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

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Abstract

When facing challenges, vertebrates activate a hormonal stress response that can dramatically alter behaviour 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 in DNA methylation. We simulated the spikes in corticosterone that accompany a stress response using non-invasive dosing in tree swallows (*Tachycineta bicolor*) and monitored the phenotypic effects 1 year later. In a subset of individuals, we characterized DNA methylation using reduced representation bisulfite sequencing shortly after treatment and a year later. The year after treatment, experimental females had stronger negative feedback and initiated breeding earlier—traits that are associated with stress resilience and reproductive performance in our population—and higher baseline corticosterone. We also found that natural variation in corticosterone predicted patterns of DNA methylation. Finally, corticosterone treatment influenced methylation on short (1–2 weeks) and long (1 year) time scales; however, these changes did not have clear links to functional regulation of the stress response. Taken together, our results are consistent with corticosterone-induced priming of future stress resilience and support DNA methylation as a potential mechanism, but more work is needed to demonstrate functional consequences. Uncovering the mechanisms linking experience with the response to future challenges has implications for understanding the drivers of stress resilience.

KEYWORDS
carryover effects, endocrinology, reduced representation bisulfite sequencing, stress, tree swallow

1 | INTRODUCTION

Wild organisms regularly encounter challenging conditions that require rapid behavioural and physiological responses. In vertebrates, the glucocorticoid-mediated stress response plays an essential role in allowing animals to successfully avoid or tolerate stressors (Sapolsky et al., 2000; Wingfield et al., 1998). While a short-term response that facilitates coping with a stressor is beneficial (Wingfield et al., 1998), unnecessary activation or prolonged elevation of glucocorticoids can result in a variety of negative consequences for health

and fitness (Korte et al., 2005). Accordingly, the dominant paradigm in behavioural ecology and endocrinology is that the immediate benefits of the stress response are balanced by long-term costs.

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

One mechanism that could play a role in the calibration of stress response systems is changes in DNA methylation. Epigenetic modification by DNA methylation can alter phenotypes by making genes or promoters more or less accessible for transcription (Anastasiadi et al., 2018; Lea et al., 2018; Sepers et al., 2019). While the majority of methylation differences may be genetically determined (Sepers et al., 2023), early life experiences can also have profound programming effects on DNA methylation patterns that often persist throughout the individuals' lifetime (McGowan et al., 2009). For example, classic work in laboratory rodents demonstrates that early life experiences regulate methylation of the gene producing the glucocorticoid receptor, which results in lifelong changes to glucocorticoid secretion in response to challenges (Liu et al., 1997; Weaver et al., 2004). A growing number of studies in wild animals also report patterns consistent with early life programming of DNA methylation in wild populations associated with dominance hierarchies (Laubach et al., 2019), brood size (Sheldon et al., 2018), temperature and weather (Metzger & Schulte, 2017; Rubenstein et al., 2016), or urbanization (von Holdt et al., 2023).

While they are less well documented, experiences during adulthood can also result in changes in DNA methylation and these adjustments can occur rapidly (Bentz et al., 2021; Metzger & Schulte, 2017). For example, brief periods of experimental competition and aggression in tree swallows (*Tachycineta bicolor*) resulted in altered DNA methylation of brain regions associated with hormone signalling, suggesting a priming effect in preparation for future aggression (Bentz et al., 2021). Conceptual models of the stress response have long recognized that the sequence, frequency, duration and intensity of stressors should change the optimal behavioural and physiological response (Romero et al., 2009; Zimmer et al., 2022). Yet, it is often unclear how the experience of challenges during adulthood would be biologically encoded to alter responses to future challenges. Altered DNA methylation is a promising mechanism because (i) it can change rapidly even during adulthood (Bentz et al., 2021; Hu et al., 2018); (ii) it might persist over moderate to long time scales (Lea et al., 2016; Zannas et al., 2015); (iii) it has been shown to change with challenging experiences (Hu et al., 2018; Metzger & Schulte, 2017); and (iv) it can directly alter an individual's phenotype (Angers et al., 2010; Sagonas et al., 2020).

We experimentally simulated a series of acute corticosterone spikes using a non-invasive dosing procedure (Vitousek, Taff, Ardia, et al., 2018) and monitored both long-term phenotypic effects and, in a subset of individuals, changes in DNA methylation. In this population, we previously found that brief increases in corticosterone have effects on behaviour and performance that persist throughout at least the length of the breeding season (Taff et al., 2018; Vitousek, Taff, Ardia, et al., 2018) and that genome-wide methylation predicts resilience to experimental challenges (Taff et al., 2019). Here, we aimed to extend those results by asking whether brief increases in corticosterone altered the regulation of the stress response and breeding decisions a full year later. We coupled this approach with reduced representation bisulfite sequencing (RRBS) and a newly improved reference genome assembled for this study to examine genome-wide patterns of DNA methylation at high resolution. We used RRBS to first assess covariation between methylation and natural variation in corticosterone regulation during an acute stress response. Next, we compared DNA methylation in corticosterone-treated females to controls to determine whether brief increases in corticosterone resulted in altered DNA methylation at either short (1–2 weeks) or long (1 year) timescales.

