**Manipulation of a social signal affects DNA methylation of a stress-related gene in a free-living bird**

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**Abstract:**

Social status directly affects the health of humans and other animals. Low status individuals receive more antagonistic encounters, have fewer supportive relationships, and have overall worse health outcomes. Epigenetic regulation of the HPA-axis, the neuroendocrine pathway that activates in response to stressors, may be one process that is sensitive to the social environment. Here, we experimentally manipulated plumage, a key social signal in female tree swallows (*Tachycineta bicolor*) and quantified methylation of four genes in the HPA axis before and after treatment. We found that dulling the white breast plumage affected methylation in one gene, CRHR1; however, the effect depended on the original brightness of the bird. Methylation in this gene was correlated with baseline corticosterone levels, suggesting that DNA methylation of CRHR1 helps regulate glucocorticoid production in this species. Methylation in two other genes, FKBP5 and GR, changed over the course of the experiment, independent of treatment. These results show that methylation of these genes is labile into adulthood and suggest that epigenetic regulation of the HPA axis could help birds respond to current environmental conditions.

**Introduction:**

The social environment affects the health of humans and other social animals. Lower status individuals have shorter lifespans and are more susceptible to disease (Alwin & Wray, 2005; Razzoli et al., 2018; Singh-Manoux et al., 2003). . The health effects of social status are partially attributed to variation in access to resources, differences in risk-aversion, and other environmental mediators of health (Singh-Manoux et al., 2003; Snyder-Mackler et al., 2019). However, increasing evidence suggests that encounters between individuals living in the same social environment may have direct and lasting physiological consequences. One potential mechanism that may mediate the effects of social interactions on health is the hypothalamic—pituitary—adrenal (HPA) axis (Creel et al., 2013). This neuroendocrine pathway underlies physiological and behavioral changes, including the production of glucocorticoid hormones, which shift energy towards immediate survival in response to an adverse event (Monaghan, 2014; Sapolsky et al., 2000; Wingfield et al., 1998). Social status can interact with the stress response in both positive and negative ways. Social bonds between high-ranking individuals can reduce glucocorticoid levels in the face of stressors (Engh et al., 2006; Young et al., 2014). For instance, male Barbary macaques (*Macaca sylvanus*) with strong social bonds had lower fecal glucocorticoid levels in response to social stressors (aggressive encounters) as well as environmental stressors (cold temperatures) (Young et al., 2014). On the other hand, low status individuals may receive frequent antagonistic attacks from conspecifics, leading to negative effects of chronically elevated glucocorticoid levels (Korte et al., 2005; Snyder-Mackler et al., 2019).

Epigenetic changes to genes involved in the HPA axis may underlie the connection between the social environment and stress-related phenotypes (Lee & Sawa, 2014; Snyder-Mackler et al., 2019; Turecki & Meaney, 2016). Epigenetic modifications, such as DNA methylation, are sensitive to the environment and can affect DNA expression and physiology (Sotnikov & Markt, 2014). A robust body of literature from primates and lab rodents demonstrates that environmental stressors may cause persistent changes in the function of genes involved with the stress response (Schartner et al., 2017; Turecki & Meaney, 2016). For instance, one study found that prenatal trauma exposure in mice led to changes in methylation of two genes in the stress axis, which were accompanied by changes in mRNA levels, corticosterone levels, and behavior (Plank et al., 2021). Another study found changes in the social environment of primates can cause changes in chromatin availability and gene expression, leading to dysregulation in the HPA axis and corresponding negative health effects (Snyder-Mackler et al., 2019).

