

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

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10 ABSTRACT

When facing challenges, vertebrates activate an evolutionarily conserved hormonal stress response that can dramatically alter behavior and physiology. Although this response can be costly, conceptual models suggest that it can also recalibrate the stress response system, priming more effective responses to future challenges. Little is known about whether this process occurs in wild animals, particularly in adulthood, and if so, how information about prior experience with stressors is encoded. One potential mechanism is hormonally-mediated changes to 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 stress-induced corticosterone
24 predicted patterns of DNA methylation, including methylation of the MC2R gene, which
25 encodes the adrenocorticotropic hormone receptor. Finally, corticosterone treatment causally
26 influenced methylation on short (1-2 weeks) and long (1 year) time scales; however, many of
27 these changes did not have clear links to functional regulation of the stress response. Taken
28 together, our results are consistent with corticosterone-induced priming of future stress
29 resilience, and support DNA methylation as a potential mechanism. Uncovering the
30 mechanisms linking experience with the response to future challenges has implications for
31 understanding the drivers of stress resilience.

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

34 SIGNIFICANCE

35 A stress response to an environmental challenge can preserve an individual's fitness and may
36 even prime them to survive similar challenges in the future. What mechanisms underlie
37 priming is unclear, but epigenetic alterations to stress-related genes are one possibility. We
38 experimentally tested whether increasing corticosterone in free-living swallows had lasting
39 phenotypic or epigenetic effects. A year after treatment, females who received corticosterone
40 had altered stress physiology and bred earlier than control birds, traits that are associated
41 with higher fitness. Treatment also altered DNA methylation and methylation of the MC2R
42 gene was correlated with stress physiology. This study adds to a growing body of literature

43 suggesting that epigenetic changes are key to animals' response to a changing environment.

44 INTRODUCTION

45 Wild organisms regularly encounter challenging conditions that require rapid behavioral and
46 physiological responses. In vertebrates, the glucocorticoid mediated stress response plays an
47 essential role in allowing animals to successfully avoid or tolerate stressors (1, 2). While an
48 appropriate response is beneficial (2), an inappropriate or prolonged elevation of
49 glucocorticoids can result in a variety of negative consequences for health and fitness (3).

50 Accordingly, the dominant paradigm in behavioral ecology and endocrinology is that the
51 immediate benefits of the stress response are balanced by long-term costs.

52 But are the long-term effects of a physiological stress response always costly? Some
53 conceptual models propose that activating the stress response system—even in
54 adulthood—primes more effective responses to future challenges (4, 5). These models predict
55 that initiating a response calibrates the stress response system, increasing organismal
56 resilience or robustness. Because physiological priming results from activating the stress
57 response system rather than from learning, it could occur even in the absence of exposure to
58 an identifiable external threat. Physiological studies have provided some evidence that
59 stressor priming can occur, including outside of critical developmental periods (6, 7).

60 However, we know little about the degree to which stressor priming operates in natural
61 populations, affecting later life behavior, physiology, and fitness. Similarly, the mechanism(s)
62 that link activation of the stress response to physiological regulation of subsequent responses
63 are not well understood.

64 One mechanism that could play a role in the calibration of stress response systems is changes
65 to DNA methylation. Epigenetic modification by DNA methylation can alter phenotypes by
66 making genes or promoters more or less accessible for transcription (8–10). Early life
67 experiences can have profound programming effects on DNA methylation patterns that often

68 persist throughout the individuals' lifetime (11). For example, classic work in lab rodents
69 demonstrates that early life experiences regulate methylation of the gene producing the
70 glucocorticoid receptor, which results in lifelong changes to glucocorticoid secretion in
71 response to challenges (12, 13). A growing number of studies in wild animals also
72 demonstrate early life programming of DNA methylation patterns in wild populations
73 resulting from dominance hierarchies (14), brood size (15, 16), temperature and weather (17,
74 or landscape features (19).

75 While they are less well documented, experiences during adulthood can also result in changes
76 to DNA methylation and these adjustments can occur rapidly (17, 20). For example, brief
77 periods of experimental competition and aggression in tree swallows (*Tachycineta bicolor*)
78 resulted in altered DNA methylation of brain regions associated with hormone signaling,
79 suggesting a priming effect in preparation for future aggression (20). Conceptual models of
80 the stress response have long recognized that the sequence, frequency, duration, and intensity
81 of stressors should change the optimal behavioral and physiological response (21, 22). Yet it
82 is often unclear how the experience of challenges during adulthood would be biologically
83 encoded to alter responses to future challenges. Altered DNA methylation is a promising
84 mechanism because i) it can change rapidly even during adulthood, ii) it can persist over
85 moderate to long time scales, iii) it has been shown to change with challenging experiences,
86 and iv) it can directly alter an individual's phenotype.

87 We experimentally simulated a series of acute corticosterone spikes using a non-invasive
88 dosing procedure (23) and monitored both long-term phenotypic effects and changes to DNA
89 methylation. In this population we previously found that brief increases in corticosterone
90 have lingering effects on behavior and performance within a breeding season (23, 24) and
91 that genome-wide methylation predicts resilience to experimental challenges (25). Here, we
92 extended those results to ask whether brief increases in corticosterone altered regulation of
93 the stress response and breeding decisions a full year later. We coupled this approach with

94 reduced representation bisulfite sequencing (RRBS) and a newly improved reference genome
95 assembled for this study to examine genome-wide patterns of DNA methylation at high
96 resolution. We used RRBS to first assess covariation between methylation and natural
97 variation in corticosterone regulation during an acute stress response. Next, we compared
98 DNA methylation in corticosterone treated females to controls to determine whether brief
99 increases in corticosterone resulted in altered DNA methylation at either short (1-2 weeks) or
100 long (1 year) timescales.

101 If activation of the stress response machinery primes future coping ability (4, 5),
102 experimentally treated females should exhibit long-term (across-year) differences in key
103 phenotypic traits. Specifically, we predicted that in the year after treatment females would
104 have a robust stress-induced increase in corticosterone and strong negative feedback, traits
105 that have been previously shown to predict stress resilience in this population (26), and
106 initiate breeding earlier, which is associated with higher reproductive performance in tree
107 swallows (27). Given previous work demonstrating a correlation between coping ability and
108 both genome-wide methylation (25) and natural variation in rapid corticosterone regulation
109 (28), we also predicted that natural variation in corticosterone (baseline, stress-induced
110 increase, and efficacy of negative feedback) would be associated with DNA methylation.
111 However, a correlation here could arise through early life programming, prior activation of
112 the acute corticosterone response, or any conditions that impact the regulation of both
113 methylation and corticosterone (e.g., body condition). In contrast, for the experimental
114 manipulation we predicted that differences in DNA methylation between control and
115 treatment groups would only be present if brief increases in corticosterone have a causal
116 effect on altering methylation patterns. We assessed the time course and persistence of any
117 such changes using comparisons 1-2 weeks after treatments and 1 year after treatments. If
118 methylation changes play a role in altering future corticosterone secretion then we expected
119 to find more differences near genes and promoters associated with endocrine regulation.