If activation of the stress response machinery primes future coping ability (Del Giudice et al., 2018; Hilker et al., 2016), experimentally treated females should exhibit long-term (across-year) differences in key phenotypic traits. Specifically, we predicted that in the year after treatment, females would have a robust stress-induced increase in corticosterone and strong negative feedback, measured as the ability to quickly downregulate corticosterone after handling stress. These traits that have been previously shown to predict stress resilience in this population (Zimmer et al., 2019). We also predicted that these females 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, et al., 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.

2 | MATERIALS AND METHODS

We studied tree swallows breeding at field sites in and around Ithaca, New York, USA from April 2014 to July 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 Days 6–7 after the beginning of incubation and again on Days 3–7 after the eggs had hatched. In the year after treatment, any returning females were captured on Days 6–7 of incubation. At each capture, we collected blood samples (<70 μL each) to measure baseline (<3 min) and stress-induced (30 min) corticosterone (Vitousek, Taff, Ardia, et al., 2018). Immediately after the second blood sample was taken, females were injected with 4.5 μL/g of dexamethasone in the pectoralis muscle (Mylan 4 mg/mL dexamethasone sodium phosphate; previously validated in Zimmer et al., 2019). This injection stimulates strong negative feedback, which is the process resulting in downregulation of circulating corticosterone after an acute increase. A final blood sample was collected 30 min after injection to measure the efficacy of negative feedback. We also collected a set of standardized morphological measurements and monitored reproductive success (Vitousek, Taff, Ardia, et al., 2018). All birds received a unique USGS aluminium band and passive integrated transponder (PIT) tag if they were not previously banded.

Between the first and second capture in Year 1, females were randomly assigned to either a control or experimental treatment group (experiment schematic and sample sizes at each stage are shown in Figure 1). We later confirmed that females in these groups did not differ significantly in any pre-treatment corticosterone measures (Welch's two-sample *t*-test for pre-treatment baseline corticosterone: $t = -1.14$, $df = 37.2$, $p = .26$; stress-induced corticosterone: $t = 0.92$, $df = 39.21$, $p = .36$; post-dexamethasone corticosterone: $t = 0.35$, $df = 12.26$, $p = .74$). In the experimental group, we simulated a brief spike-in corticosterone once per day on three to six consecutive days between the two captures (see details below). To accomplish this, we applied two 60 μL doses of corticosterone dissolved in DMSO gel 1 h apart to a fake egg anchored in the nest cup at a randomly chosen time during the day when females were absent from the nest. Upon returning, females incubated the clutch and absorbed the corticosterone across the skin on their brood patch.

We previously validated that this dosing method results in a brief (<180 min) increase in corticosterone within the range of natural