Many bird species are highly social; however, the epigenetic markers of their social landscape have not been well studied. A recent study found that increased competition in tree swallows (*Tachycineta bicolor*) has epigenetic and transcriptomic effects in brain tissue after just two days (Bentz et al., 2021). Such changes could underlie documented effects of social interactions on the stress physiology of birds (Deviche et al., 2014; Landys et al., 2010; Potticary & Duckworth, 2020). For example, female bluebirds (*Sialia mexicana*) living in dense populations have higher circulating levels of glucocorticoids, which may be transmitted to their eggs, affecting the aggression and dispersal behaviors of their offspring (Potticary & Duckworth, 2020). Still, it is largely unclear whether epigenetic mechanisms mediate a physiological response to the social environment in birds, and over what time scales these processes operate.

In this study, we experimentally manipulated a key plumage signal in tree swallows and measured its effects on methylation of four genes involved in the HPA-axis. Plumage is an important social signal in birds, conveying information about condition, parasite load, and social dominance (Mason & Bowie, 2020; Mougeot et al., 2010; Taff, Zimmer, et al., 2019). In female tree swallows, brighter white females secrete more corticosterone in response to stress, have stronger immune function, more social interactions with conspecifics and are less likely to abandon their nests under stressful conditions (Beck et al., 2015; Taff, Zimmer, et al., 2019). Methylation in some areas of the genome is correlated with plumage brightness and stress resilience, suggesting that epigenetic processes connect this plumage signal to physiology (Taff, Campagna, et al., 2019). Experimental dulling of this plumage alters the social interaction, microbiome, and glucose levels of female swallows, and dulled females invest more in reproduction compared to controls (Taff et al., 2021). This shift in behavior and physiology in response to an altered social landscape could be mediated by epigenetic changes to the HPA-axis. Here, we test whether plumage dulling of female tree swallows has an epigenetic signature in genes involved in the HPA axis, and secondly, whether DNA methylation of HPA-associated genes is correlated with corticosterone levels.

**Methods:**

We studied breeding tree swallows in Ithaca, New York, USA, during April to July of 2017 (42 degrees 30’11” N, 76 degrees 26’ 13” W). Females at each nest were captured three times during the breeding season (day 6-7 of incubation, day 3-4 after hatching, and day 7-8 after hatching). At the first capture, females were assigned randomly either to a plumage dulling treatment or to a control treatment after balancing treatments by female age (second year vs. after second year). We dulled plumage by uniformly coloring the feathers from the throat to the legs using a light gray nontoxic marker (Faber-Castell PITT Artist Pen ‘Big Brush’ Warm Grey III 272), following methods in (Taff et al., 2021). Females in the control treatment were marked in the same way with a colorless marker (Prismacolor Premier Colorless Blender PB-121). The marking treatment was re-applied at the second and third captures. We quantified the effects of dulling through spectrophotometry (Supplementary Materials). In total, the dulled group included 34 females and the control group included 36 females. Females did not differ significantly in initial brightness (average percent reflectance in the control group = 39.85, dulled group = 41.05; *P* = 0.491). Experimental dulling significantly reduced plumage brightness for all individuals in the treatment (Taff et al., 2021).

At the first and third captures (hereafter “pre-treatment” and “post-treatment”) we took a small blood sample within three minutes of capture via brachial venipuncture to measure stress physiology and quantify DNA methylation. At the pre-treatment capture we took two additional blood samples: the first was collected after 30 minutes to measure maximal corticosterone elevation (“stress-induced corticosterone”). Immediately after taking the stress-induced sample, we injected birds with 4.5 ml/g; Mylan® 4 mg/ ml dexamethasone sodium phosphate, a synthetic glucocorticoid that induces negative feedback, and then took a final blood sample 30 minutes later (“dexamethasone-controlled corticosterone”) (Zimmer et al., 2019). Within three hours erythrocytes and plasma were separated by centrifugation and stored separately at -30°C. Corticosterone was measured in the plasma using commercially available microplate kits that have been validated in this population (Zimmer et al., 2019; detailed methods for corticosterone quantification are in the Supplemental Methods). Data on the behavior, microbiome, corticosterone regulation, and reproductive success of adults in this experiment have been published previously (Taff et al., 2021). Here, we focus on the epigenetic effects of plumage manipulation and their connection to glucocorticoid levels.

