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 measurement

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 four feathers on an index card and taped them in place before smoothing the barbs. We used a fibre-optic UV/VIS probe positioned in a holster that blocked external light and measured spectra at a fixed distance of 5 mm from the feather sample. Then, we collected and saved four separate spectra for each feather stack; each spectra had a 10 scan average, 20 nm boxcar width and 60 ms integration time. The probe was removed from the feather patch and replaced between measurements. For each female in the study, we measured four sets of feathers (two from the first capture and one each from the second and third captures). Raw 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 total breast brightness as the average reflectance from 300 to 700 nm ('B2' in the 'pavo' package). We then averaged the four repeated measurements from each feather sample to calculate a final brightness measure.

Corticosterone quantification

We measured baseline, stress-induced, and post-dexamethasone corticosterone levels in blood plasma samples using commercially available enzyme immunoassay (EIA) kits (DetectX Corticosterone, K014eH5, Arbor Assays, Ann Arbor, MI, U.S.A.) that were previously validated for use in tree swallows (Taff, Zimmer et al., 2019). Briefly, corticosterone was extracted from 5 μl of plasma using a triple ethyl acetate extraction. Samples were then run in duplicate following the EIA kit manufacturer's protocol. Extraction efficiency was assessed by spiking samples with a known amount of corticosterone; average extraction efficiency was 89.7%. The lower detection limit was 0.8 ng/ml. Inter-assay variation was 5.7%, and intra-assay variation was 10.6%.

Methylation Assay Development

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.

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			
σ^2	0.04		
$\tau_{00\ Band}$	0.05		
$\tau_{00\ cpg}$	0.26		
ICC	0.88		
$N_{\ Band}$	68		
$N_{\ cpg}$	12		
Observations	1401		
Marginal R^2 / Conditional R^2	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			
σ^2	0.13		
$\tau_{00\ Band}$	0.27		
$\tau_{00\ cpg}$	0.17		
ICC	0.78		
$N_{\ Band}$	69		
$N_{\ cpg}$	11		
Observations	1272		
Marginal R^2 / Conditional R^2	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			
σ^2	0.19		
$\tau_{00\ Band}$	0.05		
$\tau_{00\ cpg}$	1.32		
ICC	0.88		
$N_{\ Band}$	68		
$N_{\ cpg}$	14		
Observations	1537		
Marginal R^2 / Conditional R^2	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			
σ^2	0.04		
$\tau_{00\ Band}$	0.08		
$\tau_{00\ cpg}$	0.25		
ICC	0.88		
$N_{\ Band}$	68		
$N_{\ cpg}$	19		
Observations	2116		
Marginal R^2 / Conditional R^2	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			
σ^2	0.05		
$\tau_{00\ Band}$	0.08		
$\tau_{00\ cpg}$	0.25		
ICC	0.88		
$N_{\ cpg}$	19		
$N_{\ Band}$	69		
Observations	2129		
Marginal R^2 / Conditional R^2	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			
σ^2	0.04		
$\tau_{00\ Band}$	0.05		
$\tau_{00\ cpg}$	0.26		
ICC	0.88		
$N_{\ cpg}$	12		
$N_{\ Band}$	69		
Observations	1412		
Marginal R^2 / Conditional R^2	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			
σ^2	0.13		
$\tau_{00\ Band}$	0.27		
$\tau_{00\ cpg}$	0.17		
ICC	0.78		
$N_{\ cpg}$	11		
$N_{\ Band}$	70		
Observations	1283		
Marginal R^2 / Conditional R^2	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			
σ^2	0.19		
$\tau_{00\ Band}$	0.05		
$\tau_{00\ cpg}$	1.32		
ICC	0.88		
$N_{\ cpg}$	14		
$N_{\ Band}$	69		
Observations	1551		
Marginal R^2 / Conditional R^2	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			
σ^2	0.04		
$\tau_{00\ Band}$	0.06		
$\tau_{00\ cpg}$	0.26		
ICC	0.87		
$N_{\ cpg}$	12		
$N_{\ Band}$	68		
Observations	810		
Marginal R^2 / Conditional R^2	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			
σ^2	0.05		
$\tau_{00\ Band}$	0.09		
$\tau_{00\ cpg}$	0.24		
ICC	0.88		
$N_{\ cpg}$	19		
$N_{\ Band}$	68		
Observations	1213		
Marginal R^2 / Conditional R^2	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			
σ^2	0.18		
$\tau_{00\ Band}$	0.29		
$\tau_{00\ cpg}$	0.18		
ICC	0.72		
$N_{\ cpg}$	11		
$N_{\ Band}$	69		
Observations	757		
Marginal R^2 / Conditional R^2	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			
σ^2	0.21		
$\tau_{00\ Band}$	0.04		
$\tau_{00\ cpg}$	1.25		
ICC	0.86		
$N_{\ cpg}$	14		
$N_{\ Band}$	68		
Observations	880		
Marginal R^2 / Conditional R^2	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			
σ^2	0.04		
$\tau_{00\ Band}$	0.06		
$\tau_{00\ cpg}$	0.26		
ICC	0.88		
$N_{\ cpg}$	12		
$N_{\ Band}$	69		
Observations	822		
Marginal R^2 / Conditional R^2	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			
σ^2	0.05		
$\tau_{00\ Band}$	0.09		
$\tau_{00\ cpg}$	0.24		
ICC	0.88		
$N_{\ cpg}$	19		
$N_{\ Band}$	69		
Observations	1232		
Marginal R^2 / Conditional R^2	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			
σ^2	0.18		
$\tau_{00\ Band}$	0.28		
$\tau_{00\ cpg}$	0.18		
ICC	0.72		
$N_{\ cpg}$	11		
$N_{\ Band}$	70		
Observations	768		
Marginal R^2 / Conditional R^2	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			
σ^2	0.20		
$\tau_{00\ Band}$	0.04		
$\tau_{00\ cpg}$	1.24		
ICC	0.86		
$N_{\ cpg}$	14		
$N_{\ Band}$	69		
Observations	894		
Marginal R^2 / Conditional R^2	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)