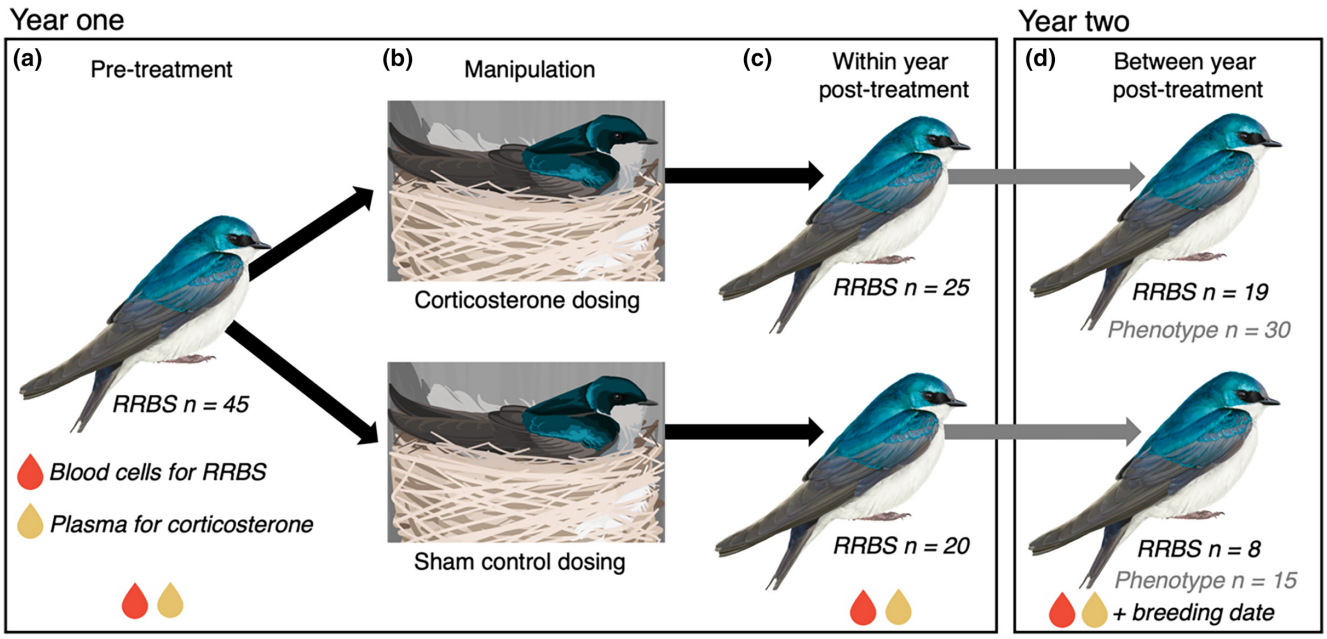

FIGURE 1 Schematic illustration of the experimental treatment and samples collected for reduced representation bisulfite sequencing (RRBS) and phenotypic comparisons. Models comparing natural corticosterone to DNA methylation used pre-treatment samples (a). After treatments were applied (b), models testing 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 and breeding timing used a larger set of females (d). See text and Table S5 for description of birds included in each group. Note that sample sizes indicate the 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 than birds sequenced. [Colour figure can be viewed at wileyonlinelibrary.com]

acute corticosterone responses (Vitousek, Taff, Ardia, et al., 2018). The elevation and time course of clearance from a single dose using this method is within the normal parameters for a natural acute corticosterone response triggered by a standard handling protocol. Control nests received either no manipulation or a sham control in which they were dosed as described above but with DMSO gel only with no corticosterone added. We previously found no difference at any time point in physiology, behaviour, reproductive success, or survival between control and sham control birds receiving this treatment (Taff et al., 2018; Vitousek, Taff, Ardia, et al., 2018), and we combined both control groups in the analyses described here. Although exogenous corticosterone clears within a few hours, repeated dosing during incubation results in altered endogenous corticosterone regulation and prolonged elevation within a breeding season. Using the doses described here, we previously demonstrated elevated corticosterone for 1–2 weeks after the completion of dosing (Taff et al., 2018; Vitousek, Taff, Ardia, et al., 2018). Samples used in this study represent a subset of those previous analyses, but we confirmed that corticosterone-treated females had higher circulating baseline and stress-induced corticosterone than control females 1–2 weeks after dosing (Welch's two-sample *t*-test baseline corticosterone: $t = -6.23$, df = 29.2, $p < .0001$; stress-induced corticosterone: $t = -2.99$, df = 22.8, $p = .007$).

For methylation analyses, we focused on a subset of females that were manipulated in 2015 and—if they returned—recaptured in 2016. These females were considered to be a part of the corticosterone treatment group if they received any of the three dose concentrations described in Vitousek, Taff, Ardia, et al. (2018): high_5d = 5 days of a once daily dose of 4 mg/mL corticosterone followed 1 h later by a sham treatment (DMSO alone); low_5d = 5 days of a once daily dose of 2 mg/mL corticosterone followed 1 h later by a sham treatment; and long_5d = 5 days of a twice daily dose of 2 mg/mL corticosterone with the two doses separated by 1 h. In analyses focused on between-year effects of treatments on later corticosterone regulation and breeding decisions, we also included additional females observed from 2015 to 2016 that experienced the same treatments but from which we do not have RRBS data, as well as a smaller number of females observed from 2014 to 2015 and from 2016 to 2017. These additional samples included slight variants on corticosterone dosing that we considered part of the corticosterone treatment group (high_6d = 6 days of a once daily dose of 4 mg/mL corticosterone or high_3d = 3 days of a once daily dose of 4 mg/mL corticosterone once per day during incubation, Taff et al., 2018). In the year after exposure, we only considered potential carryover effects of treatment on the timing of clutch initiation and corticosterone regulation at Day 6 of incubation because most females were subsequently entered into unrelated experiments that could have influenced later season measures. The slight variations on corticosterone exposure all result in categorically different regulation of corticosterone when compared to control treatments (Figure S4), and given our limited sample size, we only considered comparisons between control and combined corticosterone exposure treatments. The detailed sample sizes for each set of analyses are included in Table S5.

2.1 | Tree swallow reference genome assembly

For this study, we improved upon a previously published reference genome sequenced from a female belonging to this study population (Taff et al., 2019) by first extracting high molecular weight DNA from this same individual. We performed a phenol-chloroform extraction followed by an ethanol precipitation and finally a bead clean-up. The Duke Center for Genomic and Computational Biology core facility used the DNA to produce a large insert library (15–20 kb), which was subsequently sequenced on three cells of a Pacific Biosciences 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 et al., 2011) to merge the reads from the difference cells and retain only those that were longer than 4500 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 et al., 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).

