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The impact of parental and developmental stress on DNA methylation in the avian hypothalamic–pituitary–adrenal axis

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Abstract

The hypothalamic–pituitary–adrenal (HPA) axis coordinates an organism's response to environmental stress. The responsiveness and sensitivity of an offspring's stress response may be shaped not only by stressors encountered in their early post-natal environment but also by stressors in their parent's environment. Yet, few studies have considered how stressors encountered in both of these early life environments may function together to impact the developing HPA axis. Here, we manipulated stressors in the parental and post-natal environments in a population of house sparrows (*Passer domesticus*) to assess their impact on changes in DNA methylation (and corresponding gene expression) in a suite of genes within the HPA axis. We found that nestlings that experienced early life stress across both life-history periods had higher DNA methylation in a critical HPA axis gene, the glucocorticoid receptor (*NR3C1*). In addition, we found that the life-history stage when stress was encountered impacted some genes (*HSD11B1*, *NR3C1* and *NR3C2*) differently. We also found evidence for the mitigation of parental stress by post-natal stress (in *HSD11B1* and *NR3C2*). Finally, by assessing DNA methylation in both the brain and blood, we were able to evaluate cross-tissue patterns. While some differentially methylated regions were tissue-specific, we found cross-tissue changes in *NR3C2* and *NR3C1*, suggesting that blood is a suitable tissue for assessing DNA methylation as a biomarker of early life stress. Our results provide a crucial first step in understanding the mechanisms by which early life stress in different life-history periods contributes to changes in the epigenome of the HPA axis.

KEYWORDS
biomarker, DNA methylation, HPA axis, parental effects, stress

1 | INTRODUCTION

How organisms react to environmental stressors can affect several aspects of their fitness. The hypothalamic–pituitary–adrenal (HPA) axis coordinates an organism's physiological and behavioural responses to environmental stressors (Wingfield & Sapolsky, 2003). Through the rapid release of glucocorticoids (GCs), the HPA axis generates a stress response that can lead to enhanced vigilance, avoidance behaviour, increased energy mobilization and suppression of growth, reproduction, digestion and immunity by diverting energy away from those processes (Boonstra, 2004; Koolhaas et al., 1999). The HPA axis is in turn regulated by negative feedback, where GCs bind to receptors in the brain (e.g. hippocampus, hypothalamus) and anterior pituitary, terminating the stress response and returning the body to homeostasis (Gjerstad et al., 2018; Lupien et al., 2009; Wingfield & Romero, 2011). The HPA axis can therefore be characterized by the activity of these two key components: (1) the release of GCs

during the stress response, and (2) subsequent negative feedback mediated by the binding of circulating GCs to receptors. The two receptors that regulate GC activity are the glucocorticoid receptor (GR), encoded by the gene *NR3C1*, and the mineralocorticoid receptor (MR), encoded by the gene *NR3C2*. MR has a higher affinity for GCs than GR, so it becomes saturated at a lower concentration and is particularly important for regulating basal levels of GCs (De Kloet et al., 1998). During the stress response, as GC levels increase, they begin to occupy more GRs, which then mediate physiological and behaviour responses to the stressor (Shoener et al., 2006). These two receptors therefore work together in an antagonistic manner to regulate GC levels in both components of HPA axis activity.

The responsiveness and sensitivity of the stress response and negative feedback are often shaped by the environment experienced by an organism immediately after birth (i.e. the post-natal environment). Through a process called developmental programming, variation in the post-natal environment causes organizational changes in physiological systems (e.g. the HPA axis) producing long-term effects (Seckl & Holmes, 2007; Welberg & Seckl, 2001). Exposure to stressors (e.g. poor nutrition, maternal separation and lack of parental care) during this early life developmental period can alter responsiveness of the HPA axis, causing elevated levels of GCs in response to acute stressors as adults (Banerjee et al., 2012; Kitaysky et al., 1999; Meaney, 2001; Schmidt et al., 2014) and a blunted, or desensitized, negative feedback response (Haussmann et al., 2012; Kapoor et al., 2006; Navarrete et al., 2007). In addition, the stress response and negative feedback system can also be shaped via parental effects (i.e. the impact of the parental environment, as well as the parental phenotype, on an offspring's phenotype) (Groothuis et al., 2005; Mousseau, 1998; Uller, 2008). For example, both maternal (Hayward & Wingfield, 2004; Plotsky & Meaney, 1993; Seckl., 2004) and paternal stress (Batchelor & Pang, 2019; Chan et al., 2018; Rodgers et al., 2013) have been shown to impact the stress response and negative feedback system of the HPA axis, as well as other aspects of development in numerous vertebrate species. Notably, however, studies looking at parental and post-natal stressors often find contrasting results, potentially due to differences in the stressors, species or methods used across studies (reviewed in Huber et al., 2022; Schoech et al., 2011).

Few studies have considered how both the parental environment and the offspring's post-natal environment function together to impact the offspring's HPA axis. The stress that is experienced by parents likely modifies an offspring's developing HPA axis, thus altering the effects of stress that the offspring experiences during its own early post-natal life (Grace et al., 2017). Studies in mammals suggest that pre- and post-natal stress can have different effects on the HPA axis (Maccari et al., 1995; Vallée et al., 1997, 1999). Similarly, a study in the Japanese quail (*Coturnix coturnix japonica*) showed that glucocorticoid exposure at different developmental stages had contrasting short- and long-term effects on the functioning of the offspring's HPA axis, with post-natal stress mitigating the effects of pre-natal stress (Marasco et al., 2012). However, a conflicting study in the same species found that pre- and post-natal stress actually had a cumulative effect on exploratory and feeding behaviour, while post-natal stress alone had no effect on stress physiology (Zimmer et al., 2013). Because of the important role that early life stressors have on shaping the HPA axis, it is critical to understand how stressors experienced during different stages of development interact. Indeed, while numerous hypotheses have been developed to ascertain whether early life stress is an adaptive programming mechanism that enhances adult fitness ('the predictive adaptive response hypothesis'; Gluckman et al., 2005), or a hindrance that lowers adult fitness ('the silver spoon hypothesis'; Grafen, 1990), these alternative hypotheses often fail to distinguish between stress experienced in the pre- and post-natal developmental stages. Furthermore, to understand the adaptive potential of early life stress at any point during development, we must first determine the mechanisms by which these different environmentally induced changes occur (Monaghan, 2008).

One of the mechanisms that integrates both parental and post-natal environmental effects into the offspring phenotype are epigenetic modifications, chemical changes to DNA that can influence gene expression (Richards, 2006). DNA methylation, the addition of a methyl or hydroxymethyl group to the C5 position of cytosine, can influence changes in DNA accessibility or chromatin structure (Jones, 2012), often leading to suppression of gene expression (Wan et al., 2015). Such epigenetic modifications provide a way for the phenotype to respond to rapidly changing environmental conditions (Mazzio & Soliman, 2012), influencing phenotypic plasticity (Hu & Barrett, 2017; Zhang et al., 2013). In particular, epigenetic mechanisms represent a crucial link between environmental conditions and HPA axis function (Matthews & McGowan, 2019). Both the pre-natal parental environment (McGowan & Matthews, 2018; Mueller & Bale, 2008) and early post-natal developmental environment (Kundakovic & Champagne, 2015; Meaney & Szyf, 2005; Weaver et al., 2004) have been shown to produce epigenetic changes in HPA-related genes (Argentieri et al., 2017; Oberlander et al., 2008; Turecki & Meaney, 2016). In particular, research has demonstrated that epigenetic changes in *NR3C1*, the glucocorticoid receptor gene, can have long-term impacts on functioning of the HPA axis, predominantly in humans and rats (Francis et al., 1999; Liu et al., 1997; Miller et al., 2009). This candidate-gene approach can provide a targeted, mechanistic understanding of the relationship between early stress and HPA axis function, but focusing on a single gene fails to reveal the full epigenetic picture underlying complex biological systems such as the HPA axis (Bick et al., 2012; Siller Wilks et al., 2023). Early life environments are likely to have broad impacts on DNA methylation patterns (Szyf, 2011). By targeting numerous components involved in the HPA axis, as well as other closely related systems like the hypothalamic–pituitary–gonadal (HPG) axis (which regulates sexual behaviour and reproduction; Schmidt et al., 2014), we may be able to gain a more nuanced understanding of the role that epigenetic modifications play in regulating developmental programming in response to parental and post-natal stress and begin to understand how this might influence future fitness.

