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SYMPOSIUM

A Tissue Comparison of DNA Methylation of the Glucocorticoid Receptor Gene (Nr3c1) in European Starlings

Stefanie J. Siller[1] and Dustin R. Rubenstein

Department of Ecology, Evolution and Environmental Biology, Columbia University, 10th Floor Schermerhorn Extension, 1200 Amsterdam Avenue, New York, NY, USA

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[1]E-mail: ss4812@columbia.edu

Synopsis Negative feedback of the vertebrate stress response via the hypothalamic–pituitary–adrenal (HPA) axis is regulated by glucocorticoid receptors in the brain. Epigenetic modification of the glucocorticoid receptor gene (Nr3c1), including DNA methylation of the promoter region, can influence expression of these receptors, impacting behavior, physiology, and fitness. However, we still know little about the long-term effects of these modifications on fitness. To better understand these fitness effects, we must first develop a non-lethal method to assess DNA methylation in the brain that allows for multiple measurements throughout an organism's lifetime. In this study, we aimed to determine if blood is a viable biomarker for Nr3c1 DNA methylation in two brain regions (hippocampus and hypothalamus) in adult European starlings (*Sturnus vulgaris*). We found that DNA methylation of CpG sites in the complete Nr3c1 putative promoter varied among tissue types and was lowest in blood. Although we identified a similar cluster of correlated Nr3c1 putative promoter CpG sites within each tissue, this cluster did not show any correlation in DNA methylation among tissues. Additional studies should consider the role of the developmental environment in producing epigenetic modifications in different tissues.

Introduction

Stress is a constant occurrence in life, and the stress response, or how an individual responds to a stressor, can influence its future behavior, physiology, and fitness. The hypothalamic–pituitary–adrenal (HPA) axis serves as the primary system for mediating the stress response. By releasing glucocorticoids from the adrenal cortex, the HPA axis coordinates physiological and behavioral responses to the environment (Love et al. 2013). After the HPA axis has been stimulated, negative feedback from circulating glucocorticoids inhibits HPA activity in the hypothalamus and pituitary, ultimately returning the body to homeostasis. Sensitivity of this feedback mechanism in response to a stressor is mediated predominantly by glucocorticoid receptors (de Kloet 1991; Zimmer and Spencer 2014). Glucocorticoid receptors are found throughout the brain but are most abundant in the hippocampus and hypothalamus (Hodgson et al. 2007; Zimmer and Spencer 2014), where a higher density of receptors facilitates enhanced sensitivity to circulating glucocorticoids, and thus a faster return to homeostasis (Liebl and Martin 2013).

Glucocorticoid receptor concentration is determined by the expression of the Nr3c1 gene, and increasing evidence suggests that changes in the expression of this gene are associated with epigenetic modifications, specifically DNA methylation in the Nr3c1 promoter (Weaver et al. 2004; Champagne 2013; Palma-Gudiel et al. 2015). DNA methylation is the modification of cytosine nucleotide bases through the addition of a methyl group, resulting in 5-methylcytosine (Ehrlich and Wang 1981). This process leads to gene silencing by either preventing transcription factors from binding to the DNA, or through the recruitment of binding proteins that condense the chromatin structure, thus effectively

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reducing gene expression (Kadonaga 1998). By altering expression of Nr3c1, DNA methylation can therefore impact the negative feedback system of the HPA axis. Indeed, changes in DNA methylation of Nr3c1 are associated with plasticity of the HPA axis and may directly impact an individual's long-term stress response (Szyf et al. 2005; Witzmann et al. 2012).

Alterations of Nr3c1 DNA methylation in early life can persist in adulthood and affect behavior (Weaver et al. 2004; Champagne and Curley 2009). However, a deeper understanding of how early life changes in DNA methylation influence behavior, physiology, and fitness later in life is restricted by two factors. First, most work in this area has been done on mammals, particularly laboratory rodents. How epigenetic modification regulates responsiveness of the HPA axis in other non-mammal, non-model species, is less well understood (Verhulst et al. 2016). Second, due to the use of primarily model laboratory species, as well as the need to euthanize organisms to assess DNA methylation of Nr3c1 in the brain, we know little about the long-term adaptive significance of these epigenetic modifications. To link DNA methylation of Nr3c1 during the early environment to adult fitness, we must develop a proxy that quantitatively estimates DNA methylation in the brain and allows for multiple measurements over an organism's lifetime.