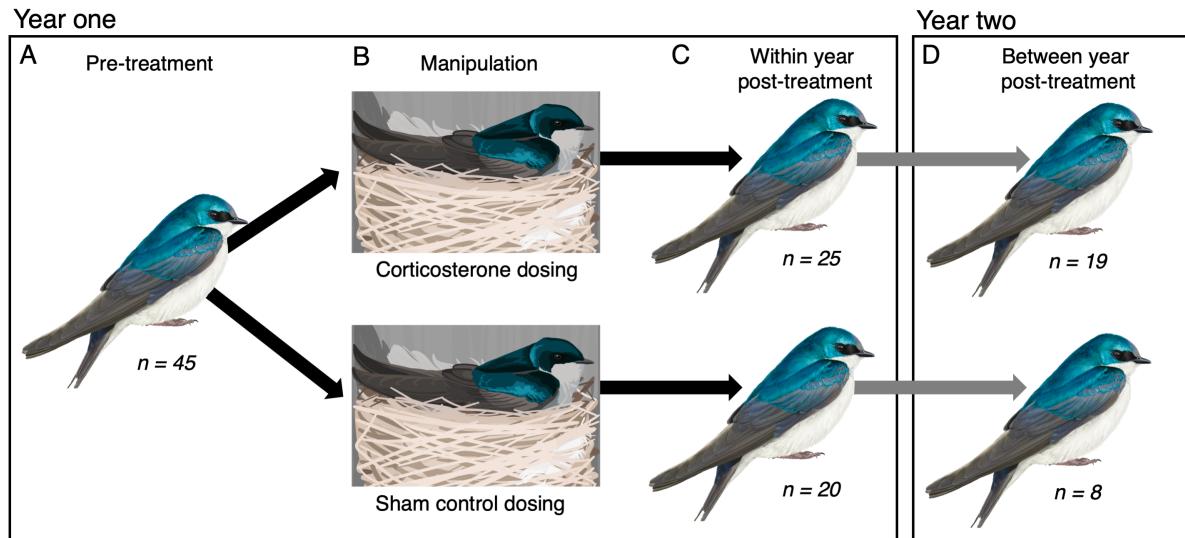


Figure 1. Schematic illustration of the experimental treatment and samples collected. Models comparing natural corticosterone to DNA methylation used pre-treatment samples (A). After treatments were applied (B), models testing for within-year effects of treatment used post-treatment samples (C), while controlling for initial methylation (A). Models testing for between-year effects used post-treatment samples (D), while controlling for initial methylation (A).

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). See text for description of birds included in treatment and control groups and additional samples used for analyses not focused on methylation. Note that sample sizes indicate number of samples used in sequencing and analysis, but are not indicative of survival in each treatment.

120 **RESULTS**

121 *Corticosterone and breeding timing*

122 Birds that were treated with corticosterone in year one had higher baseline corticosterone in
123 year two (corticosterone treatment $\beta = 3.34$; 95% confidence interval = 1.43 to 5.24; Figure
124 2A; Table S2). Prior year treatment was not related to stress-induced corticosterone (Table
125 S2), but females that were previously corticosterone-exposed had lower post-dexamethasone
126 corticosterone, indicating more robust negative feedback ($\beta = -4.86$; 95% confidence interval
127 = -9.01 to -0.70; Figure 2B; Table S2). Finally, corticosterone-exposed females initiated their
128 nesting attempt earlier in the following year ($\beta = -2.34$; 95% confidence interval = -4.49 to
129 -0.20; Figure 2C; Table S2).

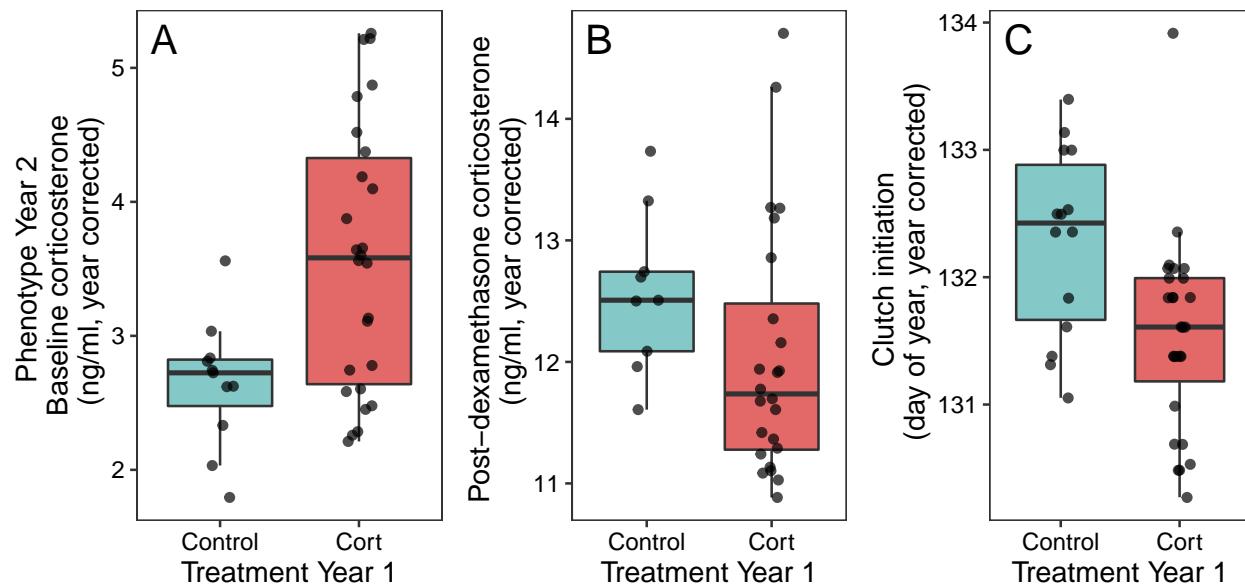


Figure 2: Effect of corticosterone treatment in one year on measures of baseline corticosterone (A), post-dexamethasone corticosterone (B), and clutch initiation date (C) in the following year. Points are partial residuals of raw data collected for average year effects. Boxes and whiskers show the median,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

130 *Association between natural or experimental corticosterone and methylation*

131 Using pre-treatment samples, we found that methylation percentage at 116 CpGs out of
132 78,143 tested was significantly associated with baseline corticosterone after FDR correction

¹³³ (Figure 3A; Table S1). For stress-induced corticosterone, we found a similar association at
¹³⁴ 356 out of 78,027 CpGs that were tested (Figure 3B; Table S1). For post-dexamethasone
¹³⁵ injection samples, we found an association between corticosterone and methylation at 735
¹³⁶ out of 69,189 CpGs tested (Figure 3C; Table S1).

¹³⁷ In models examining the causal effect of corticosterone treatment, we found that for samples
¹³⁸ collected within the same breeding season 1-2 weeks after treatment, 111 out of 48,070 CpGs
¹³⁹ tested showed evidence of differential methylation after FDR correction (Figure 3D; Table
¹⁴⁰ S1). We had fewer individuals and fewer CpGs that passed filtering for comparisons one year
¹⁴¹ after treatment, but we found that 49 out of 6,787 CpGs tested were differentially methylated
¹⁴² between treatment and control groups after one year (Figure 3E; Table S1). Although we
¹⁴³ were primarily interested in treatment effects, these models also showed that pre-treatment
¹⁴⁴ methylation at a given CpG site generally predicted post-treatment methylation both within
¹⁴⁵ a year (Figure S3A) and for samples collected one year later (Figure S3B).

¹⁴⁶ *Association between differentially methylated CpGs and genes*

¹⁴⁷ We found that CpGs that were significantly associated with baseline corticosterone,
¹⁴⁸ stress-induced corticosterone, and post-dexamethasone corticosterone were located in or near
¹⁴⁹ a total of 32, 176, and 236 identifiable genes, respectively (Table S3). When comparing
¹⁵⁰ differentially methylated CpGs after treatment effects, within-year and between-year CpGs
¹⁵¹ were located in or near 52 and 16 genes, respectively (Table S3). A subset of these genes
¹⁵² were identified in two or three different comparisons (Figure 4). Because of our filtering
¹⁵³ process many genes were not tested in each comparison (i.e., the background set of possible
¹⁵⁴ genes tested differed for each comparison).

