**Supplementary Material**

**McNew et al. 2023**

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

**Methods:**

*Plumage manipulation*

The coloration of female breast plumage was quantified following methods developed previously in this system [1,2]. We collected feathers from the center of the breast at each capture to quantify initial female brightness. Reflectance was measured with an Ocean Optics FLAMES-UV-VIS spectrophotometer with PX-2 pulsed Xenon light source and WS-1 white standard in OceanView v.1.5.2 (Ocean Optics, Dunedin, FL, U.S.A.). For each individual sample, we stacked and taped four feathers on an index card and then smoothed the barbs to create a patch large enough for measurement. We used a fibre-optic UV/VIS probe in a holster that blocked external light and took measurements at a fixed distance of 5 mm from the feather sample. Then, we collected spectra with a 10 scan average, 20 nm boxcar width and 60 ms integration time. Four separate spectra were taken for each feather stack. The probe was removed between measurements. For each female in the study, we measured four sets of feathers (two from first capture and one each from second and third capture). Reflectance spectra generated by OceanView were processed in R v.3.3.3 (R Core Team, 2016) using the package ‘pavo’ (Maia, Eliason, Bitton, Doucet, & Shawkey, 2013). We calculated mean breast brightness as the average reflectance from 300 to 700 nm (‘B2’ in the ‘pavo’ package). The four repeated measurements from each feather sample were averaged to arrive at a final brightness measure.

*Corticosterone quantification*

We measured baseline corticosterone concentration in blood plasma samples using commercially available enzyme immunoassay (EIA) kits (DetectX Corticosterone, K014eH5, Arbor Assays, Ann Arbor, MI, U.S.A.). We previously validated these kits in tree swallows, and extensive validation and protocol details are available in Taff, Zimmer et al. (2019). Briefly, we used 5 ml of plasma in a triple ethyl acetate extraction and then ran the resulting samples in duplicate following the manufacturer's protocol. Extraction efficiency was determined using samples spiked with a known amount of corticosterone; average extraction efficiency with this method was 89.7%. When starting with 5 ml of plasma, the lower detection limit was 0.8 ng/ml. Interplate variation was assessed using a plasma pool run across plates and was 5.7%. Intraplate variation was assessed using duplicate wells and averaged 10.6%.

*Methylation Quantification*

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.

Initially, we sent a set of 36 tree swallow samples (that were not part of the experiment reported in this study) to develop assays for targeted methylation analysis of our four candidate genes (GR, CRHR1, FKBP5, and CRH). For these 36 samples, EpigenDx used targeted next generation bisulfite sequencing (tNGBS) to assay methylation percentages for CpGs near each of the genes with a focus on coverage of CpG rich regions immediately upstream of the TSS and in the exons of each gene body. Sequencing was accomplished by scaffolding primer pairs along the gene and flanking regions. Using this approach, we received data for a total of 145 CpGs for GR from 23 primer pairs, 171 CpGs for CrH from 24 primer pairs, 130 genes for FKBP5 from 96 primer pairs, and 67 CpGs for CRHR1 from 96 primer pairs.

We used the tNGBS data from these 36 samples to select a smaller subset of primer pairs to pursue with pyrosequencing in the experiment reported here. To make this selection, we first excluded primer pairs in which the CpGs had very low between-individual variation (usually cases in which all CpGs were near 0 or 100% methylation). We also excluded pairs that yielded data on only a small number of CpGs or that failed to amplify consistently. In sum, we focused our targets to areas of the genome that appeared to have high between-individual variation and good amplification rates. Using those criteria, we selected three primer pairs in GR and one primer pair each in CRH, CRHR1, and FKBP5 to target for pyrosequencing in our experiment. We sent a total of 121 samples from 70 individual birds to EpigenDx for pyrosequencing to quantify methylation in the selected areas of the four target genes.

Pyrosequencing procedures followed standard methods developed by EpigenDx (Hopkinton, MA). Briefly, 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.

**Supplemental Tables**

Table S1: Summary data for each of the CpGs characterized in this study including minimum, median, mean, and maximum methylation, and the number of samples sequenced at that site (Excel file).