Biomarkers, molecular signatures that can be easily accessed and quantified in periphery tissues, such as blood and saliva, are often used to study epigenetic markers in tissues of interest like the brain that are not always accessible (Masliah et al. 2013; Farré et al. 2015). For example, DNA methylation of Nr3c1 in blood has been related to circulating glucocorticoid levels and types of stressful events in humans (Lee et al. 2011; Labonté et al. 2014; Van der Knaap et al. 2014; Palma-Gudiel et al. 2015; Yehuda et al. 2015). However, these studies often assume that DNA from peripheral tissues or cells mirror the target central nervous system regions (Ewald et al. 2014). Because DNA methylation is tissue specific and diverges with cell type, it is important to verify that peripheral tissues are in fact quantitatively related to target tissues (Razin and Riggs 1980; Razin and Szyf 1984; Ewald et al. 2014; Farré et al. 2015). While this has been demonstrated in some specific genes (see Kundakovic et al. 2015; Verhulst et al. 2016), it has not to our knowledge been verified in Nr3c1. If levels of DNA methylation between peripheral and target tissues are uncorrelated, this may limit our ability to use these tissues as quantitative biomarkers.

Here, we examine the relationship between DNA methylation of Nr3c1 in blood and brain tissue to assess the potential of using blood as a quantitative biomarker of DNA methylation in the hippocampus and hypothalamus. We quantified DNA methylation at CpG sites, where a cytosine nucleotide is followed by a guanine nucleotide, across the Nr3c1 putative promoter of free-living adult European starlings (*Sturnus vulgaris*). Chronic stress in this species has been shown to lower glucocorticoid receptor mRNA expression in the hypothalamus and hippocampus (Dickens et al. 2009), suggesting that Nr3c1 may play an important role in regulating stress reactivity. Although DNA methylation of the glucocorticoid receptor gene has not yet been studied in the European starling, DNA methylation in the Nr3c1 putative promoter of the related superb starling (*Lamprotornis superbus*) was reduced in male offspring born under harsher environmental conditions (Rubenstein et al. 2016). By studying DNA methylation in this gene across different tissues of the European starling, we can assess if blood is an effective biomarker of DNA methylation changes in Nr3c1 within the brain.

Methods

Study area and collection

Adult European starlings were captured between 8:00 AM and 2:00 PM using baited pull-string traps at the Hudson Highlands Nature Museum in Cornwall, NY, between July 23 and August 20, 2017. We collected 22 birds, but only 16 produced tissue of high enough quality for subsequent analysis. After capture, we measured a bird's wing and tarsus and weighed each individual in a cloth bag before a blood sample was collected from the alar wing vein into a 1.5-mL Eppendorf tube using a heparinized capillary tube. Birds were then euthanized using isoflurane, and brains were removed within 5 min of capture, placed directly on dry ice, and stored in −80°C until further preparation.

Tissue extraction and preparation

Brain samples were sliced on a cryostat at 30 μm to reveal the hypothalamic paraventricular nucleus and hippocampus, located using a zebra finch (*Taeniopygia guttata*) brain atlas (Nixdorf-Bergweiler and Bischof 2007). We removed tissues using punches of the hippocampus and hypothalamus and stored them at −80°C until further extraction, at which time they were homogenized using a Qiagen TissueLyser II. We used 20–30 mg of tissue material in 600 μL of Buffer RLT Plus with 6 μL β-mercaptoethanol. DNA was then extracted from brain tissues using the AllPrep DNA/RNA Mini Kit

(Qiagen, Valencia, CA, USA). Genomic DNA was extracted from blood preserved in 2% SDS Queen's lysis buffer using a DNeasy Blood & Tissue Kit (Qiagen). We bisulfite-converted 20 μL of each sample type using an Epitect Fast Bisulfite Conversion Kit (Qiagen).