Here, we experimentally examined the impact of stress experienced by parents during offspring production and/or by the offspring during post-natal development on offspring DNA methylation. We studied a free-living population of house sparrows (*Passer domesticus*), a species that has been studied extensively for both maternal (Mazuc, 2003; Partecke & Schwabl, 2008; Schwabl, 1997) and paternal effects (Schwagmeyer et al., 2005; Voltura et al., 2002), particularly exposure of hormones to developing offspring (Strasser & Schwabl, 2004). In addition, studies in house sparrows have shown that post-natal developmental conditions (e.g. resource availability and brood size) impact offspring development and individual stress responses (Killpack et al., 2015; Lendvai et al., 2009), and are correlated to changes in DNA methylation in HPA axis genes during the developmental period (Siller Wilks et al., 2023). By experimentally manipulating stress in both the parents during offspring production and directly in the offspring during post-natal development, we utilized a full factorial design to assess the differential impacts of these stressors on DNA methylation across a suite of 25 functionally relevant genes to the HPA axis, HPG axis or that have been shown previously to be impacted by either parental or post-natal stress. We analysed differentially methylated regions (DMRs) in two brain regions critical to functioning of the HPA axis—the hypothalamus and hippocampus—as well as in the blood to test the possibility that blood may be used as a biomarker for DNA methylation of genes in target central nervous system regions (Palma-Gudiel et al., 2015; Siller & Rubenstein, 2019; Yehuda et al., 2015). Furthermore, we assessed gene expression across our gene suite in both brain regions to verify the predicted negative relationship between DNA methylation and gene expression (Wan et al., 2015), and thus better ascertain the phenotypic impacts of these methylation changes. Notably, by studying these changes in a free-living bird, we can expand what we know about stress-induced changes in DNA methylation beyond humans and captive rodents to assess how these mechanisms function in vertebrates more broadly.

First, we assessed the influence of early life stress across both life-history periods by comparing nestlings in parental and post-natal control groups to those in parental and post-natal stress groups. Based on numerous previous studies of the impacts of early life stress (reviewed in Sosnowski et al., 2018), we predicted that nestlings in the stress treatment groups would have higher DNA methylation in the highly studied GR gene *NR3C1*. Furthermore, because of the competitive relationship between GRs and MRs in binding GCs, we also predicted that early stress would lead to lower DNA methylation (and higher expression) of the MR gene *NR3C2*. Next, we assessed whether the timing of early stress matters by comparing DNA methylation marks in nestlings whose parents experienced stress to nestlings who experienced stress themselves during post-natal development. We predicted that the effects of parental stress would be different from those of post-natal stress, with different genes in the HPA axis responding to each of the stressor types. In particular, we predicted that we would see differential methylation in genes central to regulating negative feedback as well as in genes involved in the stress response in nestlings exposed to parental versus post-natal developmental stress. Finally, we examined the potential interaction between stressors encountered in both life-history periods by comparing nestlings who experienced stress in both environments to those who only encountered stress in one (either the parental or post-natal) environment. If the interaction between these environments is additive, then we would expect that nestlings experiencing stress during both periods would have DNA methylation changes in the same genes as nestlings experiencing one stressor, but to a larger degree. However, if post-natal stress mitigates parental stress, then we would expect to see similar DNA methylation levels between nestlings experiencing no stressors and those experiencing both stressors, as compared to nestlings experiencing one stressor. Ultimately, our results contribute to a more complete understanding of the mechanisms by which early life stress at different stages of development program the offspring phenotype in potentially adaptive ways (Figure 1).

2 | MATERIALS AND METHODS

2.1 | Study site and experimental design

We studied a free-living population of house sparrows that bred in nest boxes from April to August 2019 at North Dakota State University in Fargo, ND (46.9 N, −96.8 W). House sparrows had been nesting in nest boxes in the study population since 2013. We monitored the boxes daily to determine nest building, onset of egg laying, clutch size, brood size and the number of nestlings that survived to 10 days post-hatching.

During nest building, we randomly assigned nests to either an experimental control or a stress parental treatment. Control and stressed nests were located at least 10 m apart and on opposite sides of buildings where possible. Nests adjacent to stress treatment nests were left unassigned to ensure that control nests were not impacted by neighbouring treatments. Control nests were not exposed to any of our planned stressors. In the stress treatment, we exposed parents at the nest to a standardized, unpredictable rotation of stressors from the onset of nest building until the first egg was laid. Stressors included predator models (American kestrel mount, *Falco sparverius*; sharp-shinned hawk mount, *Accipiter striatus*; grey squirrel mount, *Sciurus carolinensis*), decoy predators (artificial cat, owl decoy, hawk decoy, rubber snake), novel objects (a sparkling pinwheel, plastic flower, stuffed owl toy, wooden chicken model) and a human standing beneath the nest box. We presented stressors in a random order three times per day, every other day, in a series of half hour sessions, separated by half hour periods with no stressors. Each stressor was shown once before repetition. We set starting times randomly each morning, and presented stressors at experimental nests for an average of 13.4 ± 9.1 days; length of stressor exposure did not significantly impact offspring telomere length or mass at day 2 post-hatching (Young et al., 2022). This experimental stress treatment has been shown to increase glucocorticoid stress hormones, oxidative stress and telomere loss in other songbirds,

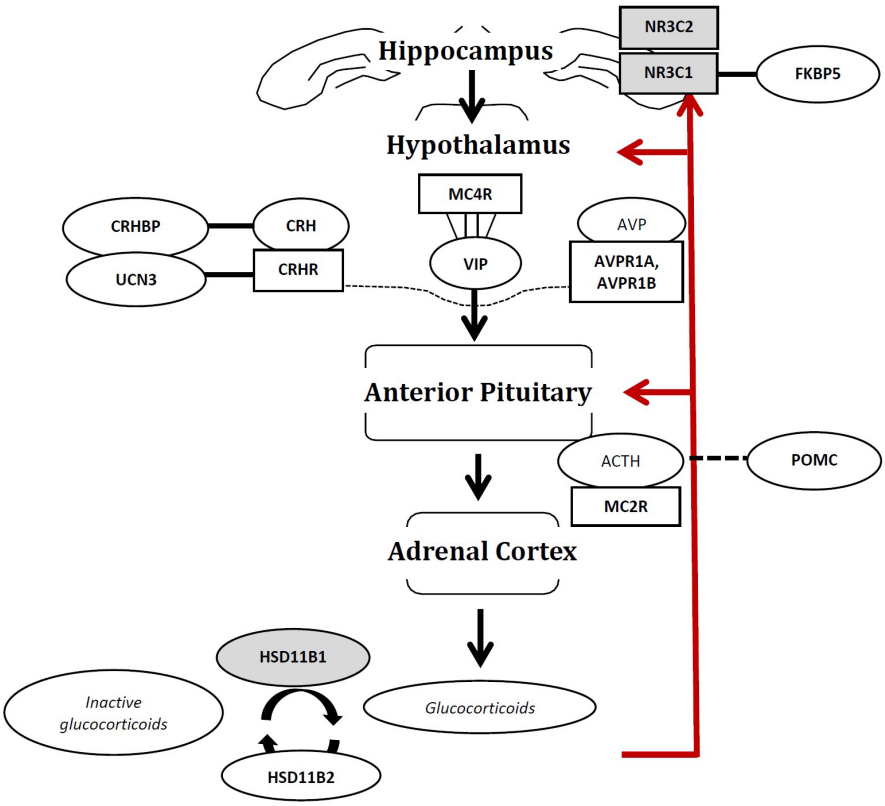

FIGURE 1 Genes in the HPA axis system impacted by early life stressors. This graphic illustrates major and peripheral genes involved in the regulation of the hypothalamic–pituitary–adrenal (HPA) axis. Genes names in boxes encode receptors, while gene names in ovals encode ligands, enzymes or other molecular products. Genes in bold are those included in our targeted gene set. The red lines illustrate the negative feedback system of the HPA axis. Genes highlighted in grey are those with differentially methylated regions (DMRs) as a result of the stress treatments in this study. [Colour figure can be viewed at wileyonlinelibrary.com]