Table S2: LMM model output predicting methylation of sites in the CRH gene

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | | **CRH** | | |
| *Predictors* | | *Estimates* | *CI* | *p* |
| (Intercept) | | -1.59 | -1.98 – -1.21 | **<0.001** |
| Treatment [Dulled] | | -0.05 | -0.17 – 0.06 | 0.353 |
| Capture [3] | | -0.02 | -0.04 – 0.00 | 0.096 |
| BibB1 | | 0.00 | -0.00 – 0.01 | 0.365 |
| **Random Effects** | | | | |
| σ2 | | 0.04 | | |
| τ00 Band | | 0.05 | | |
| τ00 cpg | | 0.26 | | |
| ICC | | 0.88 | | |
| N Band | | 68 | | |
| N cpg | | 12 | | |
| Observations | | 1401 | | |
| Marginal R2 / Conditional R2 | | 0.004 / 0.883 | | |
| Variance partitioning (repeatability): | |  | | |
|  | Band [CI] | 0.153 [0.081, 0.308] | | |
|  | CpG [CI] | 0.729 [0.491, 0.847] | | |
|  | Fixed effects [CI] | 0.004 [0.001, 0.036] | | |

Table S3: LMM model output predicting methylation of sites in the FKBP5 gene

|  |  |  |  |
| --- | --- | --- | --- |
|  | **FKBP5** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | 0.58 | -0.03 – 1.20 | 0.063 |
| Treatment [Dulled] | -0.11 | -0.36 – 0.14 | 0.382 |
| Capture [3] | -0.10 | -0.14 – -0.06 | **<0.001** |
| BibB1 | -0.00 | -0.01 – 0.01 | 0.943 |
| **Random Effects** | | | |
| σ2 | 0.13 | | |
| τ00 Band | 0.27 | | |
| τ00 cpg | 0.17 | | |
| ICC | 0.78 | | |
| N Band | 69 | | |
| N cpg | 11 | | |
| Observations | 1272 | | |
| Marginal R2 / Conditional R2 | 0.010 / 0.780 | | |
| Variance partitioning (repeatability): | |  | |
|  | Band [CI] | 0.48 [0.345, 0.624] | |
|  | CpG [CI] | 0.298 [0.121, 0.47] | |
|  | Fixed effects [CI] | 0.01 [0.004, 0.075] | |

Table S4: LMM model output predicting methylation of sites in the GR gene

|  |  |  |  |
| --- | --- | --- | --- |
|  | **GR** | | |
| *Predictors* | *Estimates* | *CI* | *p* | |
| (Intercept) | -0.94 | -1.59 – -0.28 | **0.005** | |
| Treatment [Dulled] | 0.10 | -0.01 – 0.21 | 0.075 | |
| Capture [3] | 0.11 | 0.06 – 0.15 | **<0.001** | |
| BibB1 | -0.00 | -0.01 – 0.00 | 0.698 | |
| **Random Effects** | | | | |
| σ2 | 0.19 | | | |
| τ00 Band | 0.05 | | | |
| τ00 cpg | 1.32 | | | |
| ICC | 0.88 | | | |
| N Band | 68 | | | |
| N cpg | 14 | | | |
| Observations | 1537 | | | |
| Marginal R2 / Conditional R2 | 0.003 / 0.878 | | | |
| Variance partitioning (repeatability): | |  | | |
|  | Band [CI] | 0.029 [0.015, 0.064] | | |
|  | CpG [CI] | 0.848 [0.698, 0.914] | | |
|  | Fixed effects [CI] | 0.003 [0.001, 0.013] | | |

Table S5: LMM model output predicting methylation of sites in the CRHR1 gene

|  |  |  |  |
| --- | --- | --- | --- |
|  | **CRHR1** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | -2.52 | -2.97 – -2.08 | **<0.001** |
| Treatment [Dulled] | 0.01 | -0.60 – 0.61 | 0.980 |
| Capture [3] | 0.13 | 0.02 – 0.24 | **0.022** |
| BibB1 | 0.00 | -0.01 – 0.01 | 0.739 |
| Treatment [Dulled] × Capture [3] | 0.11 | -0.06 – 0.29 | 0.209 |
| Treatment [Dulled] × BibB1 | 0.00 | -0.01 – 0.02 | 0.795 |
| Capture [3] × BibB1 | 0.00 | -0.00 – 0.00 | 0.795 |
| (Treatment [Dulled] × Capture [3]) × BibB1 | -0.01 | -0.01 – -0.00 | **0.020** |
| **Random Effects** | | | |
| σ2 | 0.04 | | |
| τ00 Band | 0.08 | | |
| τ00 cpg | 0.25 | | |
| ICC | 0.88 | | |
| N Band | 68 | | |
| N cpg | 19 | | |
| Observations | 2116 | | |
| Marginal R2 / Conditional R2 | 0.010 / 0.884 | | |
| Variance partitioning (repeatability): | |  | |
|  | Band [CI] | 0.212 [0.124, 0.35] | |
|  | CpG [CI] | 0.67 [0.479, 0.797] | |
|  | Fixed effects [CI] | 0.01 [0.007, 0.05] | |