DNA amplification and pyrosequencing

We identified the putative promoter of the European starling using a genomic reference from the European starling (KQ728588, BioSample SAMN04029017; Burt et al. 2015). We also sequenced nine additional individuals from tissue samples provided by the American Museum of Natural History's Vertebrate Zoology database, and checked the genome by conducting LASTZ alignments against the KQ reference, as well as our previously annotated superb starling genome (Rubenstein et al. 2016), in Geneious v10.0.4 (Supplementary Fig. S1). We designed pairs of forward and reverse primers to target CpG sites in the putative promoter of Nr3c1 (about 1 kb upstream from the translation start site), and flanking and sequencing primers (Supplementary Table S1) for pyrosequencing using Pyromark Assay Design Software v2.0 (Qiagen).

Polymerase chain reactions (PCRs) (25 μL) were prepared using a Pyromark PCR Kit (Qiagen) with 12.5 μL PyroMark PCR Master Mix, 2.5 μL CoralLoad Concentrate, 0.5 μL of each primer, and 0.5 μL bisulfite-converted DNA. Amplifications were carried out on an Eppendorf PCR cycler using the following profile: initial denaturation at 95°C for 15 min; 45 cycles of 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 10 min. All amplicons were visualized on 2% agarose gels prior to pyrosequencing. Each 96-well pyrosequencing assay contained up to 48 samples run in duplicate on a PyroMark Q96 (Qiagen); duplicates that differed by >5% were rerun.

Statistical analysis

All statistical analyses were conducted in R v3.5.2 (R Core Team 2018). We first created a Pearson correlation matrix to identify differentially methylated clusters of CpG sites within each tissue. Using the cluster of CpG sites identified, we determined the "total cluster DNA methylation" by summing the percent methylation for each CpG site within the correlated cluster. We determined "total promoter DNA methylation" by summing the percent methylation for each CpG site across the entire putative promoter.

We compared total promoter DNA methylation across the Nr3c1 putative promoter between sample types (blood, hippocampus, and hypothalamus) using a generalized linear mixed model (GLMM), with tissue as a fixed factor and controlling for individual as a random effect, using *lme4* (Bates et al. 2015). We used *lmerTest* to compute components via the Satterthwaite's degrees of freedom method (Kuznetsova et al. 2017). We also compared total cluster DNA methylation within the identified CpG site cluster between sample types using the same method. We checked all models for normality of residuals.

To test for correlations of DNA methylation in the Nr3c1 putative promoter among blood and brain regions, we ran Pearson's correlation tests between total promoter DNA methylation of the hippocampus, hypothalamus, and blood. We also tested for correlations between the cluster of CpG sites for each tissue type using the same method. We additionally tested for correlations between individual CpG sites of the sample types using a randomized permutation Pearson's correlation test based on 999 simulations with *jmuOutlier* (Garren 2018) to address multiple comparisons.

Results

We successfully pyrosequenced 12 CpG sites in the Nr3c1 putative promoter, from positions −948 to −825 relative to translational start site. Across the 12 CpG sites in the putative promoter region of Nr3c1, DNA methylation increased as the sites became closer to the translation start site for all three sample types (Fig. 1). We were unable to pyrosequence CpG sites −814, −800, or −775 relative to translational start site.

Within the putative starling promoter, we found groups of CpG sites whose DNA methylation was positively correlated within each tissue. Within blood, hippocampus, and hypothalamus, CpG sites −853, −848, −830, and −825 relative to the translational start site were positively correlated to each other (Fig. 2a–c). In the hippocampus, CpG sites −896, −890, and −878 relative to the translational start site were also positively correlated with each other (Fig. 2b). Because of the appearance of this similar cluster of CpG sites −853 to −825 from the translational start site within each tissue, we used this cluster for subsequent between-tissue analyses.