We investigated DNA methylation of four genes: Corticotropin Releasing Hormone (CRH), Corticotropin Releasing Hormone Receptor 1 (CRHR1), FKBP Prolyl Isomerase 5 (FKBP5), and Glucocorticoid Receptor (GR, also called NR3C1), all of which form part of the HPA axis. Epigenetic dysregulation of these genes is associated with stress-related pathologies in humans and rodents (Lee & Sawa, 2014). We submitted extracted DNA samples to EpigenDx (Worcester, MA) for methylation analysis using pyrosequencing, resulting in measures of methylation at between 11 and 19 CpG sites per gene. Primer development, assay validation, and pyrosequencing was conducted at EpigenDX.

We extracted whole genomic DNA from frozen erythrocytes using Qiagen DNEasy Blood and Tissue Kits (Valenica, CA) following the manufacturer’s protocol. We assayed DNA concentration and purity on a NanoDrop Spectrophotometer (ThermoFisher Scientific, Waltham, MA) and then shipped purified DNA to EpigenDx (Hopkinton, MA) for methylation quantification.

Between 23 and 96 primer pairs were designed per gene to assay methylation in each of the four target genes (See Supplemental Information for extended details on assay development). Based on initial tests with a separate set of 36 tree swallow samples, a subset of primer pairs with good amplification rates were selected to maximize coverage of regions with high CpG density and high variation in methylation levels. With those criteria, we used three primer pairs to assay methylation in GR, and one primer pair each in CRH, CRHR1, and FKPB5. A total of 121 samples from 70 individual birds were then pyrosequenced.

Pyrosequencing procedures followed standard methods developed by EpigenDx (Hopkinton, MA). For each sample 500 ng of genomic DNA was bisulfite treated using the EZ DNA Methylation kit (Zymo Research, Inc., CA). Bisulfite treated DNA was purified according to the manufacturer’s protocol and eluted to a final volume of 46 µL. Then, target regions were amplified in PCR reactions containing 1 µL of bisulfite treated DNA and 0.2 µM of each primer. One primer was biotin-labeled and HPLC purified (for subsequent purification with Sepharose beads).

PCR product was bound to Streptavidin Sepharose HP (GE Healthcare Life Sciences), after which the immobilized PCR products were purified, washed, denatured with a 0.2 µM NaOH solution, and rewashed using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Qiagen), as per the manufacturer’s protocol. Next, 0.5 µM of sequencing primer was annealed to the purified single stranded PCR products. 10 µL of the PCR products were sequenced by Pyrosequencing on the PSQ96 HS System (Pyrosequencing, Qiagen) following the manufacturer’s instructions.

The methylation status of each CpG site was determined individually as an artificial C/T SNP using QCpG software (Pyrosequencing, Qiagen). The methylation level at each CpG site was calculated as the percentage of the methylated alleles divided by the sum of all methylated and unmethylated alleles. Each experiment included non-CpG cytosines as internal controls to detect incomplete bisulfite conversion of the input DNA. In addition, a series of unmethylated and methylated DNA are included as controls in each PCR. Furthermore, PCR bias testing was performed by mixing unmethylated control DNA with in vitro methylated DNA at different ratios (0%, 5%, 10%, 25%, 50%, 75%, and 100%), followed by bisulfite modification, PCR, and Pyrosequencing analysis.