Table S6: LMM output modeling the relationship between baseline corticosterone and methylation in CRHR1.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **CRHR1** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | -2.34 | -2.57 – -2.10 | **<0.001** |
| Baseline cort | -0.01 | -0.01 – -0.00 | **<0.001** |
| **Random Effects** | | | |
| σ2 | 0.05 | | |
| τ00 Band | 0.08 | | |
| τ00 cpg | 0.25 | | |
| ICC | 0.88 | | |
| N cpg | 19 | | |
| N Band | 69 | | |
| Observations | 2129 | | |
| Marginal R2 / Conditional R2 | 0.003 / 0.877 | | |

Table S7: LMM output modeling the relationship between baseline corticosterone and methylation in CRH.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **CRH** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | -1.52 | -1.81 – -1.22 | **<0.001** |
| Baseline cort | 0.00 | -0.00 – 0.00 | 0.967 |
| **Random Effects** | | | |
| σ2 | 0.04 | | |
| τ00 Band | 0.05 | | |
| τ00 cpg | 0.26 | | |
| ICC | 0.88 | | |
| N cpg | 12 | | |
| N Band | 69 | | |
| Observations | 1412 | | |
| Marginal R2 / Conditional R2 | 0.000 / 0.882 | | |

Table S8: LMM output modeling the relationship between baseline corticosterone and methylation in FKBP5.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **FKBP5** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | 0.46 | 0.19 – 0.74 | **0.001** |
| Baseline cort | 0.00 | -0.00 – 0.01 | 0.251 |
| **Random Effects** | | | |
| σ2 | 0.13 | | |
| τ00 Band | 0.27 | | |
| τ00 cpg | 0.17 | | |
| ICC | 0.78 | | |
| N cpg | 11 | | |
| N Band | 70 | | |
| Observations | 1283 | | |
| Marginal R2 / Conditional R2 | 0.001 / 0.775 | | |

Table S9: LMM output modeling the relationship between baseline corticosterone and methylation in GR.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **GR** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | -0.89 | -1.49 – -0.28 | **0.004** |
| Baseline cort | -0.00 | -0.01 – 0.01 | 0.893 |
| **Random Effects** | | | |
| σ2 | 0.19 | | |
| τ00 Band | 0.05 | | |
| τ00 cpg | 1.32 | | |
| ICC | 0.88 | | |
| N cpg | 14 | | |
| N Band | 69 | | |
| Observations | 1551 | | |
| Marginal R2 / Conditional R2 | 0.000 / 0.877 | | |

Figure 1: A) Mean methylation across all interrogated CpG sites before and after treatment

Table S10: LMM output modeling the relationship between stress-induced corticosterone and methylation in CRH.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **CRH** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | -1.40 | -1.72 – -1.08 | **<0.001** |
| stress | -0.00 | -0.01 – 0.00 | 0.087 |
| **Random Effects** | | | |
| σ2 | 0.04 | | |
| τ00 Band | 0.06 | | |
| τ00 cpg | 0.26 | | |
| ICC | 0.87 | | |
| N cpg | 12 | | |
| N Band | 68 | | |
| Observations | 810 | | |
| Marginal R2 / Conditional R2 | 0.007 / 0.876 | | |

Table S11. LMM output modeling the relationship between stress-induced corticosterone and methylation in CRHR1.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **CRHR1** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | -2.41 | -2.69 – -2.13 | **<0.001** |
| stress | -0.00 | -0.01 – 0.01 | 0.959 |
| **Random Effects** | | | |
| σ2 | 0.05 | | |
| τ00 Band | 0.09 | | |
| τ00 cpg | 0.24 | | |
| ICC | 0.88 | | |
| N cpg | 19 | | |
| N Band | 68 | | |
| Observations | 1213 | | |
| Marginal R2 / Conditional R2 | 0.000 / 0.876 | | |