2.2 | Sample processing

Blood samples collected in the field were immediately stored on ice in a cooler and processed in the laboratory within 3 h 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). For consistency, we selected the second (stress-induced) sample from each individual for cryopreservation and RRBS. 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 laboratory (Vitousek, Taff, Ardia, et al., 2018).

2.3 | Corticosterone and breeding timing data analysis

We used general linear models to compare corticosterone and the timing of breeding between control and experimental females 1 year after corticosterone manipulations. We fit four models in total with either the date of clutch initiation or circulating corticosterone levels (baseline, stress-induced or post-dexamethasone injection) as the response variable. Predictors included treatment and year as a categorical fixed effect. The model for stress-induced corticosterone also included baseline corticosterone as a predictor because baseline levels can influence the amount of stress-induced increase. The model for post-dexamethasone corticosterone also included stress-induced corticosterone as a predictor because the stress-induced level directly influences post-dexamethasone levels.

2.4 | Reduced representation bisulfite sequencing

We prepared our samples for reduced representation bisulfite sequencing (RRBS) in house using the Diagenode Premium RRBS Kit and closely following the manufacturer's protocol (Veillard et al., 2016). Briefly, DNA samples were diluted to 3.85 ng/μL and 26 μL of diluted sample was used for library preparation. The process included enzymatic digestion with MspI and size selection to increase coverage of CpG-rich regions, such as CpG islands and enhancers. Individual samples received one of 24 unique barcodes and were pooled in randomized groups of eight before bisulfite conversion. We also included a methylated and unmethylated spike-in control with each sample to confirm the efficiency of bisulfite conversion.

From the available samples, we selected 120 samples to process from 61 unique birds (three samples per bird $n=14$, two samples $n=31$, one sample $n=16$, Figure 1). We prioritized the selection of individuals that had data from both years and that had complete data for the planned analyses. Because our downstream analyses often required pre-treatment methylation measures or corticosterone values, ultimately not every individual that was sequenced was included in models examining methylation patterns. Prior to RRBS processing, these 120 samples were randomly sorted to account for any batch effects. Libraries were prepared with the Diagenode kit in two batches (one set of 24 and one of 96). Prepared libraries were checked for the expected size distribution on a fragment Bioanalyzer prior to sequencing. Sequencing was performed at the Cornell BRC using NextSeq sing-end 1×75 with 20% PhiX and 85% of the normal cluster density. In total, we ran our samples on five sequencing lanes with 24 samples per lane.

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

2.5 | General methylation patterns

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

Among 45 pre-treatment samples from unique individuals, we had sufficient coverage to estimate methylation at 524,297 CpGs (minimum of 10 reads from a minimum of 10 individuals). In total, the average percentage methylation across all sites was 35.5% ± 34.0 with a wide distribution (Figure S2A). After assigning CpGs hierarchically to promoter (within 2 kb upstream of a Transcription Start Site) > exon > intron, we found that 12.1% of sites were in promoters, 7.9% in exons, 11.8% in introns and 68.1% in intergenic regions. At the level of genomic features, promoters had the lowest methylation (median = 5.3%, mean ± SEM = 20.5% ± 0.5), introns had intermediate methylation (median = 43.5%, mean ± SEM = 41.0% ± 0.5), and exons had the highest methylation (median = 54.3%, mean ± SEM = 46.7% ± 0.7). However, each of these features had a wide distribution of methylation percentages across different genes (Figure S2B).

2.6 | RRBS data analysis

Output data from the sequence processing described above were analysed in R version 4.1.1 (R Core Team, 2020). We processed the aligned sequence data with MethylKit version 1.14.2 (Akalin et al., 2012). Using MethylKit, we extracted the number of total aligned reads and number of methylated or unmethylated reads for each CpG site. As recommended by the package authors, we filtered

out CpGs above the 99.5th percentile of read depth to account for potential PCR bias.

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

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

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

For within-year ($n = 48{,}070$ CpGs) and between-year ($n = 6787$ CpGs) comparisons after treatments, we fit a single GLMM for each included CpG with the number of methylated and unmethylated reads as the binomial response variable. Predictors included pre-treatment methylation percentage at the CpG being modelled, a fixed effect of treatment (control vs. corticosterone) and a random effect for female identity. In each model, the significance of the comparison between control and corticosterone-treated birds was assessed using the emmeans version 1.5.3 package in R (Lenth, 2019), which employs the Satterthwaite approximation method for binomial mixed models. We also evaluated the stability of methylation within individuals in these models by summarizing the regression coefficient of pre-treatment methylation percentage on post-treatment methylation.

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

2.7 | Annotation of differentially methylated CpGs

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

Starting with the list of genes associated with each comparison set, we used the DAVID functional annotation tool (Huang et al., 2007) to test whether our genes were enriched in any molecular functions or biological processes in the Gene Ontology knowledgebase (Ashburner et al., 2000; Gene Ontology Consortium, 2021). For each comparison, we used the custom background list generated above. This background list is important for interpretation because we were only able to test CpGs near a subset of genes in each comparison (number of genes included in testing for baseline corticosterone = 4143; stress-induced corticosterone = 4146; post-dexamethasone corticosterone = 3863; within-year treatment = 2913; between-year treatment = 452).