Total promoter DNA methylation varied among blood, hippocampus, and hypothalamus ($F_{2,\ 30} = 23.93$, $P < 0.001$) (Fig. 3a, Table 1). Specifically, total promoter DNA methylation differed between blood and hippocampus ($t_{30} = 6.85$, $P < 0.001$), between blood and hypothalamus ($t_{30} = 2.58$, $P = 0.015$), and between hippocampus and hypothalamus

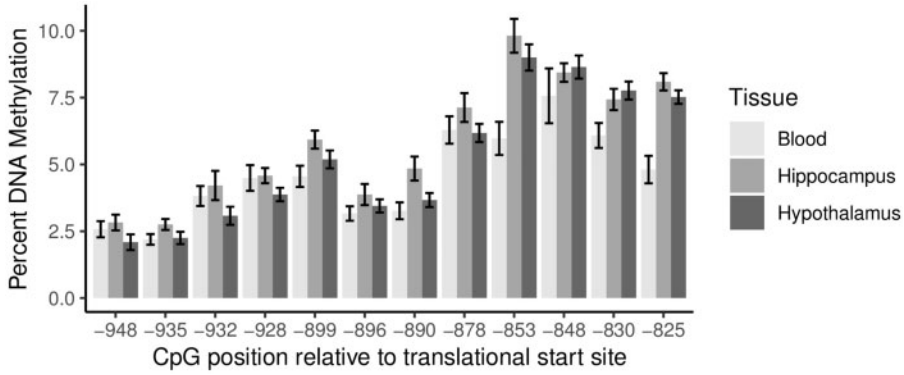

Fig. 1 Percent DNA methylation of CpG sites in the promoter of Nr3c1 in blood, hippocampus, and hypothalamus.

(t_{30} = 4.27, $P < 0.001$). Despite differences in mean DNA methylation levels among tissues types, there was no significant correlation in total promoter DNA methylation between blood and hippocampus ($R = 0.35$, $P = 0.18$), blood and hypothalamus ($R = -0.18$, $P = 0.50$), or hippocampus and hypothalamus ($R = -0.01$, $P = 0.97$).

Total cluster DNA methylation (CpG sites −853 to −825) also varied among blood, hippocampus, and hypothalamus ($F_{2,\ 30}$ = 13.96, $P < 0.001$) (Fig. 3b, Table 1). Specifically, total cluster DNA methylation differed between blood and hippocampus (t_{30} = 5.12, $P < 0.001$), and between blood and hypothalamus (t_{30} = 3.70, $P < 0.001$), but did not significantly differ between hippocampus and hypothalamus (t_{30} = 1.41, $P = 0.17$). Again, there was no significant correlation between blood and hippocampus ($R = -0.22$, $P = 0.41$), blood and hypothalamus ($R = 0.20$, $P = 0.45$), or hypothalamus and hippocampus ($R = 0.17$, $P = 0.53$).

When examining individual CpG sites, only CpG site −935 from the translational start site showed a significant negative correlation between blood and hypothalamus ($R = -0.50$, $P = 0.05$) (Supplementary Fig. S2).

Discussion

We examined DNA methylation in the Nr3c1 putative promoter region in blood, hippocampus, and hypothalamus to determine the potential of blood as a quantitative biomarker of DNA methylation in the brain. We found that DNA methylation of CpG sites across the Nr3c1 putative promoter differed significantly between tissue types. DNA methylation was highest in the hippocampus, followed by the hypothalamus, and then blood. There was no correlation between DNA methylation in the Nr3c1 putative promoter between blood and regions of the brain. We identified a cluster of CpG sites, −853 to −825, that were significantly correlated within each tissue type. DNA methylation of the CpG cluster significantly differed between blood and hippocampus, as well as between blood and hypothalamus, but did not significantly differ between hypothalamus and hippocampus. Furthermore, there was no correlation of total cluster DNA methylation between tissues. Overall, blood does not appear to be a viable quantitative biomarker when considering total DNA methylation in the Nr3c1 putative promoter, or just in the CpG site cluster identified.