Table S12: LMM output modeling the relationship between stress-induced corticosterone and methylation in FKBP5.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **FKBP5** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | 0.53 | 0.13 – 0.93 | **0.009** |
| stress | -0.00 | -0.01 – 0.01 | 0.940 |
| **Random Effects** | | | |
| σ2 | 0.18 | | |
| τ00 Band | 0.29 | | |
| τ00 cpg | 0.18 | | |
| ICC | 0.72 | | |
| N cpg | 11 | | |
| N Band | 69 | | |
| Observations | 757 | | |
| Marginal R2 / Conditional R2 | 0.000 / 0.716 | | |

Table S13: LMM output modeling the relationship between stress-induced corticosterone and methylation in GR.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **GR** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | -0.86 | -1.47 – -0.26 | **0.005** |
| stress | -0.00 | -0.01 – 0.00 | 0.242 |
| **Random Effects** | | | |
| σ2 | 0.21 | | |
| τ00 Band | 0.04 | | |
| τ00 cpg | 1.25 | | |
| ICC | 0.86 | | |
| N cpg | 14 | | |
| N Band | 68 | | |
| Observations | 880 | | |
| Marginal R2 / Conditional R2 | 0.001 / 0.863 | | |

Table S14. LMM output modeling the relationship between dexamethasone-suppressed corticosterone and methylation in methylation of CRH.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **CRH** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | -1.48 | -1.77 – -1.18 | **<0.001** |
| dex | -0.00 | -0.00 – 0.00 | 0.155 |
| **Random Effects** | | | |
| σ2 | 0.04 | | |
| τ00 Band | 0.06 | | |
| τ00 cpg | 0.26 | | |
| ICC | 0.88 | | |
| N cpg | 12 | | |
| N Band | 69 | | |
| Observations | 822 | | |
| Marginal R2 / Conditional R2 | 0.005 / 0.876 | | |

Table S15. LMM output modeling the relationship between dexamethasone-suppressed corticosterone and methylation in methylation of CRHR1.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **CRHR1** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | -2.39 | -2.63 – -2.15 | **<0.001** |
| dex | -0.00 | -0.00 – 0.00 | 0.387 |
| **Random Effects** | | | |
| σ2 | 0.05 | | |
| τ00 Band | 0.09 | | |
| τ00 cpg | 0.24 | | |
| ICC | 0.88 | | |
| N cpg | 19 | | |
| N Band | 69 | | |
| Observations | 1232 | | |
| Marginal R2 / Conditional R2 | 0.003 / 0.877 | | |

Table S16. LMM output modeling the relationship between dexamethasone-suppressed corticosterone and methylation in methylation of FKBP5.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **FKBP5** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | 0.56 | 0.27 – 0.86 | **<0.001** |
| dex | -0.00 | -0.01 – 0.00 | 0.351 |
| **Random Effects** | | | |
| σ2 | 0.18 | | |
| τ00 Band | 0.28 | | |
| τ00 cpg | 0.18 | | |
| ICC | 0.72 | | |
| N cpg | 11 | | |
| N Band | 70 | | |
| Observations | 768 | | |
| Marginal R2 / Conditional R2 | 0.006 / 0.717 | | |

Table S17. LMM output modeling the relationship between dexamethasone-surpressed corticosterone and methylation in methylation of GR.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **GR** | | |
| *Predictors* | *Estimates* | *CI* | *p* |
| (Intercept) | -0.96 | -1.55 – -0.37 | **0.001** |
| dex | 0.00 | -0.00 – 0.00 | 0.164 |
| **Random Effects** | | | |
| σ2 | 0.20 | | |
| τ00 Band | 0.04 | | |
| τ00 cpg | 1.24 | | |
| ICC | 0.86 | | |
| N cpg | 14 | | |
| N Band | 69 | | |
| Observations | 894 | | |
| Marginal R2 / Conditional R2 | 0.001 / 0.863 | | |

**References**

1. Taff CC, Zimmer C, Scheck D, Ryan TA, Houtz JL, Smee MR, Hendry TA, Vitousek MN. 2021 Plumage manipulation alters associations between behaviour, physiology, the internal microbiome and fitness. *Animal Behaviour* **178**, 11–36. (doi:10.1016/j.anbehav.2021.05.012)

2. Taff CC, Zimmer C, Vitousek MN. 2019 Achromatic plumage brightness predicts stress resilience and social interactions in tree swallows (Tachycineta bicolor). *Behavioral Ecology* **30**, 733–745. (doi:10.1093/beheco/arz010)