Using DAVID we identified a set of GO terms associated with biological processes or molecular functions that were overrepresented in the list of significant CpGs compared with the background list for that comparison (Gene Ontology Consortium, 2021). We filtered this list to include only GO terms with p-values $< .05$ after applying a FDR correction. We initially visualized the GO terms for each comparison using REVIGO (Supek et al., 2011); however, our study identified a relatively small number of GO terms and no clearly identifiable clusters of terms were identified in REVIGO. Therefore, we report the complete list of genes and GO terms associated with CpGs in each comparison.

3 | RESULTS

3.1 | Corticosterone and breeding timing

Birds that were treated with corticosterone in year one had higher baseline corticosterone in Year 2 (corticosterone treatment $\beta = 3.34$; 95% confidence interval = 1.43–5.24; Figure 2a; Table S2). Corticosterone treatment in Year 1 was not related to stress-induced corticosterone in Year 2 (Table S2), but females that were exposed to corticosterone in Year 1 had lower post-dexamethasone corticosterone in Year 2, indicating more robust negative feedback ($\beta = -4.86$; 95% confidence interval = −9.01 to −0.70; Figure 2b; Table S2). Finally, corticosterone-exposed females initiated their nesting attempt earlier in the following year ($\beta = -2.34$; 95% confidence interval = −4.49 to −0.20; Figure 2c; Table S2).

3.2 | Association between natural or experimental corticosterone and methylation

Using pre-treatment samples, we found that methylation percentage at 116 CpGs out of 78,143 tested was significantly associated with baseline corticosterone after FDR correction (Figure 3a; Table S1). For stress-induced corticosterone, we found a similar significant association at 356 out of 78,027 CpGs that were tested (Figure 3b; Table S1). For post-dexamethasone injection samples, we found a significant association between corticosterone and methylation at 735 out of 69,189 CpGs tested (Figure 3c; Table S1).

In models examining the effect of corticosterone treatment, we found that for samples collected within the same breeding season 1–2 weeks after treatment, 111 out of 48,070 CpGs tested showed significant evidence of differential methylation between treatment and control groups after FDR correction (Figure 3d; Table S1). We had fewer individuals and fewer CpGs that passed filtering for comparisons 1 year after treatment, but we found that 49 out of 6787 CpGs tested were significantly differentially methylated between treatment and control groups after 1 year (Figure 3e; Table S1). Although we were primarily interested in treatment effects, these models also showed that pre-treatment methylation at a given CpG site generally predicted post-treatment methylation both within a year (Figure S3A) and for samples collected 1 year later (Figure S3B).

3.3 | Association between differentially methylated CpGs and genes

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

In examining the function of genes identified in this process, we found no clear evidence for direct links to genes known to be involved in the regulation of the HPA axis. Stress-induced