while minimizing habituation (Cyr & Michael Romero, 2007; Hau et al., 2015; Lattin & Romero, 2014). In addition, repeated exposure to a novel stressor has been shown to impact HPA axis functionality both physiologically and behaviourally in house sparrows (Gormally & Romero, 2018; Lendvai et al., 2011). There was no significant difference between control and stressed parents in latency to lay, clutch size, hatching success, brood size, number of nestlings at day 10 or hatch date (Young et al., 2022). Although we did not measure parental stress directly in this study due to concerns over nest abandonment after handling birds and inducing stress in control birds, behavioural observations made at the nests indicated that parents reacted to all objects with behavioural signs of stress (increased movements, flying away or at the object, alarm calling, agitated movements, tail flicking) (B.J. Heidinger, personal communication, 2023). In addition, nestlings produced by stressed parents had significantly shorter telomere length at day 2 than nestlings in control nests (Young et al., 2022), which is in line with other studies that have shown that parental stress exposure leads to shorter telomeres at birth (Entringer et al., 2011; Marchetto et al., 2016). Together, this evidence suggests that the stress treatment was effective.

House sparrow nestlings hatched asynchronously over the course of a single day. As nestlings hatched, we marked them with a coloured, non-toxic marker for individual identification. Within a nest, we randomly assigned nestlings to a post-natal developmental control or a stress treatment. For the stress treatment, we used a standardized stress protocol, placing nestlings in a cloth bag for 30 min every day until they were 10 days old. Control nestlings were not handled except for brief measurements taken of all nestlings (see *Data collection and extraction*). Using this full factorial design, we produced four treatment groups for nestlings based on the environment experienced by their parents and their own early life environment: 'Parental Control – Developmental Control' (PC-DC), 'Parental Control – Developmental Stress (PC-DS)', 'Parental Stress – Developmental Control' (PS-DC) and 'Parental Stress – Developmental Stress' (PS-DS).

2.2 | Sample collection and preparation

On days 2, 6, 8 and 10 post-hatching, we recorded mass, beak length, beak depth, tarsus length, wing chord and length of the rectrices of each nestling. Blood samples were collected on days 2 and 10 post-hatching from the alar vein using a heparinized capillary tube and stored on wet ice for transport to the lab. Plasma and red blood cell fractions were separated with centrifugation and stored at −80°C until further analysis. We extracted DNA from 4 μL of packed red blood cells using the NucleoSpin Blood kit (Machery Nagel, 740951), and extracts were frozen at −80°C until further preparation.

We collected 30 offspring between June 6 and 26, 2019 in the morning on day 11 post-hatching for additional tissue analysis. We randomly selected two individuals from each nest, using only nests with more than four surviving nestlings to increase likelihood of survival for the remaining nestlings. Selected birds were placed in a cloth bag and immediately returned to the laboratory (within 10 min where possible, and under a maximum of 20 min), where they were euthanized using isoflurane. Brains were removed, weighed and

placed directly on dry ice within 5 min of death, and stored in −80°C until further preparation. Brain samples were sliced on a cryostat at 30 μm. We located the hypothalamic paraventricular nucleus (PVN) and hippocampus using a zebra finch (*Taeniopygia guttata*) brain atlas (Nixdorf-Bergweiler & Bischof, 2007) and sampled punches that were stored at −80°C before homogenization using a Qiagen TissueLyser II and 20–30 mg of tissue material in 600 μL of Buffer RLT Plus with 6 μL β-mercaptoethanol. We then extracted DNA and RNA from the brain tissues using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA). This study was performed under the approval of North Dakota State University IACUC (protocol A17035).

2.3 | TEEM-Seq DNA probe development, library preparation and sequencing

To assess DNA methylation, we used the TEEM-seq protocol (Rubenstein & Solomon, 2023), which pairs hybridization capture using custom biotinylated RNA probes with enzymatic methyl-sequencing (EM-seq) (Vaisvila et al., 2021), providing a targeted, flexible next-generation sequencing approach that focuses only on complete genomic regions of interest. In particular, EM-seq is an alternative to bisulphite sequencing that minimizes DNA damage and reduces GC bias (Hoppers et al., 2020). EM-seq libraries have been shown to perform better than bisulphite-converted libraries in terms of coverage, duplication and sensitivity (Vaisvila et al., 2021). In addition, EM-seq has been shown to be more consistent and able to detect more CpGs at a higher depth than whole genome bisulphite sequencing (Hoppers et al., 2020).

As described in (Siller Wilks et al., 2023), we used the NCBI house sparrow genome as a reference (Ravinet et al., 2018) to identify target sequences for 25 genes selected for their known roles in functioning of the HPA axis, the HPG axis or for their responsiveness to changes in stress based on a literature review (Table 1). We also sampled two genes for the enzymes that control DNA methylation (*DNMT3a* and *DNMT3b*). We targeted the 4-kb upstream region of the transcription start site, which was likely to contain putative promoters, as well as exons (excluding introns) for a subset of genes. To ensure that we had reliable EM-Seq conversion estimates for all samples, we also specifically targeted pUC10 and lambda control sequences in our bait set (Rubenstein & Solomon, 2023). Biotinylated RNA probes were then commercially prepared using myBaits v4.01 Custom 1-20K 16 Reaction Kits for target enrichment via hybridization-based capture (Daicel Arbor BioSciences). We submitted 53 target sequences totalling 159,560 bp, as well as the first 1 kb of pUC19 and the first 2 kb of lambda NEB reference fastas (available at https://www.neb.com/tools-and-resources/interactive-tools/dna-sequences-and-maps-tool), for 80 nt probe design at 2X tiling density. For each probe, myBaits designed 8 additional potential methylation schemes (all methylated, a random 50% CpGs methylated, the other 50% CpGs methylated, unmethylated and sense/antisense for each version), producing a total of 19,575 probe candidates. Probe candidates were quality-assessed and filtered based on likely performance, including possible-off-target capture and excluding low-complexity probes post-conversion. Briefly, candidates were filtered against original sequences based on: masked repeats and low complexity repeat regions using repeatmasker, resulting in 1.4% of the total sequence being masked; GC content; and BLAST hits on the zebra finch (*Taeniopygia guttata*), collared flycatcher (*Ficedula albicollis*), great tit (*Parus major*) and European starling (*Sturnus vulgaris*) reference genomes (as well as against sense strand and anti-sense strand unmethylated converted versions of each genome). We used this multispecies genome-wide BLAST screen to check for probe candidates that were likely to be very non-specific in general, which could contribute to the capturing of undesired off-target reads (see Rubenstein & Solomon, 2023 for more detail).

We used the NEBNext Enzymatic Methyl kit (New England BioLabs Inc.) to detect 5-mC and 5-hmC in our sample genomes. We ran extracted DNA on an Invitrogen Qubit 3.0 Fluorometer to determine concentration, then sheared DNA on a Covaris S220 Focused-ultrasonicator to 250–270 bp in 130 μL 0.1 mM EDTA 1X TE Buffer, with 1–3 μL each of NEB Control DNA CpG methylated pUC19 (0.1 ng/μL) and Control DNA CpG unmethylated Lambda (2 ng/μL). After concentrating DNA down to 80–100 ng in 33 μL using an Eppendorf Vacufuge, we end-repaired sheared DNA using NEBNext Ultra II reagents, ligated DNA libraries to the EM-seq adaptor, and oxidized 5-mC and 5-hmC sites in a TET2 reaction. We then denatured the EM-Seq DNA using Formamide, and deaminated unmodified cytosines to uracils in an APOBEC reaction. We PCR amplified the EM-Seq library for 12 cycles using NEBNext Q5U. EM-seq libraries were pooled at 20 ng per sample, with 96 samples in each pool to be run on the HiSeq 4000, and 48 samples in each pool to be run on the NovaSeq 6000.

