; LOC100218246; LOC100218887; LOC100220115; LOC100220137; LOC100227878; LOC105758845; LOC105758846; LOC105758847; LOC105758850; LOC105758859; LOC105760754; LOC115494114; LOC115496608; LOC115497065; LOC115497066; LOC115497067; LOC115497068; LOC115497975; LOC115498396; LOC115498513; LOC115498523; LOC116809297; NTN1; NTN3; NUAK1; NUAK2; PCDH15; RIMS3; TMPRSS6; VDAC1; VDAC2
Treatment between years	ACVR1B; ACVR1; ACVRL1; BMP1; BMPR1A; BMPR1B; LOC115495659; LOC115495665; LOC115495661; LOC115490544; MMADHC; NETO1; TBKBP1; TGFBR1; TLL2; TLL1

Table S4: GO terms identified using DAVID after correction to a false discovery rate of 0.05 for each comparison set.

GO Term	FDR	Function
Baseline corticosterone		
GO:0009881	1.23e-04	photoreceptor activity
GO:0016037	4.43e-04	light absorption
GO:0018298	4.43e-04	protein-chromophore linkage
GO:0009583	4.43e-04	detection of light stimulus
GO:0007602	4.43e-04	phototransduction
GO:0009582	9.60e-03	detection of abiotic stimulus
GO:0009581	9.60e-03	detection of external stimulus
GO:0016038	9.60e-03	absorption of visible light
GO:0031110	2.20e-03	regulation of microtubule polymerization or depolymerization
GO:0051606	9.60e-03	detection of stimulus
GO:0007601	3.88e-03	visual perception
GO:0009605	1.88e-02	response to external stimulus
GO:0050953	1.98e-02	sensory perception of light stimulus
GO:0009416	4.79e-02	response to light stimulus
Stress-induced corticosterone		
GO:0004675	4.13e-04	transmembrane receptor protein serine/threonine kinase activity
GO:0097159	5.86e-05	organic cyclic compound binding
GO:1901363	6.08e-05	heterocyclic compound binding
GO:0043565	6.12e-04	sequence-specific DNA binding
GO:0003700	5.06e-04	transcription factor activity, sequence-specific DNA binding
GO:0001071	9.66e-04	nucleic acid binding transcription factor activity
GO:0008270	4.78e-03	zinc ion binding

Table S4: GO terms identified using DAVID after correction to a false discovery rate of 0.05 for each comparison set. (*continued*)

GO Term	FDR	Function
GO:0038023	9.15e-04	signaling receptor activity
GO:0003676	6.34e-03	nucleic acid binding
GO:0004977	5.48e-03	melanocortin receptor activity
GO:0004674	5.48e-03	protein serine/threonine kinase activity
GO:0004871	1.60e-03	signal transducer activity
GO:0003677	3.92e-02	DNA binding
GO:0005524	1.14e-02	ATP binding
GO:0004872	3.85e-03	receptor activity
GO:0032559	1.21e-02	adenyl ribonucleotide binding
GO:0030554	1.21e-02	adenyl nucleotide binding
GO:0060089	3.58e-03	molecular transducer activity
GO:0046914	2.43e-02	transition metal ion binding
GO:0004888	4.35e-02	transmembrane signaling receptor activity
GO:0004930	4.62e-02	G-protein coupled receptor activity
GO:0031625	3.98e-02	ubiquitin protein ligase binding

Post-dexamethasone corticosterone

GO:0038023	2.65e-03	signaling receptor activity
GO:0004871	4.04e-03	signal transducer activity
GO:0042813	4.62e-03	Wnt-activated receptor activity
GO:0004888	5.10e-03	transmembrane signaling receptor activity
GO:0035586	5.10e-03	purinergic receptor activity
GO:0060089	5.10e-03	molecular transducer activity
GO:0004872	5.10e-03	receptor activity
GO:0004675	8.21e-03	transmembrane receptor protein serine/threonine kinase activity

Table S4: GO terms identified using DAVID after correction to a false discovery rate of 0.05 for each comparison set. (*continued*)

GO Term	FDR	Function
GO:0099600	9.77e-03	transmembrane receptor activity
GO:0017147	2.78e-02	Wnt-protein binding
Within-year treatment		
GO:0005200	1.06e-03	structural constituent of cytoskeleton
GO:0005198	1.89e-02	structural molecule activity
Between-year treatment		
GO:0004675	1.05e-06	transmembrane receptor protein serine/threonine kinase activity
GO:0004674	3.87e-05	protein serine/threonine kinase activity
GO:0019199	1.62e-04	transmembrane receptor protein kinase activity
GO:0004672	7.78e-04	protein kinase activity
GO:0016772	1.12e-03	transferase activity, transferring phosphorus-containing groups
GO:0016773	1.12e-03	phosphotransferase activity, alcohol group as acceptor
GO:0016301	1.12e-03	kinase activity
GO:0016740	3.56e-03	transferase activity
GO:0004888	3.56e-03	transmembrane signaling receptor activity
GO:0099600	3.56e-03	transmembrane receptor activity
GO:0004871	2.65e-03	signal transducer activity
GO:0060089	2.65e-03	molecular transducer activity
GO:0038023	3.56e-03	signaling receptor activity
GO:0004872	3.56e-03	receptor activity
GO:0032559	4.35e-03	adenyl ribonucleotide binding
GO:0030554	4.35e-03	adenyl nucleotide binding
GO:0005524	4.35e-03	ATP binding