Our results are consistent with the idea that DNA methylation of Nr3c1 differs significantly among tissue types. DNA methylation patterns are cell and tissue specific (Christensen et al. 2009; Ziller et al. 2013). For example, Davies et al. (2012) showed a hierarchy of genome-wide DNA methylation levels between parts of the central nervous system and blood based on the early developmental pathway of those tissues. In particular, Nr3c1 gene expression is regulated in a tissue-specific manner, and tissue-specific differences in promoter activity have been shown in the liver, hippocampus, and thymus (McCormick et al. 2000). The significantly lower levels of DNA methylation in blood compared to regions of the brain found here may be related to the generally lower abundance of glucocorticoid receptors in blood compared to the hippocampus or hypothalamus (Zimmer and Spencer 2014).

Despite this tissue specificity, numerous studies have shown correlations in DNA methylation among diverse cell and tissue types, specifically in particular genes of interest. There is evidence for partial correlation in gene expression and DNA methylation between genes in the brain and blood (Sullivan et al. 2006; Davies et al. 2012; Masliah et al. 2013). For instance, Ewald et al. (2014) found a correlation in mice between DNA methylation in FKBP5 (which functions within the glucocorticoid receptor regulatory network) in blood and FKBP5 DNA

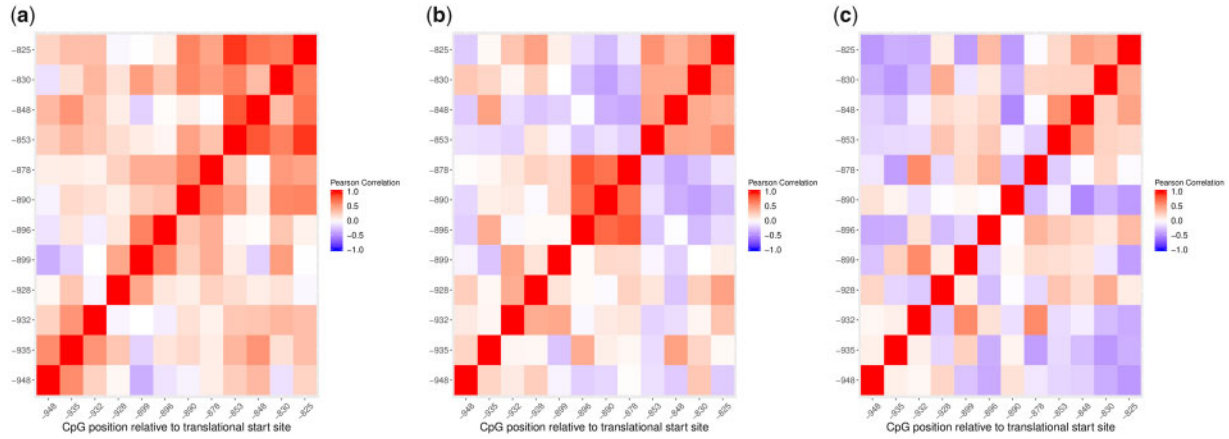

Fig. 2 Heat maps showing positive or negative correlations of percent DNA methylation between (**a**) blood, (**b**) hippocampus, and (**c**) hypothalamus at different CpG sites. CpG sites −853 to −825 were summed for each tissue for further analysis.

methylation and expression changes in the hippocampus, as well as with circulating glucocorticoid levels. Following these studies, we expected to find a correlation between total promoter DNA methylation in the Nr3c1 of the brain and blood. However, we found no evidence of this relationship, either across the entire putative promoter or within the cluster of correlated CpG sites.

Although total promoter DNA methylation was not correlated among tissues, we did find a relationship between blood and hypothalamus in CpG site −935. This region shows the same pattern of lower DNA methylation in blood than hypothalamus, which is characteristic across the entire promoter region, and lies outside the correlated cluster of CpG sites. Such CpG site specificity could be important, as distinct regions of a gene may be affected differently by DNA methylation.

Furthermore, there were similar within-tissue patterns of DNA methylation at CpG sites in the putative promoter. All tissues showed correlations between CpG sites −853 to −825. Similarly, Rubenstein et al. (2016) showed correlations between CpG sites −869 to −848 and sites −830 to −775 relative to the translational start site in the superb starling, and found that only DNA methylation over CpG sites −830 to −775 relative to the translational start site was positively correlated with pre-breeding rainfall, a significant factor in starling behavior, physiology, and reproductive life history. Notably, this similar cluster is located closer to the transcription start site, a region identified as important to gene regulation and function in other DNA methylation studies in birds (Verhulst et al. 2016). Unfortunately, a number of CpG sites further upstream in this particular region were excluded from our analysis because of failure to pyrosequence (most likely because of tertiary genome structure in this region). Although uncorrelated between tissues, this region near the transcription start site may be of similar regulatory importance in both the brain and the blood.