We followed the myBaits hybridization capture for targeted NGS protocol v4.01 for whole bait capture (Arbor BioSciences). We bound hybridization beads to the pooled library-blocker mix, cleaned with three washes of buffer, and amplified the resuspended bead-bound DNA with 16 PCR cycles in a KAPA HiFi reaction at 60°C annealing temperature and a 1-min extension step at 72°C. We cleaned the amplified capture pool with AMPure XP beads. Finally, we sequenced brain ($n = 59$) and blood ($n = 37$) samples at 2 × 150 bp with 5 percent PhiX in one full lane (110G) of an Illumina HiSeq 4000, and we sequenced additional blood samples ($n = 96$) in one partial lane (150G) of a NovaSeq 6000, at Novogene (Sacramento, CA).

2.4 | Data alignment, coverage and validation

We trimmed sequencing data using the Trim Galore v0.4.2 (Krueger et al., 2021) wrapper of Cutadapt v1.12 (Martin, 2011) with standard parameters. We aligned trimmed reads to the house sparrow bisulphite genome reference generated by Bismark v0.19.0 (Krueger & Andrews, 2011). Alignments were deduplicated, and CpG coverage files with methylation percentages (100 * methylated cytosines/total of methylated plus unmethylated cytosines in CpG context) were extracted from alignments using Bismark (see Rubenstein &

TABLE 1 Targeted genes for house sparrow probes.

Gene	Name	Role	Representative reference[b]
AR[a]	Androgen receptor	HPG axis	Pfannkuche et al. (2011)
AVPR1A[a]	Arginine vasopressin receptor 1A	HPA axis—stress response	Lesse et al. (2017)
AVPR1B[a]	Arginine vasopressin receptor 1B	HPA axis—stress response	Dempster et al. (2007)
CRH[a]	Corticotropin releasing hormone	HPA axis—stress response	Kertes et al. (2016)
CRHBP	CRH binding protein	HPA axis – stress response	Kertes et al. (2016)
CRHR	Corticotropin-releasing hormone receptor	HPA axis—stress response	Maras and Baram (2012)
DNMT3a	DNA methyltransferase 3a	DNA methyltransferase	Catale et al. (2020)
DNMT3b	DNA methyltransferase 3b	DNA methyltransferase	Urb et al. (2019)
EGR1[a]	Early growth response 1	Synaptic plasticity and neuronal activity	Xie et al. (2013)
ESR2	Oestrogen receptor 2	HPG axis	Bentz et al. (2016)
FKBP5[a]	FK506 binding protein 5	HPA axis—negative feedback	Yehuda et al. (2015)
GNRHR2	Gonadotropin-releasing hormone receptor	HPG axis	Khor et al. (2016)
HSD11B1	11β-Hydroxysteroid dehydrogenase type 1	HPA axis—stress response	Verstraeten et al. (2019)
HSD11B2	11β-Hydroxysteroid dehydrogenase type 2	HPA axis—stress response	Peña et al. (2012)
HTR1A	5-hydroxytryptamine receptor 1A	Serotonergic system	Ahmed et al. (2014)
MC2R[a]	Melanocortin receptor 2	HPA axis—stress response	Lewis et al. (2021)
MC4R[a]	Melanocortin receptor 4	HPA axis—stress response; metabolism	Ryan et al. (2014)
NLRC5	NOD-like receptor family CARD domain containing 5	Immune system	Murani et al. (2022)
NR3C1[a]	Glucocorticoid receptor	HPA axis—negative feedback	Witzmann et al. (2012)
NR3C2[a]	Mineralocorticoid receptor	HPA axis—negative feedback	Madison et al. (2018)
NR4A1	Nerve growth factor IB	Nuclear hormone receptor	Kember et al. (2012)
POMC[a]	Proopiomelanocortin	HPA axis—stress response	Wu et al. (2014)
SIK2	Salt inducible kinase 2	Metabolism	Liu et al. (2012)
UCN3	Urocortin – 3	HPA axis—stress response	Alcántara-Alonso et al. (2017)
VIP	Vasoactive intestinal peptide	HPA axis—stress response (circadian)	Loh et al. (2008)

[a]Genes targeted exons in addition to the putative promoter.

[b]Examples of studies that demonstrate the role of the gene in modulating responses to stress, or where possible, show the impact of early life stress via changes in expression or epigenetic regulation on the gene (for full reviews, see Sosnowski et al., 2018, van Bodegom et al., 2017 and Argentieri et al., 2017).

Solomon, 2023 for more on deduplication methods for EM-seq). Bismark coverage files were intersected with 4 kb 'promoter' and exon ranges using Bedtools v2.29.2 (Quinlan & Hall, 2010). Coverage files were then converted to unfiltered 0-based coordinate bedGraph files and combined using unionbedg to compare coverage across samples (see DMR analysis). We used SAMtools coverage to calculate mean read coverage stats in probe target ranges (Danecek et al., 2021).

We found that the control probes were highly specific, targeting the first 1 kb for pUC19 and the first 2 kb for lambda (Rubenstein & Solomon, 2023). We excluded samples from our analysis that did not amplify ($n=6$) or that failed a control check (<90% methylation in pUC19, or >4% methylation in lambda) ($n=16$). In addition, we excluded samples with <25 times mean coverage after deduplication ($n=5$). On the HiSeq 4000, for blood samples that amplified and passed control checks ($n=35$), the mean lambda was 0.31%, and the mean pUC19 was 96.42%, and for brain samples that amplified and passed control check ($n=59$), the mean lambda was 0.20% and the mean pUC10 was 96.93%. On the NovaSeq 6000, for blood samples that amplified and passed control checks ($n=75$), the mean lambda was 0.69%, and the mean pUC19 was 94.98%. As a subset of our blood samples were used for another analysis, our final data set for this analysis included samples from the blood ($n=48$), hypothalamus ($n=29$) and hippocampus ($n=30$).

2.5 | DMR analysis

We used Metilene v0.2-7 (Jühling et al., 2016) to identify differentially methylated regions (DMRs). Metilene uses circular binary segmentation and a two-dimensional Kolmogorov–Smirnov (2D-KS) test to determine DMRs between dichotomous groups, which constrained our analysis to pairwise comparisons. We analysed six pairwise comparisons in three different tissues for day 10 (blood) and day 11 (brain) samples of each treatment group: 'Parental Control – Developmental Control' (PC-DC) (hypothalamus $n=9$; hippocampus $n=9$; blood $n=12$); 'Parental Stress – Developmental Stress' (PS-DS)

(hypothalamus $n=7$; hippocampus $n=8$; blood $n=12$); 'Parental Control – Developmental Stress (PC-DS)' (hypothalamus $n=6$; hippocampus $n=6$; blood $n=13$); and 'Parental Stress – Developmental Control' (PS-DC) (hypothalamus $n=7$; hippocampus $n=7$; blood $n=11$). Due to the design of our experiment, some of the nestlings were related, but our sample sizes were too small to take this into account for our analysis. We set the minimum number of CpGs in a DMR to 3, and filtered the results by an absolute mean methylation difference of more than 2.0% between groups; we used this threshold because it is unknown what percentage of change is needed to affect gene expression, and studies indicate that it may be very low (<10%) (Laine et al., 2022), and because we focused on fewer target regions it made sense to have a narrow DMR window (Bentz et al., 2021). We then used a cut-off of Mann–Whitney *U* (MWU)-test *p*-value <.01 and *q*-value <0.1 corrected for multiple comparisons using Benjamini–Hochberg; *q*-values and MWU-test *p*-values are reported. We used these particular cut-offs to be more inclusive in our analysis and to capture DMRs despite our relatively small sample size (Laine et al., 2022). Since we found no DMRs in the exon regions of any genes analysed in any tissues, we focused our analysis only on putative promoter regions.