*Analysis*

We modeled methylation in each gene separately using linear mixed effects models in R (version 4.2.2). Each model predicted per-CpG methylation as a function of capture (pre- or post-treatment), treatment, and initial plumage brightness. We first created an interaction model of the three main effects (i.e. capture\*brightness\*treatment). When interactions were not significant, they were removed, and we present estimates from the additive model instead. Models also included the random effect of CpG site and the random effect of individual. We logit transformed methylation data prior to modeling it following best-practices for percentage data (Stevens et al., 2016; Warton & Hui, 2011). We partitioned the variance in methylation among the fixed effects and random effects using rptR to compare how much variation in methylation was explained by experimental variables vs. between-individual differences (Stoffel et al., 2017). Secondly, we tested for a relationship between corticosterone levels and methylation at each gene by modeling methylation as a function of corticosterone, using methylation and corticosterone data from both captures. These models included the random effects of individual and CpG identity. Conceptually, we predicted that DNA methylation controls gene expression in the HPA axis and thus affects blood corticosterone levels (i.e. corticosterone depends on DNA methylation). However, due to the hierarchical structure of the data, we found it more appropriate to model DNA methylation as the dependent variable (with corticosterone as a fixed effect and CpG as a random effect).

**Results:**

We quantified methylation data at 56 CpG sites in the four focal genes (CRH, FKBP5, GR, and GRHR1). We received methylation data from between 85 and 120 samples at each CpG. The mean methylation per site varied from 2.6% to 76.8%.

Methylation of the CRH gene did not significantly differ between treatments or between captures and was not associated with initial female brightness (Figure 1, Table 1). Methylation in the FKBP5 gene was significantly higher pre-treatment compared to post-treatment (Figure 1, Table 1). However, there was no significant difference between treatments, and no relationship between initial brightness and methylation. In contrast, methylation in the GR gene was significantly lower pre-treatment compared to post-treatment (Figure 1, Table 1). Again, methylation did not significantly differ between treatments and was uncorrelated with initial brightness. Finally, we found that methylation of the CRHR1 gene depended on the three-way interaction between treatment, capture, and brightness (Figure 2, Table 1). Females in the dulled treatment tended to lose methylation in CRHR1 following experimental dulling. The decrease in methylation post-treatment was strongest for females that were originally bright. In contrast, for females in the control group, methylation tended to increase slightly post-treatment and did not depend on initial brightness.

In all genes the random effects of individual and CpG explained substantially more variation than did the fixed effects of treatment, capture number, and initial brightness (i.e. Conditional R2 >> Marginal R2; Table 1). Individual bird identity alone explained between 2.9% and 48% of variation in methylation at each gene (Table 1).

Methylation of CRHR1 was significantly associated with baseline corticosterone levels. There was a significant negative relationship between baseline corticosterone and methylation in CRHR1 (LMM P < 0.001; Figure 2; Table S1). Baseline corticosterone was not associated with methylation of any other of the three genes (Table S1). There was no significant association between pre-treatment stress-induced corticosterone and methylation of any of the four genes (Table S2). There was also no significant association between pre-treatment dexamethasone-controlled corticosterone and methylation in any of the four genes (Table S3).

**Discussion**

In this study we tested the effects of manipulating a key social plumage signal on HPA-axis methylation in tree swallows. We found that experimentally dulling the white breast plumage of tree swallows resulted in changes in the methylation of the corticotropin releasing hormone receptor 1 (CRHR1) gene. The effect of dulling on methylation was strongest for females who were initially bright, suggesting that high-status females experienced the strongest consequences of social environment change. CRHR1 binds corticotropin releasing hormone (CRH), triggering the release of the adrenocorticotropic hormone which leads to the release of corticosteroids (Schartner et al., 2017). Decreased methylation of CRHR1 and associated upregulation of this gene are associated with anxiety-related phenotypes in humans and rodent models (Plank et al., 2021; Schartner et al., 2017; Sotnikov & Markt, 2014). Baseline corticosterone values in our tree swallows were negatively associated with methylation across this gene. Thus, the decrease in methylation that we observed in females is consistent with the upregulation of this gene and activation of the HPA-axis in response to plumage dulling and the concomitant changes to the social environment (Taff et al., 2021).

