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

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**Abstract:**

**Introduction:**

The social environment affects the health of humans and other social animals. Lower status individuals generally have shorter lifespans and are more prone to disease (Alwin & Wray, 2005; Razzoli et al., 2018; Singh-Manoux et al., 2003). Part of this variation in health is due to correlates of social status, such as access to resources. However, increasing evidence suggests that the social environment directly affects individual physiology and fitness (Snyder-Mackler et al., 2019).

One potential mechanism that may mediate the effects of social interactions on health is hypothalamic—pituitary—adrenal (HPA) axis, the neuroendocrine pathway that regulates the production of glucocorticoid hormones (Creel et al., 2013). The production of glucocorticoid hormones is part of a suite of physiological and behavioral changes that help individuals cope with unpredictable biotic and abiotic stressors (Monaghan, 2014; Sapolsky et al., 2000; Wingfield et al., 1998). Although the production of glucocorticoids is part of an adaptive response to acute stressors, chronically high levels of circulating glucocorticoids are associated with negative health effects (Clinchy et al., 2004; Korte et al., 2005). Low status individuals may experience the negative effects of chronic stress as the targets of antagonistic interactions from conspecifics and/or a lack of social support (Snyder-Mackler et al., 2019). On the other hand, social bonds between individuals can also reduce glucocorticoid levels in the face of adverse environmental events, predation and/or aggressive encounters (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 and environmental stressors (Young et al., 2014).

Epigenetic changes to genes involved in the HPA axis may underlie the connection between the social environment and stress-related disorders (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). Increasing evidence suggests 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).

Outside of primates and laboratory models, relatively little is known about the interaction between the social environment and stress, and the epigenetic mechanisms that could connect these processes. Many birds live in highly complex social environments. Social interactions between conspecifics can raise glucocorticoid levels (Deviche et al., 2014; Landys et al., 2010; Potticary & Duckworth, 2020). For example, female bluebirds (*Sialia mexicana*) living in dense populations where competitive interactions are common 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). Moreover, a recent study found that increased competition in tree swallows (*Tachycineta bicolor*) has epigenetic and transcriptomic effects on brain tissue, which may mediate the physiological response to this stressor (Bentz et al., 2021).

In this study, we experimentally manipulated a key plumage signal in tree swallows and measured its effects on glucocorticoid levels and methylation of four genes involved in the stress response. 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 et al., 2019). In female tree swallows, brighter white females 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 et al., 2019). Experimental manipulation of this plumage alters social interactions, glucose levels, and breeding success (Taff et al., 2021). We predicted that experimentally dulling this white plumage would lead to decreased methylation and corresponding activation of the HPA axis. We secondarily predicted that the effects of plumage dulling would be strongest in females who were originally brightest.

**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 (Appendix). In total, the dulled group included 34 females and the control group included 36 females. Females did not differ significantly in initial brightness (average % 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. The change in brightness averaged -0.99% immediately after treatment to -0.49% 10-14 days post-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. Within three hours erythrocytes and plasma were separated by centrifugation and stored separately at -30. Baseline corticosterone was measured in the plasma using commercially available microplate kits that have been validated in this population (Taff et al., 2019, 2021). Data on the behavior, microbiome, and reproductive success of adults in this experiment have been published previously (Taff et al., 2021). Here, we focus on the effects of experimental manipulation of the breast plumage on DNA methylation and stress physiology.

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), 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). Methylation was quantified using pyrosequencing at between 11 and 19 CpG sites per gene (Appendix).

*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 also tested for a relationship between corticosterone levels and methylation at each gene by modeling methylation as a function of corticosterone. These models also 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 is dependent 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).

**Talk about preregistration?**

**Results:**

We quantified methylation data at 56 CpG sites in the four focal genes (GRH, KFPB5, GR, and GRHR1). After filtering, we had methylation data from between 85 and 120 samples at each CpG (Table S1). The mean methylation per site varied from 2.6% to 76.8% (Table S1).

Methylation the CRH gene did not significantly differ between treatments or between captures and was not associated with initial female brightness (Figure 1, Table S2). Methylation in the KFBP5 gene was significantly higher pre-treatment compared to post-treatment (Figure 1, Table S3). However, there was no significant difference between treatments, and no relationship between initial brightness and methylation. In contrast, methylation in the GRn gene was significantly lower pre-treatment compared to post-treatment (Figure 1, Table S4). Again, methylation did not significantly differ between treatments and was uncorrelated with initial brightness. Finally, we found that methylation of the GRHR1 gene depended on the three-way interaction between treatment, capture, and brightness (Figure 2, Table S5). Females in the dulled treatment tended to lose methylation in GRHR1 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.

Baseline corticosterone levels did not differ significantly between treatments or between captures and were not associated with initial plumage brightness (Table S6). However, methylation of GRHR1 was significantly associated with corticosterone levels. There was a significant negative relationship between baseline cort and methylation in GRHR1 (Figure 2; Table S7). Cort was not associated with methylation of any other of the three genes (Tables S8 – S10).