¹⁵⁵ In examining the function of genes identified in this process, only one was obviously directly
¹⁵⁶ connected to regulation of the hypothalamic-pituitary-adrenal (HPA) axis. We found that
¹⁵⁷ individuals with higher stress-induced corticosterone in the observational dataset had higher
¹⁵⁸ methylation at a CpG associated with the MC2R gene, which is responsible for making the

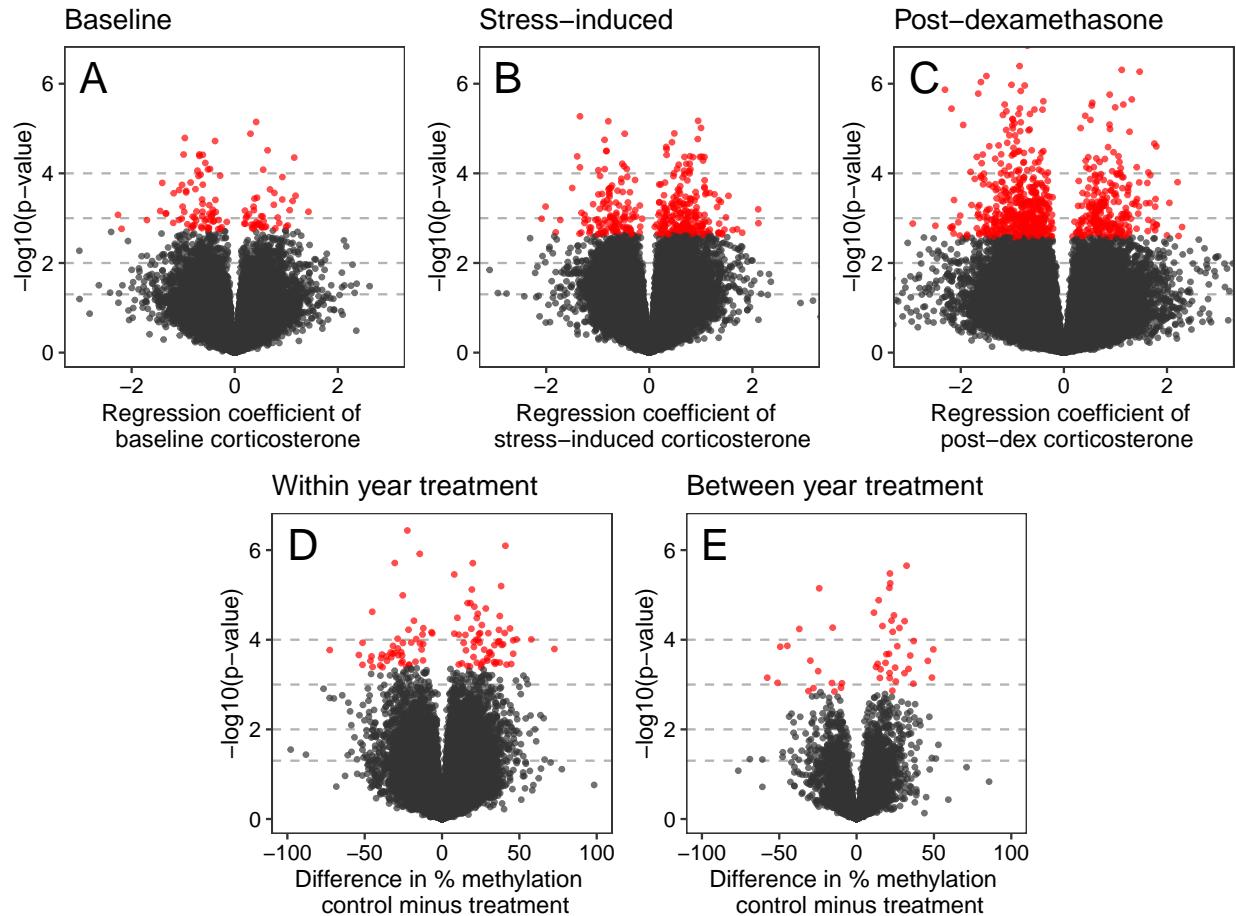


Figure 3: Association between DNA methylation and corticosterone from GLMMs based on observational and experimental study components. Panels A, B, and C show the pre-treatment regression coefficient for baseline corticosterone, stress-induced corticosterone, and post-dexamethasone corticosterone on methylation 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.

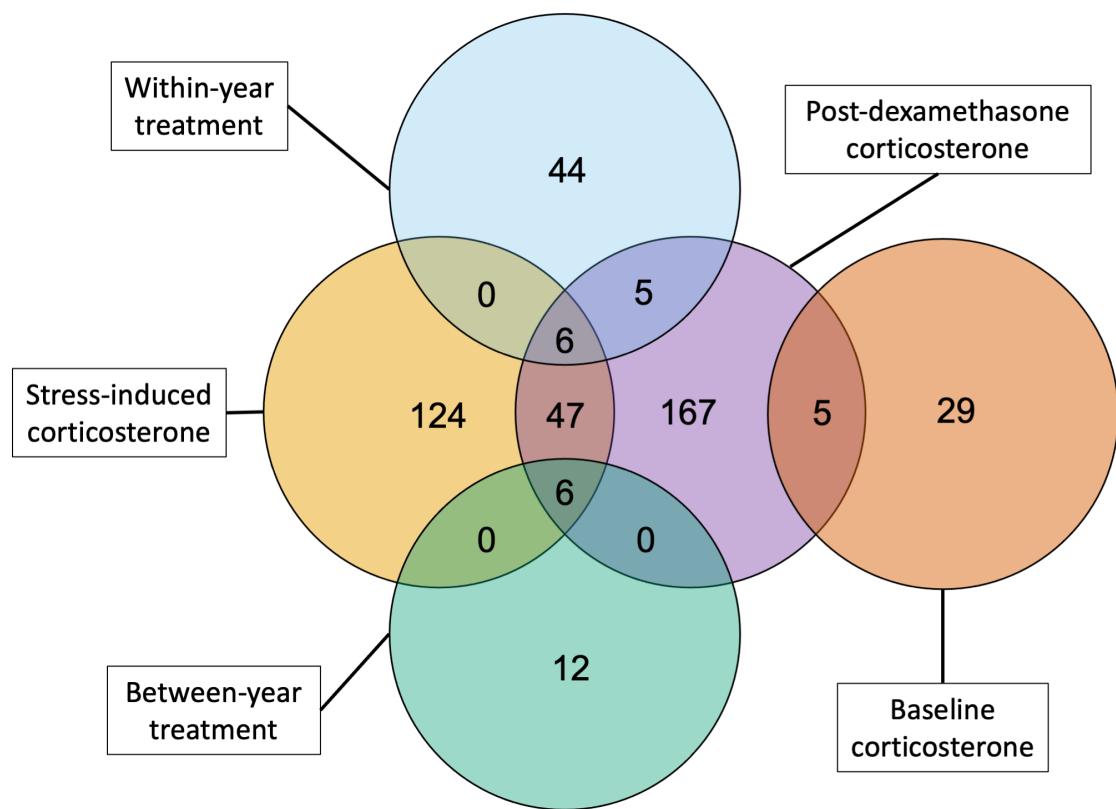


Figure 4: Number of genes near CpGs that were significantly associated with natural variation in corticosterone (baseline, stress-induced, and post-dexamethasone) or with experimental corticosterone elevation (within-year and between-year). No identified genes were shared between the comparisons with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

₁₅₉ ACTH receptor (Figure S4). Several other genes known to be associated with the HPA axis
₁₆₀ (e.g., CRH, CRHR1, FKBP5) did not have any CpGs near them in the background set, so
₁₆₁ we could not test for differences associated with these genes.