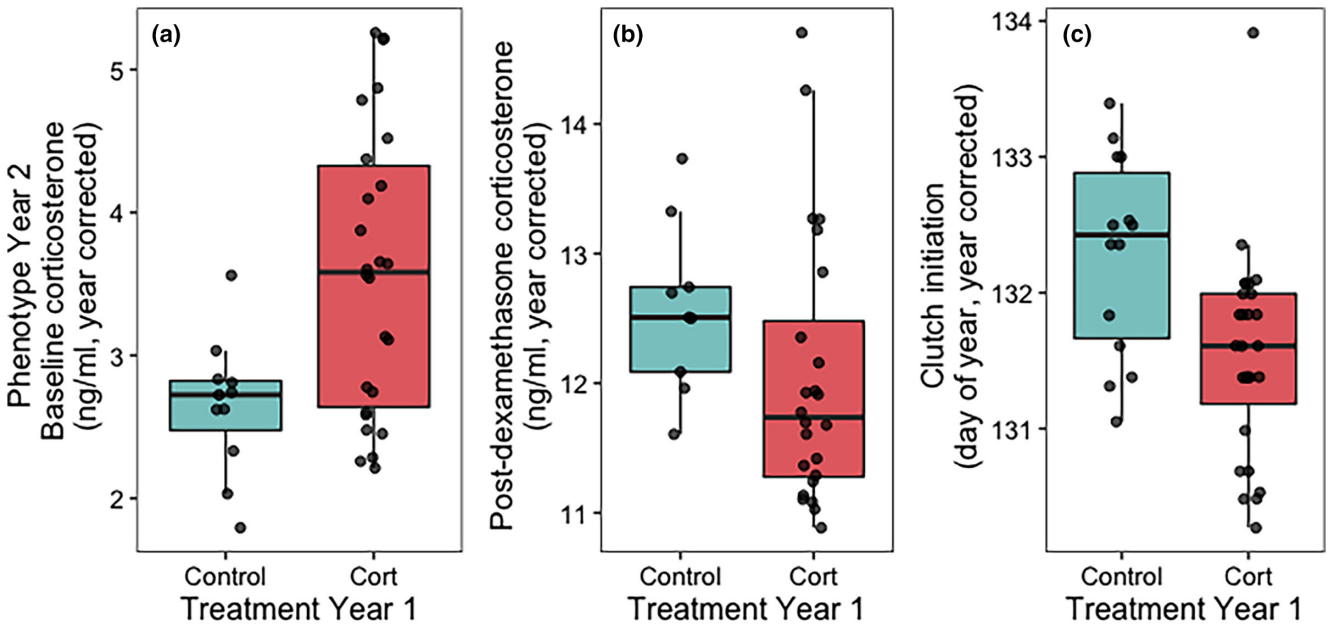

FIGURE 2 Effect of corticosterone treatment in 1 year on measures of baseline corticosterone (a), post-dexamethasone corticosterone (b) and clutch initiation date (c) in the following year. Points are partial residuals of raw data collected for average-year effects. Boxes and whiskers show the median, interquartile range and 1.5 times IQR. [Colour figure can be viewed at wileyonlinelibrary.com]

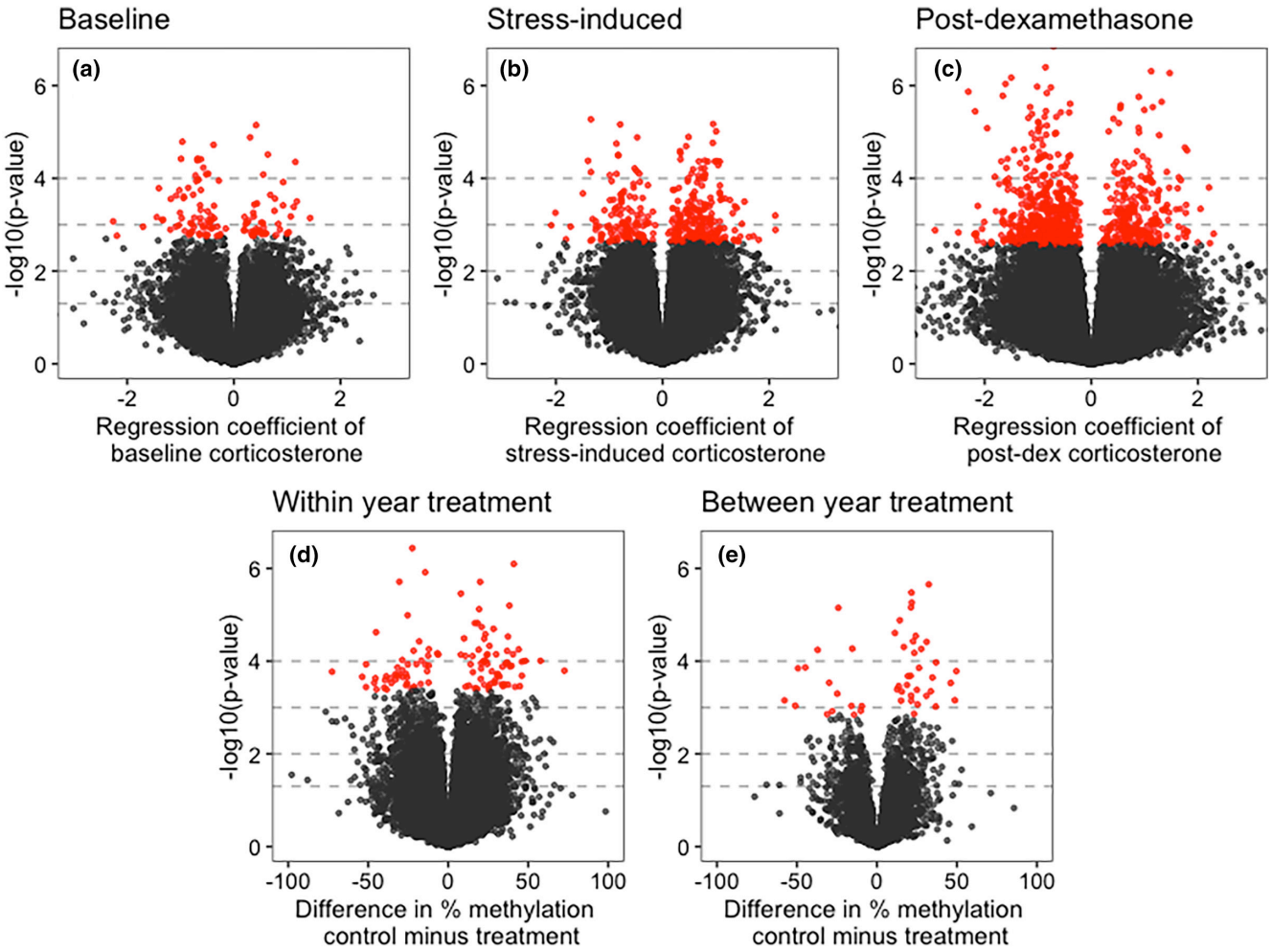

FIGURE 3 Association between DNA methylation and corticosterone from generalized linear mixed models (GLMMs) based on observational and experimental study components. Panels (a–c) show the pre-treatment regression coefficient for baseline corticosterone, stress-induced corticosterone and post-dexamethasone corticosterone on methylation percentage, respectively. Panels (d) and (e) show the difference in methylation for control versus 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 .05, .01, .001 and .0001 moving from the bottom to top of each plot to aid in interpretation. [Colour figure can be viewed at wileyonlinelibrary.com]