**Discussion**

In this study we tested the effects of manipulating a key social plumage signal on HPA-axis methylation and 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. These changes varied with initial female brightness, suggesting that the effect of the plumage manipulation was dependent on the initial quality of the female and/or her position in the social environment.

CRHR1 binds corticotropin releasing hormone (CRH), triggering the release of the adrenocorticotropic hormone (ACTH), leading to the excretion 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 mouse models (Plank et al., 2021; Schartner et al., 2017; Sotnikov & Markt, 2014). We did not measure ACTH in the birds in our study; however, baseline corticosterone was negatively associated with methylation across this gene. Thus, the decrease in methylation that we observed in females in the dulling treatment is consistent with the upregulation of this gene and thus activation of the HPA axis in response to alterations in the social environment.

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 (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 analysis of this experimental manipulation found that plumage dulling had effects on the behavior, microbiome, and glucose levels of the females in our study (Taff et al., 2021). However, many of these effects were dependent on nestling stage and initial female brightness. The fact that we did not detect significant differences in corticosterone levels between treatments or between sampling points further indicates that the effects of our signal manipulation may have been relatively minor, and/or context dependent. More work is needed to understand exactly how manipulation of white plumage in tree swallows affects their social environment.

The methylation of two genes (FKBP and GR) did change significantly between the two time points in the study (pre-treatment: days 6-7 of incubation and post-treatment: days 7-8 after hatching). The glucocorticoid receptor (GR) is an intracellular transcription factor. Upon binding to glucocorticoid hormones, GRs move into the nucleus and affect the transcription of a number of proteins involved in the stress response (Guidotti et al., 2013; Zannas et al., 2016). The FK506-binding protein 41 (FKBP5) is a negative regulator of GR signaling and has important role in the modulation of the HPA axis. (Menke et al., 2013; Zannas & Binder, 2014). Disregulation of FKBP5 is associated with a number of psychiatric disorders in humans and stress-related phenotypes in laboratory models (Zannas et al., 2016).

The change in methylation of GR and FKBP5 demonstrate that methylation in genes involved in the HPA axis is labile over short time periods (approximately 14 days in this study). We do not know for sure what factors were responsible for this shift. However, female tree swallows lose body mass over the course of incubation and nestling period (Taff et al., 2022). Thus, reproduction and parental care may itself be a source of stress. In our study, methylation of FKBP5 decreased in tree-swallows over the course of the study period. Decreased methylation of FKBP5 is expected to cause upregulation of expression of this gene and corresponding inhibition of glucocorticoid receptor signaling (Zannas et al., 2016). Correspondingly, the change that we observed in our birds could be related to an effort to downregulate the stress response during an energetically costly life stage.

In contrast to FKBP5, methylation of GR increased over the study period. Since methylation is typically associated with lower gene expression, this change could also signal a shift towards downregulation or modulation of the HPA axis. A previous study of superb starlings (*Lamprotornis superbus*), which live in a highly unpredictable semi-arid environment, found that methylation in the promoter of GR was positively correlated with environmental conditions (rainfall) early in life (Rubenstein et al., 2016). The data in that study suggested that epigenetic mechanisms mediate adaptive priming of individuals to match variable environmental conditions. Our data could similarly reflect effects of environmental conditions on stress physiology of tree swallows. However, Rubenstein et al. (2016) found that GR methylation was programmed early in life, and remained largely stable through adulthood. In contrast, here we document intra-individual changes in GR methylation within the course of a single breeding season. Individual CpGs may vary in the stability of their methylation and the CpG sites interrogated in our study and Rubenstein et al. do not (2016) overlap entirely.

A growing number of studies are investigating 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 (Anderson et al., 2021; Rubenstein et al., 2016). Thus, 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 consistent changes across all the genes we studied, the change in methylation in CRHR1 following plumage dulling suggests that epigenetic mechanisms can mediate effects of the social environment on stress physiology of social animals.

**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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**Extra text**

*Alternate approach to studying cort x methylation 1 : base\_cort ~ average\_methylation*

We tested for an effect of average percent methylation in each gene on baseline corticosterone. There was no significant effect of methylation on baseline corticosterone for any gene.

*Alternate approach cort x methylation 2: base\_cort ~ Per\_cpg\_methylation*

We tested for an effect of percent methylation at each CpG in the GRHR1 gene on baseline corticosterone. We modeled baseline corticosterone as a function of methylation, including capture number and treatment as fixed effects and individual band as a random effect, and creating a separate model for each CpG. We adjusted P-values using a Bonferroni correction to account for multiple comparisons. After Bonferroni adjustment there were no significant associations between CpG methylation and baseline corticosterone.

DNA methylation is the epigenetic factor that is most widely studied in non-model organisms. However, other factors, including microRNAs (miRNAs) and histone modifications, likely are also important epigenetic regulators of the HPA axis (Sotnikov & Markt, 2014).