2.6 | RNA sequencing analysis (TagSeq)

We analysed extracted RNA from the hypothalamus and hippocampus on an Agilent 4200 TapeStation to determine concentration and RINe for RNA integrity, using a cutoff of 7.6 (mean RINe = 9.44). We then sequenced RNA using TagSeq (Meyer et al., 2011) on an Illumina NovaSeq S1 (SR100) at the Genomic Sequencing and Analysis Facility, University of Texas, Austin. TagSeq works by only priming the 3′ end of mRNA fragments and generating a single tag read for each transcript, making it an efficient and low-cost alternative to whole mRNA seq for differential gene expression analysis (Lohman et al., 2016).

We sequenced 29 hypothalamus and 30 hippocampus RNA samples. We trimmed and aligned files to the great tit (*Parus major*) reference genome (Ensembl 2021) using STAR v2.7.9. We used the great tit genome rather than the house sparrow genome because it was better at identifying our genes of interest (Table 1). For differential expression analysis of gene counts between each treatment group, we used DESeq2, under Bioconductor v3.10 in R 3.6.2 (Love et al., 2014). We examined differential expression using Wald tests and an FDR-adjusted *p* value ($p<.1$ indicating differential expression).

3 | RESULTS

3.1 | TEEM-Seq metrics

Target coverage ranged from a mean of 149–631X, with an overall mean of 393X before deduping for samples sequenced on the HiSeq 4000. After deduplication, the target coverage ranged from a mean of 28–242X, with an overall mean of 130x. For samples that amplified, passed control checks and had over 28X deduplication coverage, sequence depth ranged from 1,149,192 to 9,794,490 reads. Similarly, target coverage ranged from a mean of 34–347X, with an overall mean of 160X before deduping for samples sequenced on the NovaSeq 6000. After deduplication, the target coverage ranged from a mean of 28–218X, with an overall mean of 112X. For samples that amplified, passed control checks and had over 28X deduplication coverage, sequence depth ranged from 1,041,387 to 5,627,204 reads.

3.2 | TagSeq metrics

For the 25 genes in our panel, most were annotated on the great tit (*Parus major*) genome and had sufficient coverage when our samples from the hypothalamus ($n=29$) and hippocampus ($n=30$) were aligned. *GNRHR2* and *POMC* were not annotated on the great tit genome but the overlapping gene was located using BLAST; *MC4R* was not annotated and we could not locate an overlapping gene. In our samples, *MC2R* and *POMC* did not have coverage in either the hypothalamus or hippocampus, and *GNRHR2* and *NLRC5* did not have coverage in the hippocampus.

The average number of input reads was 1,504,969.75, and the average mean input read length was 74.56. For unique reads, the average number of uniquely mapped reads was 933,463.88, or 61.75%; the average mean mapped length was 73.75, and the average number of total splices was 78,689.32. For multi-mapping reads, the average number of reads that mapped to multiple loci was 42,119.64, or 2.78%, and the average number of reads that mapped to too many loci was 1020.83, or 0.06%. For unmapped reads, the average number of unmapped reads due to mismatches was 0, or 0%; the average number of unmapped reads due to being too short was 528,285.44, or 35.4%; and the average number of unmapped reads due to 'other' was 79.95 (<0.01%).

3.3 | The influence of early life stress across development

To assess the influence of early life stress across both environmental periods, we compared DNA methylation within each tissue between nestlings that experienced stress in both the parental and post-natal developmental period (PS-DS) and nestlings that did not experience any stress (PC-DC). In the hypothalamus, we found one DMR in the putative promoter of *NR3C1* (Table 2); DNA methylation was higher in the PS-DS group compared to the PC-DC group. We found no DMRs in the hippocampus or the blood.

We also assessed gene expression in the hippocampus and hypothalamus in order to verify the predicted negative relationship between DNA methylation and gene expression. While there were no significant differences in gene expression between the PS-DS and PC-DC groups, we looked specifically for trends in any genes within tissues with significant DMRs. In *NR3C1* in the hypothalamus, we

found that expression levels were similar between the PC-DC group and the PS-DS group, with greater variation in the PS-DS group (Figure 2b).

3.4 | The timing of early life stress: Parental stress versus post-natal stress

To assess whether the timing of early life stress differentially influences DNA methylation, we compared DNA methylation within each tissue between nestlings that experienced only parental stress (PS-DC) and those that experienced only post-natal developmental stress (PC-DS). We found two DMRs in the hippocampus in the putative promoter of *NR3C2* (Table 3a). DNA methylation was higher in the nestlings that experienced post-natal developmental stress (PC-DS) compared to those that experienced parental stress (PS-DC) in both DMRs. We found no DMRs in the hypothalamus or the blood.

In our gene expression analysis, we found a marginally significant difference in gene expression in the gene *ZPF36L1*, which had a trend for lower expression in the PC-DS group compared to the PS-DC group in the hypothalamus (p_{adj} = .068; Figure 2a), and significantly lower expression in the PC-DS group compared to the PS-DC group in the hippocampus (p_{adj} = .002; Figure 3a). Looking specifically at *NR3C2* in the hippocampus, there was a non-significant trend for higher expression (and greater variation) in the PS-DC group compared to the PC-DS group (Figure 3b).

TABLE 2 The influence of early life stress across development. Differentially methylated regions (DMRs) comparing nestlings in the overall control (PC-DC) versus overall stress (PS-DS) treatment groups. The base pair start and stop location is provided, as well as number of CpG sites within the DMR, and the mean DNA methylation for each group. The subscript number after the gene name indicates the probe used to capture that distinct region of the gene if multiple probes were used either due to length of the gene or non-consecutive target hits in the genome during probe development. Direction indicates which sample mean (PC-DC or PS-DS) has higher DNA methylation.

Tissue	Gene	Start	Stop	CpG#	PC-DC mean	PS-DS mean	Mean methylation difference	Direction	*p*-Value	*q*-Value
Hypothalamus	*NR3C1*$_7$	2064	2094	3	34.10	65.93	−31.83	PS-DS	<.001	0.045