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

3.4 | GO term analysis

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

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

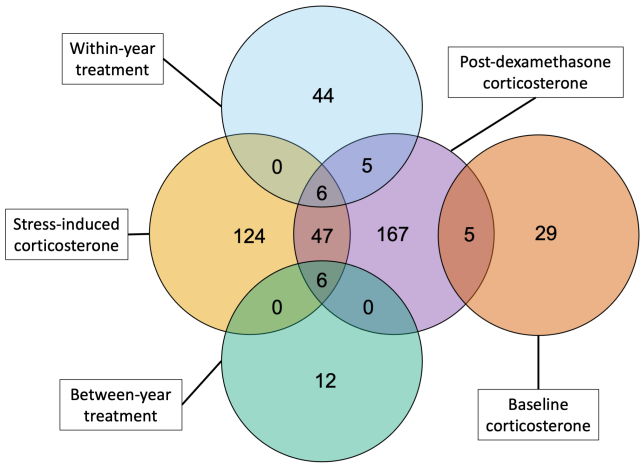

FIGURE 4 Number of genes near CpGs that were significantly associated with natural variation in corticosterone (baseline, stress-induced and post-dexamethasone) or with experimental corticosterone elevation (within-year and between-year). No identified genes were shared between the comparisons with circles that do not overlap. [Colour figure can be viewed at wileyonlinelibrary.com]

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

4 | DISCUSSION

We found that experimental increases in corticosterone induced long-term phenotypic changes. Females that experienced a few brief spikes in exogenous corticosterone had stronger negative feedback in the HPA axis and bred earlier in the subsequent year; these characteristics are typically associated with high stress resilience and reproductive success in this population. Furthermore, natural variation in corticosterone was correlated with DNA methylation, and experimental treatments altered DNA methylation patterns. Importantly, regulation of DNA methylation in response to corticosterone occurred rapidly in adults (within days) and resulted in detectable changes at least 1 year after treatment, paralleling the changes in physiological and behavioural phenotypes. Taken together, these results support the idea that the activation of the stress response machinery changes traits associated with stress resilience, and thus may prime future responses to challenges. DNA methylation could act as a key mechanism linking the prior experience of stressors—including during adulthood—to subsequent coping ability. However, we did not identify any clear functional consequences of methylation changes in this study. Rapid endocrine flexibility and adaptive calibration of the stress response have emerged as key determinants of resilience to challenges (Grindstaff et al., 2022; Hau & Goymann, 2015; Taff & Vitousek, 2016) and understanding the mechanistic basis of these patterns is an important step in predicting when flexibility is sufficient for coping with changing conditions.

The changes in phenotype that we detected 1 year after experimentally elevating corticosterone partially matched our predictions if exposure altered phenotype in ways that would increase future stress resilience. We found that, compared with controls, experimental females initiated breeding earlier and had stronger negative feedback in the subsequent year. In tree swallows, clutch initiation date is a strong predictor of both seasonal and lifetime reproductive success and is often considered a proxy for individual quality or condition (Winkler et al., 2020). Similarly, the strength of negative feedback is consistently the best physiological predictor of coping ability and reproductive success both under natural conditions and after imposing experimental challenges (Taff et al., 2018; Zimmer et al., 2019). However, contrary to our prediction, treatment had no effect on stress-induced corticosterone the following year. We also found that compared with control females, corticosterone-dosed females had higher baseline corticosterone and no difference in stress-induced corticosterone 1 year after treatment. Baseline corticosterone does not predict stress resilience in this population (Zimmer et al., 2019). However, because baseline corticosterone often increases in preparation for periods of high energetic demands, including the demands of reproduction (the Cort-Adaptation hypothesis, Bonier et al., 2011; Casagrande et al., 2018; Hau et al., 2010), these results might reflect an increased allocation to breeding in subsequent years in corticosterone-treated females. For example, female European starlings (*Sturnus vulgaris*) manipulated to increase parental investment increased their baseline corticosterone during incubation (Love et al., 2014). Similarly, tree swallows that increase baseline corticosterone more over the reproductive period provision offspring at higher rates (Bonier et al., 2011). Thus, our results might represent a combination of priming effects coupled with the immediate energetic demands of breeding earlier. Alternatively, corticosterone treatment in year one could have influenced measurements in year two indirectly, rather than by priming responses to later challenges.