₁₆₂ *GO term analysis*

₁₆₃ Using the gene lists from Table S3 as input, we identified GO terms that were significantly
₁₆₄ associated with each comparison. With the false discovery rate set at 0.05, we identified 14
₁₆₅ GO terms associated with baseline corticosterone, 22 terms associated with stress-induced
₁₆₆ corticosterone, 10 terms associated with post-dexamethasone corticosterone, 2 terms for the
₁₆₇ within-year treatment effect, and 27 terms for the between-year treatment effect (Table S4).
₁₆₈ None of these lists resulted in any clear clustering of processes using the REVIGO
₁₆₉ visualization tool and many terms were repetitive and attributable to the same few gene
₁₇₀ associations.

₁₇₁ Baseline corticosterone was associated with photoreceptor activity and response to light,
₁₇₂ which was primarily driven by opsin and rhodopsin gene associations (OPN1SW, RHO,
₁₇₃ LWS). Stress-induced corticosterone was associated with a wider range of processes
₁₇₄ connected to a larger set of genes. These included a variety of cell signaling and receptor
₁₇₅ pathways, such as the ACTH association described above (MC2R). Post-dexamethasone
₁₇₆ corticosterone was primarily associated with signaling receptor activity driven by a relatively
₁₇₇ large number of associated genes (Table S4).

₁₇₈ Differentially methylated CpGs for within-year corticosterone treatment were only related to
₁₇₉ two GO terms associated with structural cell components and attributable to genes of
₁₈₀ unknown function. Between-year corticosterone treatment was associated with a variety of
₁₈₁ GO terms having to do primarily with transmembrane receptor signaling, but nearly all of
₁₈₂ these terms were selected from the same set of gene associations (BMPR1A and B, ACVR1,
₁₈₃ and TGFBR1).

¹⁸⁴ **DISCUSSION**

¹⁸⁵ We found that experimental increases in corticosterone induced long-term phenotypic
¹⁸⁶ changes. Females that experienced a few brief spikes in exogenous corticosterone had
¹⁸⁷ stronger negative feedback in the HPA axis and bred earlier in the subsequent year; these
¹⁸⁸ characteristics are typically associated with high stress resilience and reproductive success in
¹⁸⁹ this population. Furthermore, natural variation in corticosterone was correlated with DNA
¹⁹⁰ methylation, and experimental treatments altered DNA methylation patterns. Importantly,
¹⁹¹ regulation of DNA methylation in response to corticosterone occurred rapidly in adults
¹⁹² (within days) and resulted in detectable changes at least one year after treatment, paralleling
¹⁹³ the changes in physiological and behavioral phenotypes. Taken together, these results
¹⁹⁴ support the idea that the activation of the stress response machinery changes traits
¹⁹⁵ associated with stress resilience, and thus may prime future responses to challenges. DNA
¹⁹⁶ methylation could act as a key mechanism linking the prior experience of stressors—including
¹⁹⁷ during adulthood—to subsequent coping ability. Rapid endocrine flexibility and adaptive
¹⁹⁸ calibration of the stress response have emerged as key determinants of resilience to
¹⁹⁹ challenges (29–31) and understanding the mechanistic basis of these patterns is an important
²⁰⁰ step in predicting when flexibility is sufficient for coping with changing conditions.

²⁰¹ The changes in phenotype that we detected one year after experimentally elevating
²⁰² corticosterone partially matched our predictions if exposure altered phenotype in ways that
²⁰³ would increase future stress resilience. We found that, compared to controls, experimental
²⁰⁴ females initiated breeding earlier and had stronger negative feedback in the subsequent year.
²⁰⁵ In tree swallows, clutch initiation date is a strong predictor of both seasonal and lifetime
²⁰⁶ reproductive success and is often considered a proxy for individual quality or condition (27).
²⁰⁷ Similarly, the strength of negative feedback is consistently the best physiological predictor of
²⁰⁸ coping ability and reproductive success both under natural conditions and after imposing
²⁰⁹ experimental challenges (24, 26). However, contrary to our prediction, treatment had no

210 effect on stress-induced corticosterone the following year. We also found that corticosterone
211 dosed females had higher baseline corticosterone and no difference in stress-induced
212 corticosterone one year after treatment. Baseline corticosterone does not predict stress
213 resilience in this population (26). However, because baseline corticosterone often increases in
214 preparation for periods of high energetic demands, including the demands of reproduction
215 (the cort-adaptation hypothesis, 32, 33, 34), these results might reflect an increased
216 allocation to breeding in subsequent years in corticosterone treated females. For example,
217 female European starlings (*Sturnus vulgaris*) manipulated to increase parental investment
218 increased their baseline corticosterone during incubation (35). Similarly, tree swallows that
219 increase baseline corticosterone more over the reproductive period provision offspring at
220 higher rates (32). Thus, our results might represent a combination of long-term priming
221 effects coupled with the immediate energetic demands of breeding earlier.

222 Our study also adds to the growing recognition of bidirectional links between coping ability
223 and DNA methylation. While this relationship has been demonstrated in laboratory-based
224 model systems (12, 13) the potential for environmental stressors to trigger methylation, and
225 affect subsequent coping ability, has only recently been explored in wild animals. Early
226 results in wild animals suggest patterns similar to those seen in laboratory rodents. For
227 example, early life maternal care and social connections in spotted hyenas (*Crocuta crocuta*)
228 predict DNA methylation and glucocorticoid regulation as an adult (14, 36). Similar effects
229 can play out in adulthood; for example, in savannah baboons (*Papio cynocephalus*) high
230 social status as an adult is associated with more rapid changes to DNA methylation
231 (epigenetic aging) as a consequence of the social stress that accompanies high status (37).
232 Our results are consistent with the results derived from lab rodents, wild mammals, and a
233 growing number of studies in wild birds (18, 38), suggesting that flexible adjustment of
234 methylation may be a general mechanism by which prior experiences of stressors are encoded
235 in order to modulate future responses to challenges.

²³⁶ While there has been a rapid increase in studies of methylation in wild birds in recent years
²³⁷ (18, 19, 39), relatively few studies have sampled the same adults multiple times. Our study
²³⁸ design allowed us to assess the stability of genome-wide DNA methylation within individuals.
²³⁹ We found that many CpGs that we interrogated had large between individual differences in
²⁴⁰ methylation and that those differences were typically stable even in samples collected one
²⁴¹ year apart. Compared to these individual differences, flexible changes in methylation were
²⁴² relatively smaller and detectable at fewer CpGs. The stable individual differences that we
²⁴³ detected might represent the consequences of early life conditions (14–16). For example,
²⁴⁴ early life climate conditions are related to lifelong methylation of the glucocorticoid receptor
²⁴⁵ gene in superb starlings (*Lamprotornis superbus*, 18). We could not assess the possibility of a
²⁴⁶ similar pattern in our study because we did not have any information on early life conditions
²⁴⁷ for our birds. Regardless of the source of these initial differences, our results clearly
²⁴⁸ demonstrate that detecting subtle adjustments of methylation in adulthood to any treatment
²⁴⁹ of interest will often require accounting for pre-treatment methylation.

²⁵⁰ The functional consequences of most of the specific methylation changes that we detected are
²⁵¹ somewhat unclear. We did find that stress-induced corticosterone was correlated with
²⁵² methylation of a CpG associated with the MC2R gene, which encodes the ACTH receptor.
²⁵³ Individuals with a more robust stress response had higher methylation at this CpG. Higher
²⁵⁴ methylation is expected to be associated with lower gene expression (8, 10), suggesting that
²⁵⁵ individuals with a more robust corticosterone response might have lower ACTH receptor
²⁵⁶ expression. It isn't clear why lower ACTH receptor expression would result in higher
²⁵⁷ stress-induced corticosterone, but because regulation of the HPA axis can occur at multiple
²⁵⁸ levels with bi-directional feedback, the result may instead reflect a downregulation of ACTH
²⁵⁹ receptor expression in response to robust activation of other components of the HPA axis.
²⁶⁰ Pairing RRBS with gene expression measurements and comparisons in different tissues would
²⁶¹ be helpful to understand these patterns (e.g., 40).