The lack of correlation in total promoter DNA methylation among tissue types in our study may be at least partially due to our sampling method. For this study, we captured free-living adult European starlings, with no information about their developmental history or background. Yet, developmental stress may be essential for producing similar DNA methylation profiles across tissues. Exposure to stress in the early life can have long-lasting impacts on behavior, pathology, and the adult stress response. The HPA axis is a principal target of this developmental programming, whereby early exposure to glucocorticoids in the pre- and postnatal environments may modify Nr3c1 expression in the hippocampus or other sites of feedback, thereby altering HPA functioning (Welberg and Seckl 2001; Matthews 2002; Kapoor et al. 2006; Cottrell and Seckl 2009; Lupien et al. 2009; Harris and Seckl 2011). Indeed, the influence of Nr3c1 expression in the hippocampus and hypothalamus on HPA sensitivity is usually studied in the context of exposure to stressors in the developmental environment, where pre- and early postnatal stress can alter HPA responsiveness through epigenetic alterations of the genes involved in HPA functioning (Mueller and Bale 2008; Banerjee et al. 2012; Ahmed et al. 2014; Kundakovic et al. 2015). Programming of the HPA axis in response to developmental stress may produce a similar effect on DNA methylation across tissues, and

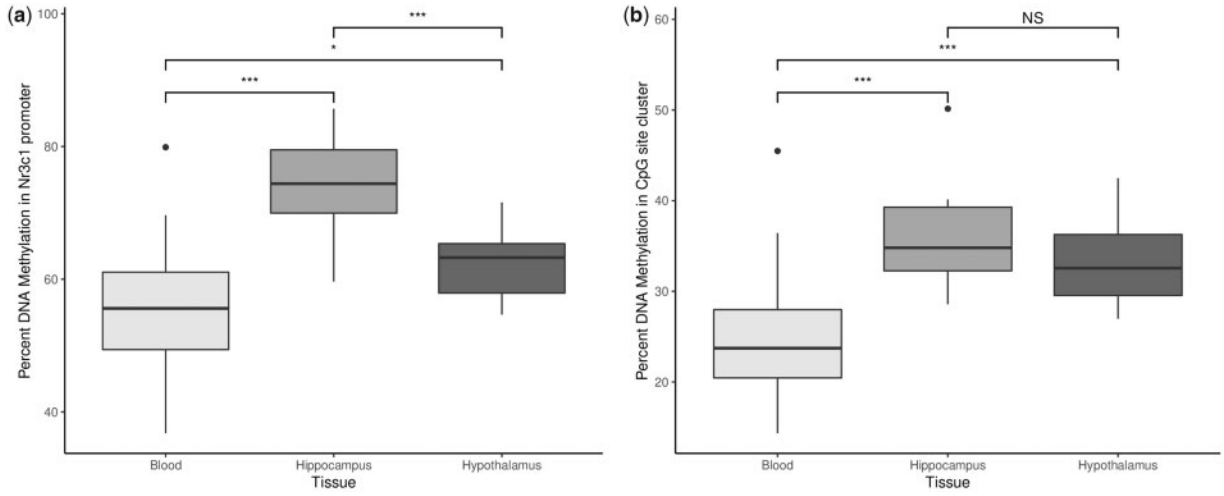

Fig. 3. Boxplots showing mean total DNA methylation (**a**) across the Nr3c1 promoter and (**b**) in the cluster of CpG sites −853 to −825 in blood, hippocampus, and hypothalamus. Boxes represent the interquartile range with median line shown, and whiskers represent quartiles 1 and 4. * indicates a significance level of $P < 0.05$, and *** indicates a significance level of $P < 0.001$.