Table S4: GO terms identified using DAVID after correction to a false discovery rate of 0.05 for each comparison set. (*continued*)

GO Term	FDR	Function
GO:0003824	3.35e-03	catalytic activity
GO:0035639	1.81e-02	purine ribonucleoside triphosphate binding
GO:0032555	1.81e-02	purine ribonucleotide binding
GO:0017076	1.81e-02	purine nucleotide binding
GO:0032553	1.86e-02	ribonucleotide binding
GO:1901265	1.86e-02	nucleoside phosphate binding
GO:0000166	1.86e-02	nucleotide binding
GO:0043167	2.60e-02	ion binding
GO:0097367	2.80e-02	carbohydrate derivative binding
GO:0036094	2.80e-02	small molecule binding

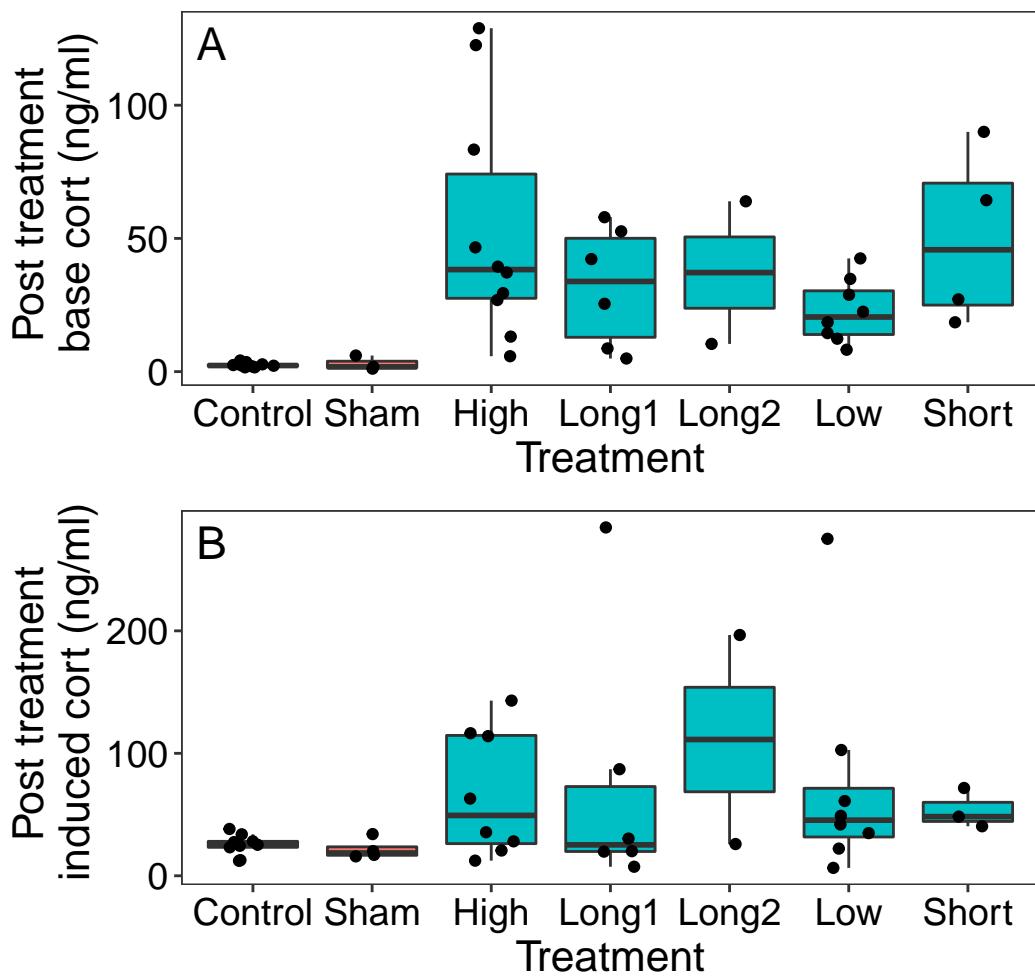


Figure S4: Effect of treatment variations on (A) baseline corticosterone and (B) stress-induced corticosterone 1-2 weeks after treatments ended. Each application of corticosterone results in a single spike in circulating levels that lasts a few hours. Repeated treatment during incubation results in a change in endogenous regulation of corticosterone levels. Individuals differ in the magnitude of this shift, but all corticosterone exposed treatments are categorically different than control treatments. For this study, corticosterone exposed and control treatments were grouped to simplify comparisons. See text for description of each treatment variation.

Table S5: Sample sizes for the comparisons in main analyses. See text for description of each treatment variation.

Treatment Category	Sample Type	Within-Year n	Between-Year n
Between-year phenotypic effects of corticosterone dosing			
Control	Sham	NA	4
Control	Full Control	NA	11
Corticosterone Exposed	High	NA	10
Corticosterone Exposed	Long1	NA	6
Corticosterone Exposed	Long2	NA	2
Corticosterone Exposed	Low	NA	8
Corticosterone Exposed	Short	NA	4
Correlation between methylation and natural corticosterone			
None	No Manipulation	45	NA
Effects of corticosterone dosing on methylation			
Control	Sham	10	3
Control	Full Control	10	5
Corticosterone Exposed	High	9	8
Corticosterone Exposed	Long1	6	4
Corticosterone Exposed	Low	10	7