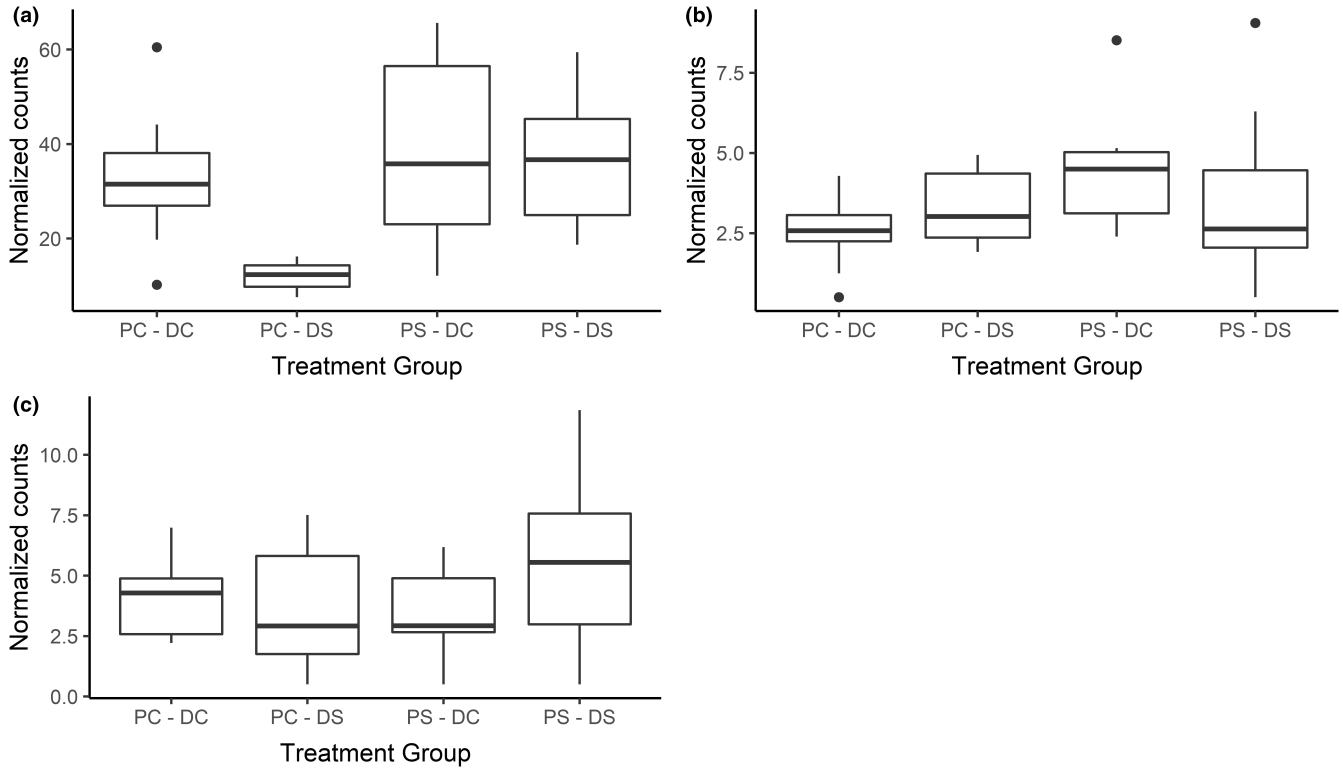

FIGURE 2 Differential gene expression in the hypothalamus for *ZPF36L1* (a), *NR3C1* (b) and *NR3C2* (c) between treatment groups. Boxplots indicate median, upper and lower quartiles, and range of the mean normalized count for each treatment group. Outliers are shown as black dots. Treatment groups are: Parental Control – Developmental Control (PC-DC), Parental Control – Developmental Stress (PC-DS), Parental Stress – Developmental Control (PS-DC) and Parental Stress – Developmental Stress (PS-DS). In *ZPF36LI* (a), expression was significantly lower in the PC-DS group compared to the PS-DC group. In *NR3C1* (b), expression was not significantly different between any treatment groups. In *NR3C2* (c), expression was higher (but not significantly so) in the PS-DS group compared to the PC-DS group.

In addition, we compared each of these groups (PS-DC; PC-DS) separately to nestlings that did not experience any stress (PC-DC). Comparing nestlings that experienced only parental stress (PS-DC) to those that did not experience any stress (PC-DC), we found one DMR in blood in the putative promoter region of *HSD11B1*, and one DMR in the putative promoter region of *NR3C1* (Table 3b). DNA methylation was higher in the nestlings that did not experience stress (PC-DC) compared to those that experienced only parental stress (PS-DC) in *HSD11B1*, while DNA methylation was higher in nestlings that experienced only parental stress (PS-DC) in *NR3C1*. We found no DMRs in the hypothalamus or hippocampus.

Comparing nestlings that experienced only post-natal developmental stress (PC-DS) to those that did not experience any stress (PC-DC), in the blood we found one DMR in the putative promoter of *HSD11B1*, and one DMR in the putative promoter of *NR3C2* (Table 3c). In the hippocampus, we found one DMR in the putative promoter of *NR3C2*. DNA methylation was higher in the nestlings that did not experience any stress (PC-DC) compared to those that experienced only post-natal developmental stress (PC-DS) in *HSD11B1*, while DNA methylation was higher in nestlings that experienced only post-natal developmental stress (PC-DS) for both *NR3C2* DMRs. We found no DMRs in the hypothalamus.

In our gene expression analysis, there were no significant differences in gene expression in the hippocampus or hypothalamus when comparing PS-DC to PC-DC. Comparing PC-DS to PC-DC, we found a significant difference in gene expression in the gene *ZPF36L1*, which had lower expression in the PC-DS group compared to PC-DC group (p_{adj} = .023) in the hippocampus (Figure 3a).

TABLE 3 The timing of early life stress. Differentially methylated regions (DMRs) comparing nestlings experiencing stress at different times: (a) Parental stress versus post-natal developmental stress (PS-DC vs. PC-DS); (b) Parental stress versus control (PS-DC vs. PC-DC); and (c) Post-natal developmental stress versus control (PC-DS vs. PC-DC). The base pair start and stop location is provided, as well as number of CpG sites within the DMR, and the mean DNA methylation for each group. The subscript number after the gene name indicates the probe used to capture that distinct region of the gene if multiple probes were used either due to length of the gene or non-consecutive target hits in the genome during probe development. Direction indicates which sample mean has higher DNA methylation.

Tissue	Gene	Start	Stop	CpG#	PC-DS mean	PS-DC mean	Mean methylation difference	Direction	*p*-Value	*q*-Value
A. Parental stress versus post-natal developmental stress (PS-DC vs. PC-DS)										
Hippocampus	$NR3C2_3$	1492	1631	14	87.57	80.33	7.24	PC-DS	<.001	0.054
Hippocampus	$NR3C2_3$	2138	2273	28	95.20	92.16	3.04	PC-DS	<.001	0.054
B. Parental stress versus control (PS-DC vs. PC-DC)										
Blood	$HSD11B1_8$	48	279	23	10.34	4.76	5.57	PC-DC	<.001	<0.001
Blood	$NR3C1_8$	1313	1340	8	1.82	3.83	−2.01	PS-DC	<.001	0.059
C. Post-natal developmental stress versus control (PC-DS vs. PC-DC)										
Blood	$HSD11B1_8$	48	492	11	12.89	4.44	8.45	PC-DC	<.001	<0.001
Blood	$NR3C2_3$	2625	2660	8	84.77	88.66	−3.89	PC-DS	<.001	0.061
Hippocampus	$NR3C2_3$	2200	2392	19	92.42	95.50	−3.08	PC-DS	<.001	0.073

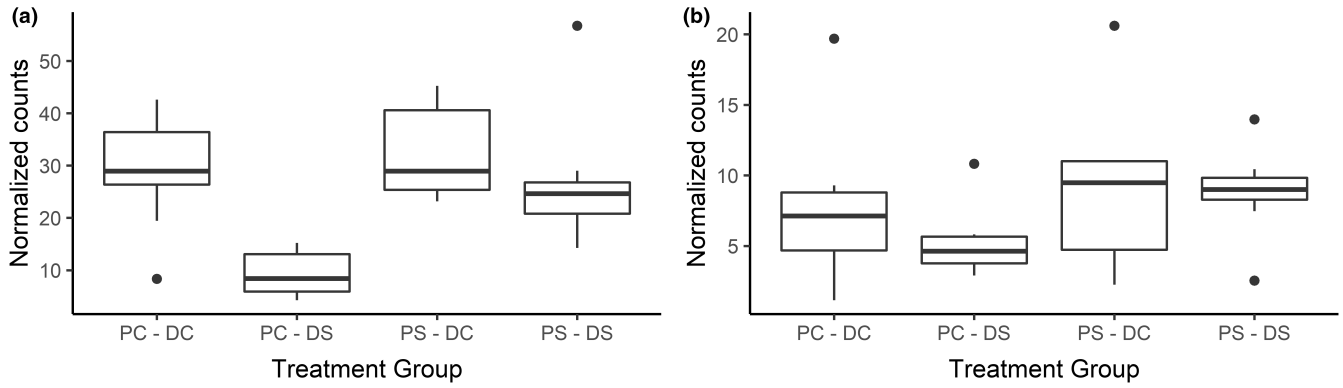

FIGURE 3 Differential gene expression in the hippocampus for *ZPF36L1* (a) and *NR3C2* (b) between treatment groups. Boxplots indicate median, upper and lower quartiles, and range of the mean normalized count for each treatment group. Outliers are shown as black dots. Treatment groups are: Parental Control – Developmental Control (PC-DC), Parental Control – Developmental Stress (PC-DS), Parental Stress – Developmental Control (PS-DC) and Parental Stress – Developmental Stress (PS-DS). In *ZPF36LI* (a), expression was significantly lower in the PC-DS group compared to all other treatment groups. In *NR3C2* (b), expression was higher (but not significantly so) in the PS-DS group compared to the PC-DS group.

Looking specifically at *NR3C2* in the hippocampus, there was a non-significant trend for higher expression (and greater variation) in the PS-DC group compared to the PC-DC group (Figure 3b).

3.5 | The interactive effect of parental and post-natal developmental stress

To assess the potential interaction between stressors encountered by parents and those encountered by nestlings in the post-natal developmental environment, we compared DNA methylation within each tissue in nestlings who experienced both stressors (PS-DS) to those that only experienced parental stress (PS-DC) and to those that only experienced post-natal developmental stress (PC-DS). Comparing PS-DS to PS-DC, we found no DMRs in the hypothalamus, hippocampus or blood.