Table 1 GLMM exploring differences in total promoter DNA methylation across the Nr3c1 putative promoter between blood and brain tissue, and total cluster DNA methylation in the identified cluster of correlated CpG sites 853 to 825 between blood and brain.

		Total promoter DNA methylation			Total cluster DNA methylation		
	Predictors	**Estimates**	**CI**	***P***	**Estimates**	**CI**	***P***
Model 1							
Fixed	(Intercept)	55.70	51.75 – 59.66	**<0.001**	25.18	22.25 – 28.11	**<0.001**
	Hippocampus	18.59	13.27 – 23.91	**<0.001**	7.76	3.65 – 11.86	**0.001**
	Hypothalamus	6.99	1.67 – 12.31	**0.015**	10.71	6.61 – 14.82	**<0.001**
Model 2							
Fixed	(Intercept)	62.70	58.74 – 66.65	**<0.001**	32.93	30.01 – 35.86	**<0.001**
	Hippocampus	11.59	6.27 – 16.91	**<0.001**	2.95	-1.15 – 7.06	0.169
	Blood	-6.99	-12.31 – -1.67	**0.015**	-7.76	-11.86 –-3.65	**0.001**
	Observations	σ^2	τ_{00}	ICC	σ^2	τ_{00}	ICC
Random	48	58.94	6.27	0.10	35.08	0.63	0.02

Notes: For fixed factors, parameter estimates and CIs are given, as well as *P*-values. Significant *P*-values are in bold. For random effects, the number of observations, the within- and between-group variance (σ^2, τ_{00}), and the ICC are given. Model 1 uses blood as the intercept, whereas Model 2 is releveled to use hypothalamus as the intercept.
ICC, Intra-class correlation coefficient; CI, confidence interval.

thus be critical for establishing similar patterns of DNA methylation in the brain and blood (Aberg et al. 2013). This makes sense considering that epigenetic modifications can be influenced by early life experiences and the environment, and environmentally-driven epigenetic modifications are a potential crucial component in mediating impacts of the early developmental environment on an individual's stress response (Weaver et al. 2004; Champagne 2013; Curley et al. 2017; Kilvitis et al. 2017).

Although our results suggest that DNA methylation in blood may not be useful as a quantitative biomarker, it may still have potential as a qualitative one. Even without correlation with the brain, epigenetic markers in the blood may be predictive of phenotypic changes or differences. Verhulst et al. (2016), for instance, found that CpG methylation levels of the DRD4 gene in great tit (*Parus major*) blood differed significantly according to personality type, despite differences in CpG site methylation between the brain and blood. Glucocorticoids, produced by the HPA axis under stressful conditions, can have wide-ranging effects on multiple cell types, including blood cells, which may produce coordinated changes in the epigenome (Turecki and Meaney 2016). A crucial future step will involve repeating this study with a controlled population of developmentally stressed and non-stressed birds to

determine the potential for DNA methylation in the blood as a qualitative biomarker for stress.

Finally, it is critical to note that the majority of studies measuring epigenetic markers in blood are on mammals, and use whole blood, often without adjusting for differences in cell composition (Houseman et al. 2015). Whole blood contains red blood cells, which in mammals are enucleated; however, in birds, each red blood cell retains its nucleus. It is possible that this divergence may impact epigenetic modifications in the blood differently in birds versus mammals. For instance, human cord blood, which has a high proportion of nucleated red blood cells, shows largescale epigenetic differences compared to other cord blood cell types, with a distinct DNA methylation profile (de Goede et al. 2015; Bakulski et al. 2016). Thus, in future studies on taxa with nucleated red blood cells, it will be important to consider how this may impact epigenetic profiles.

Ultimately, our results suggest that DNA methylation of the Nr3c1 putative promoter differs significantly between tissue types but is uncorrelated among tissues. As such, blood from adult European starlings is unlikely to serve as a viable quantitative biomarker for studying DNA methylation of the Nr3c1 in the hippocampus or hypothalamus. However, DNA methylation in the blood may still be useful as a qualitative biomarker, and further focus should be placed on determining the role of the developmental environment in producing epigenetic modifications across tissues.

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Supplementary data

Supplementary data available at *ICB* online.

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