262 In contrast to stress-induced corticosterone, none of the genes or GO terms associated with
263 natural variation in baseline corticosterone, post-dexamethasone corticosterone, or with
264 treatments had clear connections to HPA axis regulation. We previously found that
265 non-specific, genome-wide methylation predicts stress resilience to experimental challenges in
266 this population (25). Thus, the differences that we detected might reflect large-scale
267 regulation of methylation rather than targeted regulation of sites with specific functional
268 consequences. Alternatively, some of the changes that we detected might have functional
269 effects on stress response calibration that are not obvious from the known effects of those
270 genes. In support of this idea, we did find some overlap between methylation in the genes
271 associated with natural variation in corticosterone and with the consequences of our
272 experimental manipulation of corticosterone. In particular, post-dexamethasone
273 corticosterone, which is a strong predictor of stress resilience in tree swallows (24, 26), had
274 the most extensive correlations between methylation and identified genes and some of these
275 genes were shared with the other corticosterone measures and with treatment effects.

276 Another potential reason for our failure to find clear links between changes in DNA
277 methylation and genes associated with the stress response may result from limitations of our
278 approach. An advantage of RRBS is that it does not rely on pre-selecting candidate genes,
279 but a disadvantage is that not all relevant genes are necessarily tested. After filtering our
280 data, many of the genes with known roles in the HPA axis were not included in comparisons
281 or had coverage at only a few CpG sites. Thus we did not directly test for methylation
282 differences for many key genes. It is possible that deeper sequencing of our libraries would
283 have improved our ability to detect functional differences. Moreover, although we used the
284 most complete reference genome available for tree swallows, many CpGs mapped to
285 predicted genes whose function is unknown. Continued improvement of assembly and
286 annotation for reference genomes of non-model organisms is important for understanding the
287 functional importance of epigenetic changes. Studying DNA methylation in non-model
288 systems is a rapidly developing field and many recent papers outline the pros and cons of

289 various approaches (9, 41, 42). One particularly promising approach that may strike a
290 balance between a focus on candidate genes and the ability to detect genome-wide
291 associations is to combine RRBS with probes that enrich sequences at a potentially large
292 number of target genes (target-enriched enzymatic methyl sequencing, 43).

293 Regardless of the functional consequences of the changes we detected, we found that brief
294 increases in corticosterone have effects on subsequent corticosterone regulation, breeding
295 decisions, and methylation a full year after dosing ended. At least some of the phenotypic
296 changes we detected support a hormone-mediated priming effect in which activation of the
297 stress response machinery improves the capacity to cope with future challenges, increasing
298 stress resilience. The fact that these changes in phenotype are coupled with changes in
299 methylation patterns implicates the regulation of DNA methylation as a potential
300 mechanism of flexibly adjusting the stress response system based on prior experiences.

301 Understanding the mechanisms that integrate experience with future stress responsiveness
302 has important consequences for predicting how and when individuals can cope with repeated
303 exposure to challenges. Conceptual models of the stress response suggest that while repeated
304 challenges can sometimes impose long-term costs (21, 44), activation of the stress response at
305 other times may prime more effective responses to future challenges (4, 45). Studying the
306 mechanisms by which stress exposure is encoded biologically will help to differentiate these
307 possibilities and shed light on when and how individuals succeed or fail through flexible
308 regulation of the physiological response to challenges.

309 MATERIALS AND METHODS

310 We studied tree swallows breeding at field sites in and around Ithaca, New York, U.S.A. from
311 April to July 2014 to 2017. This population of tree swallows has been continuously studied
312 since 1986 and we followed well-established monitoring protocols (for details see 27). Adult
313 females were captured on day 6 to 7 after the beginning of incubation and again on day 3 to

314 7 after eggs had hatched. In the year after treatment, any returning females were captured
315 on day 6 to 7 of incubation. At each capture we collected blood samples ($< 70\mu\text{l}$ each) to
316 measure baseline (< 3 minutes) and stress-induced (30 minutes) corticosterone (23).
317 Immediately after the second blood sample was taken, females were injected with $4.5\ \mu\text{l/g}$ of
318 dexamethasone in the pectoralis muscle, which stimulates strong negative feedback (Mylan
319 4mg ml^{-1} dexamethasone sodium phosphate; previously validated in 26). A final blood
320 sample was collected 30 minutes after injection to measure the efficacy of negative feedback.
321 We also collected a set of standardized morphological measurements and monitored
322 reproductive success (23). All birds received a unique USGS aluminum band and passive
323 integrated transponder (PIT) tag if they were not previously banded.
324 Between the first and second capture in year one, females were randomly assigned to either a
325 control or experimental treatment group (experiment schematic and sample sizes at each
326 stage are shown in Figure 1). In the experimental group, we simulated a brief spike in
327 corticosterone once per day on five consecutive days between the two captures. To
328 accomplish this, we applied two $60\ \mu\text{l}$ doses of corticosterone dissolved in DMSO gel one
329 hour apart to a fake egg anchored in the nest cup at a randomly chosen time during the day
330 when females were absent from the nest. Upon returning, females incubated the clutch and
331 absorbed the corticosterone across the skin on their brood patch. For the purposes of this
332 study, we considered females as part of the corticosterone treatment group if they received
333 any of the three dose concentrations described in Vitousek et al. 2018 (high = $4\ \text{mg ml}^{-1}$
334 corticosterone once plus sham once per day; low = $2\ \text{mg ml}^{-1}$ once plus sham once per day;
335 long = $2\ \text{mg ml}^{-1}$ twice per day).
336 We previously validated that this dosing method results in a brief (< 180 minutes) increase
337 in corticosterone within the range of natural acute corticosterone responses (23). Control
338 nests received either no manipulation or a sham control in which they were dosed as
339 described above but with DMSO gel only with no corticosterone added. We previously found

³⁴⁰ no difference in physiology, behavior, reproductive success, or survival between control and
³⁴¹ sham control birds receiving this treatment (23, 24) and we combined both control groups in
³⁴² the analyses described here.

³⁴³ For methylation analyses, we focused on the set of females that were manipulated in 2015
³⁴⁴ and—if they returned—recaptured in 2016. In analyses focused on between year effects of
³⁴⁵ treatments on later corticosterone regulation and breeding decisions, we also included a
³⁴⁶ smaller number of females observed from 2014 to 2015 and from 2016 to 2017. These
³⁴⁷ additional samples included slight variants on corticosterone dosing that we considered part
³⁴⁸ of the corticosterone treatment group (six doses or three doses of 4 mg ml⁻¹ corticosterone
³⁴⁹ once per day during incubation, 24). In the year after exposure, we only considered potential
³⁵⁰ carryover effects of treatment on corticosterone regulation at the first capture and on the
³⁵¹ timing of clutch initiation because some females were subsequently entered into unrelated
³⁵² experiments that could have influenced later season measures.

³⁵³ *Tree swallow reference genome assembly*

³⁵⁴ For this study, we improved upon a previously published reference genome sequenced from a
³⁵⁵ female belonging to this study population (25) by first extracting high molecular weight
³⁵⁶ DNA from this same individual. We performed a phenol-chloroform extraction followed by
³⁵⁷ an ethanol precipitation and finally a bead cleanup. The Duke Center for Genomic and
³⁵⁸ Computational Biology core facility used the DNA to produce a large insert library (15 to 20
³⁵⁹ kb), which was subsequently sequenced on 3 cells of a Pacific Biosciences RSII instrument.
³⁶⁰ This produced a total of 9.6 Gbp of data with an average read length of 12,053 bp and an
³⁶¹ N50 subread length of 15,643 bp. We used bamtools version 2.5.1 (46) to merge the reads
³⁶² from the difference cells and retain only those that were longer than 4,500 bp (47.6% of the
³⁶³ original raw reads). We improved our first assembly with the PBJelly2 module of PBSuite
³⁶⁴ version 15.8.24 (47), which uses long reads to fill or reduce gaps. This pipeline produced an
³⁶⁵ assembly which was moderately improved from the previous version (25). The total length of

366 the assembly was 1.22 Gb (previously 1.14 Gb) and was contained in 49,278 scaffolds
367 (previously 92,148), with an N50 of 82.9 kb (originally 34 kb) and 1.9% Ns (vs. 5.8%). We
368 assessed the completeness of our assembly by running BUSCO version 5.2.2 (48), using the
369 passeriformes dataset of 10,844 conserved genes. We found 80.5% of these genes in a single
370 and complete copy, 3.9% were fragmented, 1.8% were duplicated, and 13.8% were missing.
371 Finally, we annotated the genome following the pipeline described in Taff et al. (25). The
372 assembly generated for this project is deposited on GenBank (BioProject ID PRJNA553513).