In contrast, the methylation of the other three genes we studied (GR, FKBP5, and CRH) was not significantly associated with treatment. We chose these candidate genes as targets because they have known epigenetic associations with stress in humans and model organisms (Lee & Sawa, 2014). Still, we interrogated relatively few sites across these specific genes and so it is possible that we did not detect some of the epigenetic effects of the plumage manipulation. Previous analyses of this experiment found that plumage dulling altered social interactions and had physiological effects on microbiome diversity and glucose levels of female birds (Taff et al., 2021). However, there was no significant effect of treatment on corticosterone levels (Taff et al., 2021). Thus, the fact that we similarly saw no treatment effect on methylation in three of the four genes we studied suggests that plumage manipulation had relatively minor effects on HPA-axis activity. Previous analysis of behavioral changes in response to plumage dulling indicated that manipulation of this signal affects the social landscape in subtle and complex ways. Indeed, many of the effects of treatment identified by Taff et al. (2021) were dependent on nestling stage and initial female brightness. More work is needed to understand exactly how manipulation of white plumage in tree swallows affects their social environment.

The methylation of two genes (GR and FKBP5) were not affected by treatment; however, methylation did change significantly between the two time points in the study (pre-treatment: days 6-7 of incubation and post-treatment: ~ 14 days later). We do not know for sure what factors affected methylation of these genes. However, HPA axis activation can inhibit reproductive success (Bókony et al., 2009; Wingfield & Sapolsky, 2003). Thus, epigenetic shifts in these genes may have helped birds downregulate these genes over the breeding period. Indeed, a previous study in this population found that birds mount a weaker corticosterone response to environmental stressors during provisioning compared to incubation (Vitousek et al., 2022).

The glucocorticoid receptor (GR, also called NR3C1) is an intracellular transcription factor that mediates the expression of several proteins involved in the stress response (Guidotti et al., 2013; Zannas et al., 2016). Methylation of GR increased over the study period in our swallows. Since methylation is typically associated with lower gene expression, this change could signal a shift towards modulation of the HPA axis during breeding. Alternatively, methylation of this gene could also be related to environmental conditions. A previous study of superb starlings (*Lamprotornis superbus*), found that methylation in the promoter of GR was positively correlated with environmental conditions (rainfall) early in life (Rubenstein et al., 2016). The change that we observed over our study shows that methylation of this gene is labile over relatively short periods (~ 2 weeks) in adults. Thus, methylation in this gene may help birds adapt to variable environments both during development and into adulthood.

The FK506-binding protein 41 (FKBP5) is a negative regulator of GR signaling (Menke et al., 2013; Zannas & Binder, 2014). Dysregulation of FBKP5 is associated with psychiatric disorders and other stress-related phenotypes in humans and laboratory models (Zannas et al., 2016; Zimmer et al., 2020). Although data from wild organisms are limited, FKBP5 expression in house sparrows (*Passer domesticus*)is correlated with HPA flexibility and exploratory behavior (Zimmer et al., 2021). FKBP5 therefore appears to be a key regulator of the HPA axis across many vertebrates (Zimmer et al., 2020). In our study, methylation of FKBP5 decreased over the course of the study period. Decreased methylation of FKBP5 is expected to upregulate this gene and inhibit glucocorticoid receptor signaling (Zannas et al., 2016). Correspondingly, the change that we observed in our birds could also be related to an effort to downregulate the stress response during breeding. FKBP5 was also notable because individual bird identity explained a substantial proportion (48%) of the variation in methylation in this gene (Table S3). The consistency in methylation of this gene across individuals suggests that perhaps some epigenetic programming of this gene occurs early in life and/or is transgenerationally inherited. In fact, another study found that methylation in FKBP5 is a heritable epigenetic marker of trauma in humans (Yehuda et al., 2016), supporting the idea that FKBP5 could mediate physiological effects of both current and historical stressors.