Comparing PS-DS to PC-DS, in the blood we found one DMR in the putative promoter of *HSD11B1* (Table 4). DNA methylation was higher in nestlings that experienced both stressors (PS-DS) compared to those that experienced only post-natal developmental stress (PC-DS). In both the hypothalamus and the hippocampus, we also found a DMR in the putative promoter of *NR3C2*; DNA methylation was higher in nestlings that experienced post-natal developmental stress (PC-DS) compared to those that experienced both stressors (PS-DS) for both DMRs.

In our gene expression analysis, there were no significant differences in gene expression in the hippocampus or hypothalamus when comparing PS-DS to PS-DC. Comparing PS-DS to PC-DS, we again found a significant difference in gene expression in the gene *ZPF36L1*, which had lower expression in the PC-DS group compared to the PS-DS group (p_{adj} = .102) in the hippocampus (Figure 3a). Looking specifically at *NR3C2* in the hypothalamus, the PS-DS group showed a non-significant trend for higher expression than the PC-DS group (Figure 2c). Similarly, in the hippocampus, there was a non-significant trend of higher expression in the PS-DS group compared to the PC-DS group (Figure 3b).

4 | DISCUSSION

To understand the role of epigenetic mechanisms in shaping developmental responses to early life stress, we examined DNA methylation in a free-living population of house sparrows. Specifically, we surveyed a suite of 25 genes related to HPA axis function, the related HPG axis, or that have been shown previously to be impacted by early life stress in order to gain a more nuanced and comprehensive understanding of how these epigenetic modifications relate to early life stress. By manipulating both parental stress and post-natal developmental stress, we were able to compare differentially methylated regions (DMRs) in offspring that experienced stress during different early life-history stages, as well as the potentially interactive effect of these different stressors. In addition, by assessing DNA methylation in both the brain and the blood, we were able to evaluate cross-tissue patterns in differentially methylated regions, testing the possibility that blood may be used as a biomarker for DNA methylation in other target regions.

Of the 25 genes that we targeted in our analysis, only three showed differences in DNA methylation (*NR3C1*, *NR3C2*, and *HSD11B1*). Moreover, these same three genes showed up repeatedly in different treatment comparisons. We found that nestlings that experienced early life stress across both the parental and post-natal developmental periods had higher DNA methylation in the putative promoter of a *NR3C1* in the hypothalamus. The hypothalamus plays an important role in both the stress response and negative feedback regulation. During the stress response, the hypothalamus releases arginine vasopressin (AVP) and corticotropin-releasing hormone (CRH), which mediate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, stimulating the release of glucocorticoids from the adrenal cortex (Meaney et al., 1996; Smith & Vale, 2006). In addition, GR, encoded by *NR3C1*, functions in the hypothalamus to regulate negative feedback of the stress response by inhibiting synthesis and/or release of CRH and AVP (Welberg & Seckl, 2001). Specifically, high levels of GR are found in neurons of the hypothalamic paraventricular nucleus (PVN), an important site for negative feedback regulation (Smith & Vale, 2006). This finding is consistent with our initial prediction that general early life stress would increase DNA methylation in *NR3C1*. Numerous studies in various taxa have shown that early life stress increases DNA methylation in the promoter region of *NR3C1* in the brain (Kember et al., 2012; McGowan et al., 2009; Weaver et al., 2004), as well as specifically in the hypothalamus (Ahmed et al., 2014; Oztürk et al., 2022). Our findings in the hypothalamus are, therefore, consistent with previous results,

TABLE 4 The interactive effect of parental and post-natal stress. Differentially methylated regions (DMRs) comparing nestlings who experienced both stressors (PS-DS) with nestlings who experienced only post-natal developmental stress (PC-DS). The base pair start and stop location is provided, as well as number of CpG sites within the DMR, and the mean DNA methylation for each group. The subscript number after the gene name indicates the probe used to capture that distinct region of the gene if multiple probes were used either due to length of the gene or non-consecutive target hits in the genome during probe development. Direction indicates which sample mean has higher DNA methylation.

Tissue	Gene	Start	Stop	CpG#	PC-DS mean	PS-DS mean	Mean methylation difference	Direction	*p*-Value	*q*-Value
Blood	$HSD11B1_8$	48	492	28	4.44	13.20	−8.76	PS-DS	<.001	<0.001
Hypothalamus	$NR3C2_3$	1967	2290	38	96.83	94.58	2.25	PC-DS	<.001	0.069
Hippocampus	$NR3C2_3$	2138	2290	32	95.10	92.39	2.71	PC-DS	<.001	0.038

demonstrating that early life stress leads to increased DNA methylation in *NR3C1* in the brain (Turecki & Meaney, 2016), which is associated with lower expression and thus decreased stress responsivity (reviewed in Champagne & Curley, 2009). Interestingly, we did not find differential methylation in other genes involved in mediating negative feedback, thus underlining the central role and responsiveness of *NR3C1* in this system. This finding suggests that we can now extend previous, laboratory-based findings on the role of *NR3C1* to a broader, free-living vertebrate system.

Next, we assessed whether the timing of early life stress between parental and post-natal developmental environments differentially influenced DNA methylation. In line with our prediction, we found that the life-history stage in which stress was encountered impacted some genes differently, including genes involved in both negative feedback and the stress response. This supports similar findings from a study in Japanese quail (*Coturnix japonica*) which showed that gene expression patterns in some genes responded similarly to both pre-natal and post-natal stressors, while others were uniquely impacted by one type of stressor (Marasco et al., 2016). We found that in *HSD11B1*, exposure to early life stress in either the parental or post-natal developmental period led to lower levels of DNA methylation in the putative promoter region, which would suggest increased gene expression. *HSD11B1* is an isoform of 11β-hydroxysteroid dehydrogenase that regenerates active glucocorticoids, thus increasing local glucocorticoid activity (Chapman et al., 2013). Exposure to glucocorticoids prenatally has been shown to increase its expression in some cells and tissues (Nyirenda et al., 2009), but not in others (reviewed in Chapman et al., 2013). To our knowledge, however, previous studies have only looked at the related *HSD11B2* gene for epigenetic responses to early life stress (Conradt et al., 2013; Marsit et al., 2012; Monk et al., 2016). We, therefore, show that DNA methylation of *HSD11B1* responds similarly to stressors encountered in either life-history period.

In contrast, *NR3C1* and *NR3C2* were differentially impacted by stress in the parental versus post-natal developmental periods. Parental (but not post-natal) stress generated a DMR in the blood in the putative promoter region of *NR3C1*, with higher DNA methylation in nestlings that experienced parental stress compared to those that did not. This suggests that parental stress leads to increased DNA methylation in the *NR3C1* putative promoter, which is maintained regardless of the post-natal environment. This finding is consistent with previous work in other birds, such as the European starling (*Sturnus vulgaris*), where pre-natal exposure to elevated levels of glucocorticoids led to a lower stress response in offspring, regardless of the developmental environment (Love & Williams, 2008). In contrast, post-natal (but not parental) stress generated a DMR in the putative promoter region of *NR3C2*, with higher DNA methylation in nestlings that experienced post-natal stress compared to those that did not. Contrary to our prediction, this suggests that post-natal stress leads to increased DNA methylation in the *NR3C2* putative promoter, regardless of the parental environment. *NR3C2* encodes the mineralocorticoid receptor (MR), which regulates HPA axis activity and negative feedback in a converse manner to GR due to its higher affinity for GCs (de Kloet et al., 2005). Exposure to stressors in the early life environment have been shown to decrease MR expression (Vazquez et al., 1996). Our results similarly show that in house sparrows, early life stress experienced in post-natal development leads to increased DNA methylation in *NR3C2*, which suggests decreased MR expression (Figure 3b). It is particularly interesting that we see different impacts of stress on *NR3C1* and *NR3C2*, as the ratio of glucocorticoid receptors (GR, encoded by *NR3C1*) to mineralocorticoid receptors (MR, encoded by *NR3C2*) are critical for mediating HPA axis feedback in general (Galbally et al., 2020), and for the stress response in house sparrows specifically (Liebl & Martin, 2014). Indeed, the MR/GR balance hypothesis suggests that it is the coordination of these receptors together that determines the effects of glucocorticoids (De Kloet et al., 1998; Liebl & Martin, 2014). Our results suggest that these genes respond to, or are sensitive to, early life stress during different life-history stages, and thus the timing of early life stress may lead to subtle changes in how the stress response is regulated due to differences in MR and GR glucocorticoid affinity.