373 *Sample processing*

374 Blood samples collected in the field were immediately stored on ice in a cooler and processed
375 in the lab within 3 hours of capture. Red blood cells were separated from plasma by
376 centrifugation and added to 1 mL of ice cold cryopreservation buffer (90% newborn calf
377 serum, 10% DMSO, 49). Samples were then frozen at a constant cooling rate with isopropyl
378 alcohol and stored at -80° C until further processing. Cryopreserved blood samples were
379 thawed and DNA was extracted using the DNeasy Blood & Tissue spin column extraction
380 kits according to the manufacturer's protocol (Qiagen Sciences Incorporated).

381 *Corticosterone and breeding timing data analysis*

382 We used general linear models to compare corticosterone and the timing of breeding between
383 control and experimental females one year after corticosterone manipulations. We fit four
384 models in total with either the date of clutch initiation or circulating corticosterone levels
385 (baseline, stress-induced, or post-dexamethasone injection) as the response variable.
386 Predictors included treatment and year as a categorical fixed effect. The model for
387 stress-induced corticosterone also included baseline corticosterone as a predictor and the
388 model for post-dexamethasone corticosterone also included stress-induced corticosterone as a
389 predictor.

390 *Reduced representation bisulfite sequencing*

391 We prepared our samples for reduced representation bisulfite sequencing (RRBS) using the
392 Diagenode Premium RRBS Kit and closely following the manufacturer's protocol (50).
393 Briefly, samples were diluted to 3.85 ng/ μ l and 26 μ l of diluted sample was used for library
394 preparation. The process included enzymatic digestion with Mspl and size selection to
395 increase coverage of CpG-rich regions, such as CpG islands and enhancers. Individual
396 samples received one of twenty-four unique barcodes and were pooled in randomized groups
397 of 8 before bisulfite conversion. We also included a methylated and unmethylated spike in
398 control with each sample to confirm the efficiency of bisulfite conversion.

399 From the available samples, we selected 120 samples to process from 61 unique birds (three
400 samples per bird n = 14, two samples n = 31, one sample n = 16). Prior to RRBS
401 processing, these 120 samples were randomly sorted to account for any batch effects.

402 Libraries were prepared with the Diagenode kit in two batches (one set of 24 and one of 96).
403 Prepared libraries were checked for the expected size distribution by digital PCR prior to
404 sequencing. Sequencing was performed at the Cornell BRC using NextSeq 1x75 with 20%
405 PhiX and 85% of the normal cluster density. In total, we ran our samples on five sequencing
406 lanes with 24 samples per lane.

407 Raw sequence data were first processed with **Trim Galore!** using the default RRBS settings.
408 Visual inspection of FastQC files confirmed high quality reads for all samples. Next, we used
409 **Bismark** to align each sequence to the prepared genome and extract the methylation status
410 for each CpG, CpH, or CHH site (51). As expected, global methylation at CpH and CHH
411 sites was extremely low (1.0% and 0.6%, respectively, Figure S1) and we only considered
412 methylation at CpG sites in our subsequent analyses. We also used **Bismark** to determine
413 the methylation conversion efficiency for each sample based on methylated and unmethylated
414 spike in controls and following the instructions in the Diagenode RRBS kit (50, 51).

415 *General methylation patterns*

416 Our process resulted in 9.8 ± 4.3 million (SD) total reads per sample (Figure S1). Across all

417 samples, we were able to align 51.1% of the total reads produced, which is comparable to
418 several recent studies in wild birds (39, 52). Spiked controls in each sample indicated that
419 our bisulfite conversion worked efficiently and within the recommended kit parameters
420 (conversion of methylated control sites = $1.9\% \pm 1.4$; conversion of unmethylated control
421 sites = $99.5\% \pm 0.6$).

422 Among 45 pre-treatment samples, we had sufficient coverage to estimate methylation at
423 148,167 CpGs. In total, the average percentage methylation across all sites was $35.5\% \pm 34.0$
424 with a wide distribution (Figure S2A). After assigning CpGs hierarchically to promoter
425 (within 2kb upstream of a TSS) > exon > intron, we found that 12.1% of sites were in
426 promoters, 7.9% in exons, 11.8% in introns, and 68.1% in intergenic regions. At the level of
427 genomic features, promoters had the lowest methylation (median = 5.3%, mean \pm SEM =
428 $20.5\% \pm 0.5$), introns had intermediate methylation (median = 43.5%, mean \pm SEM =
429 $41.0\% \pm 0.5$), and exons had the highest methylation (median = 54.3%, mean \pm SEM =
430 $46.7\% \pm 0.7$). However, each of these features had a wide distribution of methylation
431 percentages across different genes (Figure S2B).

432 *RRBS Data Analysis*

433 Output data from the sequence processing described above was analyzed in R version 4.1.1
434 (53). We processed the aligned sequence data with **MethylKit** (54). Using **MethylKit**, we
435 extracted the number of total aligned reads and number of methylated or unmethylated
436 reads for each CpG site.

437 For analyses of corticosterone and treatment associations, we filtered these CpGs to include
438 only those that met the following criteria. First, we required a minimum coverage of 10 reads
439 per sample to retain data for that sample at a given CpG. We further filtered the dataset to
440 remove any CpGs that were mostly invariant (i.e., more than half of samples had
441 methylation percentage of 0 or 100%) as well as CpGs that had extremely low variation (SD
442 less than 5% across all samples, 55, 56). For models comparing treatment effects, we

⁴⁴³ required that females have data at a given CpG from both pre- and post-treatment sampling
⁴⁴⁴ points to be included. For basic descriptions of methylation patterns, we used all CpGs that
⁴⁴⁵ had 10 reads or more in the pre-treatment samples.

⁴⁴⁶ The built-in differential methylation techniques in **MethylKit** are designed for two group
⁴⁴⁷ comparisons with limited flexibility in modeling options. Because we had repeated measures
⁴⁴⁸ before and after treatments for both groups, we could not specify the necessary models
⁴⁴⁹ within **MethylKit** itself. Therefore, we exported and combined the filtered CpG records for
⁴⁵⁰ all groups so that we could fit generalized linear mixed models (GLMMs) for each CpG site
⁴⁵¹ (as in 38) using the `glmer` function in R package `lme4` (57). We fit a separate set of models
⁴⁵² for natural corticosterone variation (baseline, stress-induced, or post-dexamethasone),
⁴⁵³ within-year treatments, and between-year treatments. Each of these datasets were
⁴⁵⁴ constructed separately since they included different subsets of both individual birds and of
⁴⁵⁵ CpGs that met the criteria described above.