Our study also adds to the growing recognition of bidirectional links between coping ability and DNA methylation. While this relationship has been demonstrated in laboratory-based model systems (Liu et al., 1997; Weaver et al., 2004), the potential for environmental stressors to trigger methylation, and affect subsequent coping ability, has only recently been explored in wild animals. Early results in wild animals suggest patterns similar to those seen in laboratory rodents. For example, early life maternal care and social connections in spotted hyenas (*Crocuta crocuta*) predict DNA methylation and glucocorticoid regulation as an adult (Laubach et al., 2019, 2021). Similar effects can play out in adulthood; for example, in savannah baboons (*Papio cynocephalus*), high

social status as an adult is associated with more rapid changes in DNA methylation (epigenetic ageing) as a consequence of the social stress that accompanies high status (Anderson et al., 2021). Our results are consistent with the results derived from laboratory rodents, wild mammals and a few studies in wild birds (e.g., Rubenstein et al., 2016), suggesting that flexible adjustment of methylation may be a general mechanism by which prior experiences of stressors are encoded in order to modulate future responses to challenges.

While there has been a rapid increase in studies of methylation in wild birds in recent years (e.g., Mäkinen et al., 2019; Rubenstein et al., 2016; Sheldon et al., 2018; von Holdt et al., 2023), relatively few studies have sampled the same adults multiple times. Our study design allowed us to assess the stability of genome-wide DNA methylation within individuals. We found that many CpGs that we interrogated had large between-individual differences in methylation and that those differences were typically stable even in samples collected 1 year apart. Compared with these individual differences, flexible changes in methylation were relatively smaller and detectable at fewer CpGs. The stable individual differences that we detected may be largely explained by genetic differences between individuals (e.g., Sepers et al., 2023), but the consequences of early life conditions may also play an important role in generating differences (Jimeno et al., 2019; Laubach et al., 2019; Sheldon et al., 2018). For example, early life climate conditions are related to lifelong methylation of the glucocorticoid receptor gene in superb starlings (*Lamprotornis superbus*, Rubenstein et al., 2016). We could not assess the possibility of a similar pattern in our study because we did not have information on early life conditions for our birds. Regardless of the source of these initial differences, our results clearly demonstrate that detecting subtle adjustments of methylation in adulthood to any treatment of interest will often require accounting for pre-treatment methylation. Our results also suggest that if changes in methylation are ecologically relevant for phenotypic flexibility during adulthood, these effects are likely to occur through modest changes at specific locations rather than through large-scale modifications to methylation patterns.

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

Another potential reason for our failure to find clear links between changes in DNA methylation and genes associated with the stress response may result from the limitations of our approach. An advantage of RRBS is that it does not rely on pre-selecting candidate genes, but a disadvantage is that not all relevant genes are necessarily tested. After filtering our data, many of the genes with known roles in the HPA axis were not included in comparisons or had coverage at only a few CpG sites. Thus, we did not directly test for methylation differences for many key genes. It is possible that deeper sequencing of our libraries or a whole-genome approach would have improved our ability to detect functional differences. Moreover, although we used the most complete reference genome available for tree swallows, many CpGs mapped to predicted genes with unknown functions. Continued improvement of assembly and annotation for reference genomes of non-model organisms is important for understanding the functional importance of epigenetic changes. Studying DNA methylation in non-model systems is a rapidly developing field and many recent papers outline the pros and cons of various approaches (Beck et al., 2022; Laine et al., 2022; Sepers et al., 2019). One particularly promising approach that may strike a balance between a focus on candidate genes and the ability to detect genome-wide associations is to combine RRBS with probes that enrich sequences at a potentially large number of target genes (target-enriched enzymatic methyl sequencing, Rubenstein & Solomon, 2023).

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

these possibilities and shed light on when and how individuals succeed or fail through flexible regulation of the physiological response to challenges.

AUTHOR CONTRIBUTIONS

Conor C. Taff and Maren N. Vitousek conducted the field-based data collection. Conor C. Taff and Maren N. Vitousek conceived the study. Conor C. Taff and Sabrina M. McNew conducted the laboratory work for RRBS. Conor C. Taff and Leonardo Campagna conducted the laboratory work for creating the reference genome and Leonardo Campagna carried out the bioinformatics for genome assembly and annotation. Conor C. Taff analysed and visualized the data with assistance from Sabrina M. McNew and Leonardo Campagna. Conor C. Taff drafted the paper with input from all authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The complete set of bioinformatic processing scripts, R code and data associated with each sample is available and permanently archived on Zenodo (https://doi.org/10.5281/zenodo.8125152). Raw sequence data from RRBS are available on GenBank (BioProject ID PRJNA953597). The genome assembly generated for this project is deposited on GenBank (BioProject ID PRJNA553513).

ETHICS STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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