A growing number of studies have investigated the plasticity of DNA methylation in response to environmental conditions in free-living animals. However, most of these studies have focused on early life epigenetic programming (McNew et al., 2021; Sheldon et al., 2020; Watson et al., 2019) and studies that sample the same individuals repeatedly are particularly rare (e.g. Anderson et al., 2021; Rubenstein et al., 2016). Our results highlight that DNA methylation patterns can change within an individual in response to specific experimental manipulation, and moreover, may be naturally labile over short periods. Although we did not see changes across all the genes we studied in response to plumage dulling, the change in methylation in CRHR1 suggests that epigenetic mechanisms may mediate the effects of social environment on the physiology of free-living social birds.

**Data Availability**

Data and scripts used in the analysis are available at <https://github.com/smcnew/tres_pryo> and will be archived permanently on zenodo upon acceptance.

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**Ethical Statement**

Work was conducted under federal and state scientific collecting permits to MNV (USGS 24129, USFWS MB42428C; New York State 215 and 2350). All procedures were approved by the Cornell University Institutional Animal Care & Use Board (IACUC protocol 2019-0023 and 2001-0051).

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**Tables:**

Table 1: Outputs of linear mixed effect models (LMMs) modeling the effects of experimental treatment, capture number, initial brightness, and their interaction on methylation of each of the study genes.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **CRH** | | | **CRHR1** | | | **FKBP5** | | | **GR** | | |
| *Predictors* | *Estimates* | *CI* | *p* | *Estimates* | *CI* | *p* | *Estimates* | *CI* | *p* | *Estimates* | *CI* | *p* |
| Intercept | -1.59 | -1.98 – -1.21 | **<0.001** | -2.52 | -2.97 – -2.08 | **<0.001** | 0.58 | -0.03 – 1.20 | 0.063 | -0.94 | -1.59 – -0.28 | **0.005** |
| Treatment[Dulled] | -0.05 | -0.17 – 0.06 | 0.353 | 0.01 | -0.60 – 0.61 | 0.980 | -0.11 | -0.36 – 0.14 | 0.382 | 0.10 | -0.01 – 0.21 | 0.075 |
| Capture number | -0.02 | -0.04 – 0.00 | 0.096 | 0.13 | 0.02 – 0.24 | **0.022** | -0.10 | -0.14 – -0.06 | **<0.001** | 0.11 | 0.06 – 0.15 | **<0.001** |
| Initial brightness | 0.00 | -0.00 – 0.01 | 0.365 | 0.00 | -0.01 – 0.01 | 0.739 | -0.00 | -0.01 – 0.01 | 0.943 | -0.00 | -0.01 – 0.00 | 0.698 |
| Treatment:Capture |  |  |  | 0.11 | -0.06 – 0.29 | 0.209 |  |  |  |  |  |  |
| Treatment:Brightness |  |  |  | 0.00 | -0.01 – 0.02 | 0.795 |  |  |  |  |  |  |
| Capture:Initial brightness |  |  |  | 0.00 | -0.00 – 0.00 | 0.795 |  |  |  |  |  |  |
| Treatment:Capture:Initial brightness |  |  |  | -0.01 | -0.01 – -0.00 | **0.020** |  |  |  |  |  |  |
| Observations | 1401 | | | 2116 | | | 1272 | | | 1537 | | |
| Marginal R2 / Conditional R2 | 0.004 / 0.883 | | | 0.010 / 0.884 | | | 0.010 / 0.780 | | | 0.003 / 0.878 | | |
| Variance partitioning (repeatability): |  | | |  | | |  | | |  | | |
| Band [CI] | 0.153 [0.081, 0.308] | | | 0.212 [0.124, 0.35] | | | 0.48 [0.345, 0.624] | | | 0.029 [0.015, 0.064] | | |
| CpG [CI] | 0.729 [0.491, 0.847] | | | 0.67 [0.479, 0.797] | | | 0.298 [0.121, 0.47] | | | 0.848 [0.698, 0.914] | | |
| Fixed effects [CI] | 0.004 [0.001, 0.036] | | | 0.01 [0.007, 0.05] | | | 0.01 [0.004, 0.075] | | | 0.003 [0.001, 0.013] | | |