⁴⁵⁶ For natural variation in corticosterone, we included only the pre-treatment samples. Using
⁴⁵⁷ these samples, we fit a GLMM for each CpG with the number of methylated and
⁴⁵⁸ unmethylated reads as the binomial response variable. We fit this set of models separately
⁴⁵⁹ with baseline, stress-induced, or post-dexamethasone corticosterone as the single continuous
⁴⁶⁰ predictor variable. The models included a random effect for female identity to account for
⁴⁶¹ repeated sequencing of the same CpG sites within each female. We excluded the results for
⁴⁶² any models that failed to converge because we could not reliably estimate effects in those
⁴⁶³ cases.

⁴⁶⁴ For within-year and between-year comparisons after treatments, we fit a single GLMM for
⁴⁶⁵ each included CpG with the number of methylated and unmethylated reads as the binomial
⁴⁶⁶ response variable. Predictors included pre-treatment methylation percentage at the CpG
⁴⁶⁷ being modeled, a fixed effect of treatment (control vs. corticosterone), and a random effect
⁴⁶⁸ for female identity. In each model, significance of the comparison between control and

⁴⁶⁹ corticosterone treated birds was assessed using the `emmeans` package in R (58). We also
⁴⁷⁰ evaluated the stability of methylation within individuals in these models by summarizing the
⁴⁷¹ regression coefficient of pre-treatment methylation on post-treatment methylation.

⁴⁷² We accounted for multiple comparisons in each of these analyses by adjusting all p-values
⁴⁷³ using the q-value approach implemented by the `qvalue` package in R with the false discovery
⁴⁷⁴ rate set at 0.05 (59). We only report and interpret estimates with q-values < 0.05.

⁴⁷⁵ *Annotation of differentially methylated CpGs*

⁴⁷⁶ After identifying CpGs that were significantly associated with either natural corticosterone
⁴⁷⁷ or experimental treatment with corticosterone, we identified genes associated with each CpG.
⁴⁷⁸ We used the `bedtoolsr` package to select genes that had a significant CpG either within the
⁴⁷⁹ gene body or within 2 kb upstream of the transcription start site (60). We generated
⁴⁸⁰ separate lists of genes associated with CpGs for baseline corticosterone, stress-induced
⁴⁸¹ corticosterone, post-dexamethasone corticosterone, within-year treatment effects, and
⁴⁸² between-year treatment effects. For each of these comparisons we also generated a complete
⁴⁸³ list of genes associated with all of the CpGs that passed the filtering criteria described above
⁴⁸⁴ to be used as a null background list (see below).

⁴⁸⁵ Starting with the list of genes associated with each comparison set, we used the DAVID
⁴⁸⁶ functional annotation tool (61) to test whether our genes were enriched in any molecular
⁴⁸⁷ functions or biological processes in the Gene Ontology knowledgebase (62, 63). For each
⁴⁸⁸ comparison we used the custom background list generated above. This background list is
⁴⁸⁹ important for interpretation because we were only able to test CpGs near a subset of genes
⁴⁹⁰ in each comparison (number of genes included in testing for baseline corticosterone = 4,143;
⁴⁹¹ stress-induced corticosterone = 4,146; post-dexamethasone corticosterone = 3,863;
⁴⁹² within-year treatment = 2,913; between-year treatment = 452).

⁴⁹³ Using DAVID we identified a set of GO terms associated with biological processes or
⁴⁹⁴ molecular functions that were over represented in the list of significant CpGs compared to

495 the background list for that comparison (63). We filtered this list to include only GO terms
496 with p-values < 0.05 after applying a false discovery rate correction. We initially visualized
497 the GO terms for each comparison using REVIGO (64); however, our study identified a
498 relatively small number of GO terms and no clearly identifiable clusters of terms were
499 identified in REVIGO. Therefore, we report the complete list of genes and GO terms
500 associated with CpGs in each comparison.

501 *Data and code availability*

502 The complete set of bioinformatic processing scripts, R code, and data associated with each
503 sample is available and permanently archived on Zenodo
504 (<https://doi.org/10.5281/zenodo.8125153>). Raw sequence data from RRBS is available on
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514 **AUTHOR CONTRIBUTIONS**

515 CCT and MNV conducted the field based data collection. CCT and MNV conceived the
516 study. CCT and SMM conducted the lab work for RRBS. CCT and LC conducted the lab
517 work for creating the reference genome and LC carried out the bioinformatics for genome

518 assembly and annotation. CCT analyzed and visualized the data with assistance from SMM
519 and LC. CCT drafted the paper with input from all authors.

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723 SUPPLEMENTARY FIGURES AND TABLES

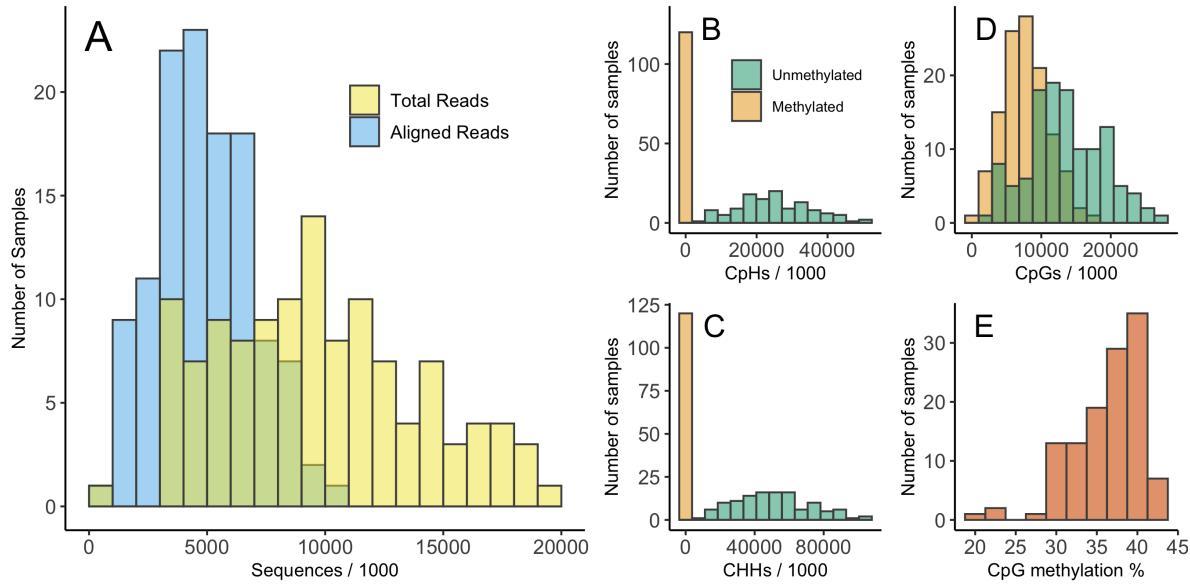


Figure S1: Summary of sequencing and methylation call results from raw sequence data. Panel A shows the distribution of the total number of sequences for each sample and number of sequences that aligned to the tree swallow genome. Panel B shows the number of CpH sites that were methylated or unmethylated 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.

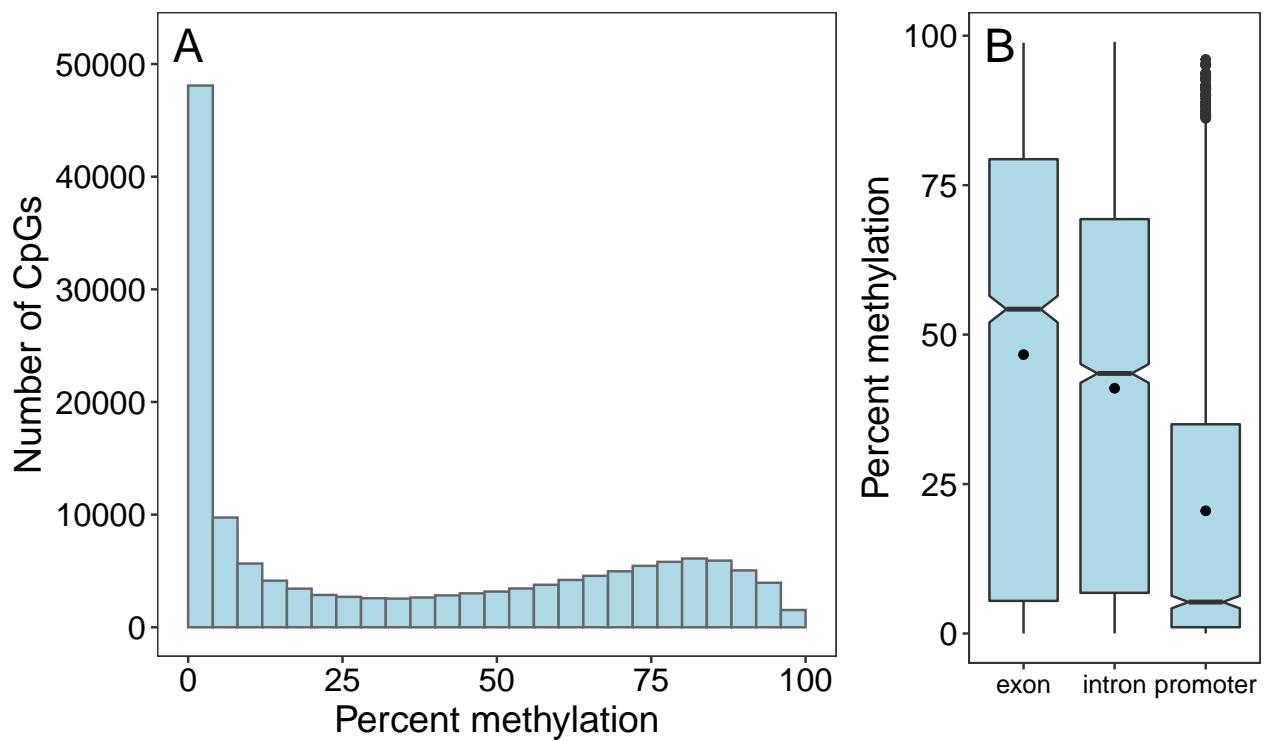


Figure S2: Distribution of methylation percentage across all CpGs and for different genomic features. Panel A shows entire distribution of methylation percentage for all 148,167 CpGs from pre-treatment samples before any filtering. Panel B shows the methylation percentage for exons, introns, and promoters 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.

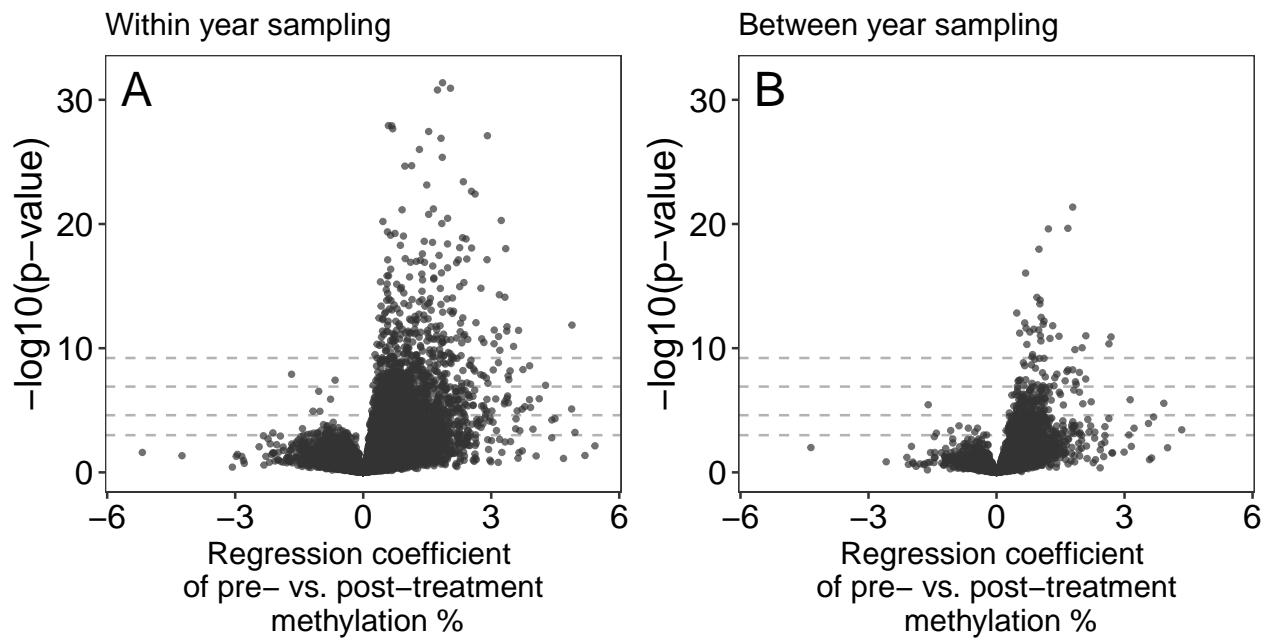


Figure S3: Association between pre-treatment methylation percentage and post-treatment methylation at each CpG for samples collected within a breeding season (panel A) and for samples collected one year apart (panel B). To help with interpretation, horizontal dashed lines indicate p-values of 0.05, 0.01,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

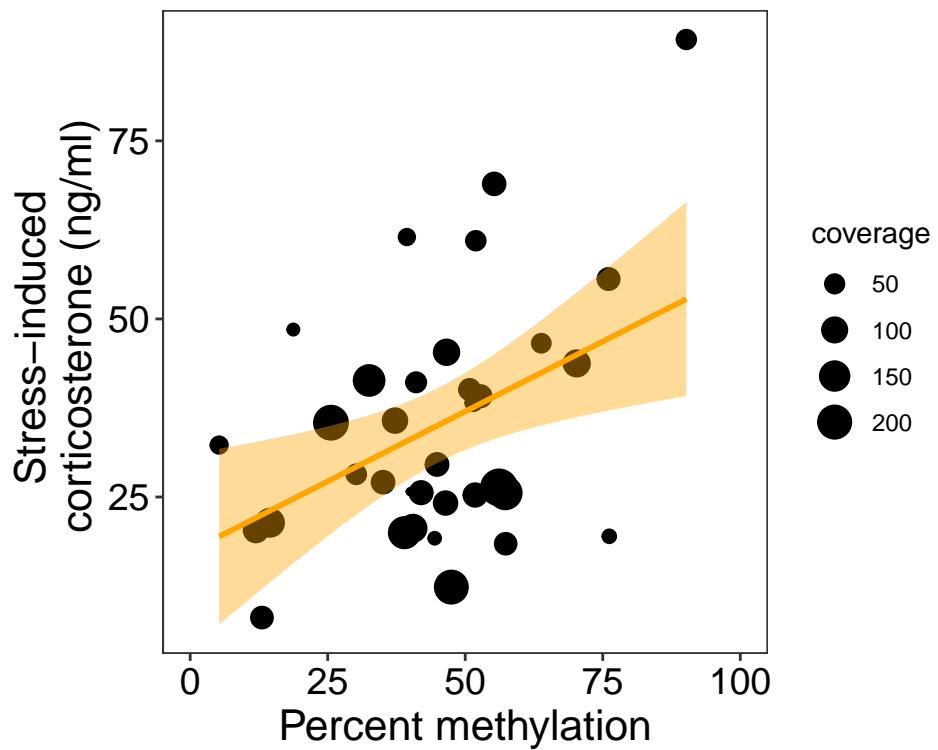


Figure S4: Percent methylation in relation to stress-induced corticosterone at the CpG near the MC2R gene that was significantly associated with corticosterone. Size of circles indicates sequence coverage for each sample. The trendline and confidence interval are shown for illustration but significance was assessed using the binomial GLMM described in the text.

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; SYNPO⁴²; 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