Finally, we examined the potential interactive effects of stressors encountered in both life-history periods. We considered two potential interactions: an additive effect of stressors, or mitigation of parental stress by post-natal stress. We primarily found evidence for mitigation of parental stress by post-natal stress, specifically in the genes *HSD11B1* and *NR3C2*. In *HSD11B1*, experiencing either parental or post-natal developmental stress decreased DNA methylation in the putative promoter region of this gene, but experiencing both stressors increased DNA methylation to similar levels as in nestlings that did not experience any early life stress (PC-DC). Similarly, in *NR3C2*, experiencing post-natal developmental stress increased DNA methylation in this gene, but experiencing both stressors decreased methylation to similar levels as in nestlings that did not experience any early life stress (PC-DC). Notably, these changes in *NR3C2* occurred across tissues, with overlapping DMRs in the hypothalamus and hippocampus, and a neighbouring DMR in the blood (see below). Thus, in both *HSD11B1* and *NR3C2* there was no significant difference in mean DNA methylation between nestlings that experienced no stress (PC-DC) and those that experienced both stressors (PS-DS). This finding explains why, unlike *NR3C1*, we did not see differential methylation in *NR3C2* when we looked at the influence of early life stress across both life-history periods. Other work in birds (Marasco et al., 2012) and especially in mammals (Maccari et al., 1995; Vallée et al., 1997, 1999) have similarly shown that post-natal stress can mitigate the effects of parental stress, where individuals experiencing combined stressors have a similar stress response as control individuals. However, such studies are rare, and ours is the first to our knowledge to investigate the epigenetic mechanisms underlying these changes in birds.

While our findings suggest an impact of timing of stress exposure on differential DNA methylation, it is important to consider that these results could also be due to differences in the types of stress (acute or chronic) experienced in each life-history period. An acute stressor works through the HPA axis to activate a physiological and

behavioural response by increasing circulating GCs, which subsequently control HPA axis activity through negative feedback. In contrast, a chronic stressor may disrupt the negative feedback system through prolonged elevation of glucocorticoids, leading to hyperactivity of the HPA axis (Zhu et al., 2014). The shift from an acute to chronic stress response may depend on the kind of stressor, the frequency of presentation, and the circumstances under which the stressor is encountered. As these factors differed in our experiment between the two life-history stages, it is possible that stressors shifted from acute to chronic in either period, and thus interfaced with different underlying physiological mechanisms. It will, therefore, be important in future work to verify the effect of the parental stress treatment on parents' physiology to determine if it induces chronic stress, and determine if the same stress paradigm used in both life-history stages produces similar results.

Our findings on the interactive effects of parental and post-natal stress are important for understanding the potential adaptive significance of stressors encountered throughout early life. As the HPA axis is critical in regulating physiological and behavioural responses to the environment, alterations in DNA methylation, and subsequently expression, of the HPA axis genes in early life can have long-term consequences for survival and fitness. Our findings indicate that experiencing stress across both early life-history stages (compared to just one) increases DNA methylation in a gene related to the stress response (*HSD11B1*), and decreases DNA methylation in a critical gene related to basal levels of GCs and negative feedback (*NR3C2*), which we predict would produce a dampened stress response. Other work on birds has suggested that for offspring experiencing a challenging or unpredictable developmental or later life environment, a lower stress response would be adaptive by avoiding frequent, costly increases in energy mobilization through repeated activation of the HPA axis, thereby enhancing survival (Grace et al., 2020; Weber et al., 2018). Parental stress may, therefore, serve as a predictive cue that, rather than hindering offspring development (as suggested by the 'silver spoon hypothesis'; Grafen, 1990), instead adaptively programming offspring for a stressful environment later in life (Love & Williams, 2008). Our results are thus consistent with predictions of the predictive adaptive response (PAR) hypothesis, which suggests that early life stress is adaptive when there is an environmental match between the developmental and adult environments (Gluckman et al., 2005; Monaghan, 2008). However, whether or not a lower stress response is adaptive will also depend on the specific stressors encountered in the developmental and adult environments, as a dampened stress response may be more adaptive in regard to some stressors (e.g. low resource availability or repeated non-lethal disturbances) than others (e.g. high predation risk) (see Schoenle et al., 2018 for review). Further study is needed to connect these changes in DNA methylation to HPA axis functionality—both in regard to the stress response and negative feedback system—to assess the potential fitness consequences of these epigenetic changes across different post-natal environments.

Although we did not find significant differences in gene expression between nestling groups in genes across our panel, we did find a trend in *NR3C2*—the most common gene to show differential DNA methylation in all of our comparisons—that suggests that DNA methylation suppresses gene expression. In both the hypothalamus and the hippocampus, there was a trend of higher expression in the Parental Stress – Developmental Stress (PS-DS) group compared to the Parental Control – Developmental Stress (PC-DS) group, corresponding with lower DNA methylation in the DMRs in the PS-DS group compared to the PC-DS group. Our inability to detect significant differences in gene expression in this or other genes is most likely due to limitations generated by our experimental design. Because we used a full factorial design, we had small sample sizes that may not have had enough power to show significant differences in gene expression. In addition, we were unable to standardize times between collection, euthanization and tissue removal of individuals, and times differed based on the distance of the field site to the laboratory and other mitigating factors which likely impacted mRNA in tissues at the time of extraction. Finally, a number of our DMRs occurred in the blood, and we were unable to collect RNA from blood during our experiment. However, it is also possible that differential DNA methylation in specific regulatory regions did not alter gene expression. While DNA methylation in promoter regions is typically inversely associated with gene expression (Bird & Wolffe, 1999; Deaton & Bird, 2011; Siegfried et al., 1999), this is not always the case (Walsh & Bestor, 1999; Warnecke & Clark, 1999), suggesting that the relationship between DNA methylation and expression is more complex (Buitrago et al., 2021). For instance, Jimeno et al. (2019) found that *NR3C1* expression was not correlated with average percent DNA methylation or the number of CpG sites but was strongly correlated at specific CpG sites close to the exon. To assess the impact of these DNA methylation marks on phenotypic outcomes, it will be important to verify this relationship between DNA methylation and expression, as well as the impact of these methylation marks on HPA axis function. We did find a significant difference in expression in *ZFP36L1*, which codes for an RNA-binding protein and is a potential tumour suppressor gene (Martínez-Calle et al., 2019); its role here requires further investigation.

By assessing DNA methylation in both the brain and the blood, we were able to evaluate cross-tissue patterns. Although DMRs in *HSD11B1* were only apparent in the blood, we found cross-tissue DMRs in both *NR3C2* and *NR3C1*. In *NR3C2*, overlapping DMRs occurred in the hypothalamus and hippocampus, and a neighbouring DMR also occurred in the blood. Similarly, we found a neighbouring DMR in *NR3C1* in both the blood and the hypothalamus. This is consistent with other studies that have reported similar changes in DNA methylation in the same genes across tissues (Davies et al., 2012; Masliah et al., 2013). While previous work did not find similar DNA methylation in *NR3C1* across tissues in the European starling (*Sturnus vulgaris*) (Siller & Rubenstein, 2019), it was suggested that this might be because cross-tissue changes in DNA methylation require exposure to developmental stress (Aberg et al., 2013). Our results support this hypothesis, as early life stress led to similar changes in DNA methylation in both *NR3C1* and *NR3C2* in multiple tissues. Our results also support previous findings in this system showing that DNA methylation marks

may occur across tissues in some genes (Siller Wilks et al., 2023), and suggests that blood may be a potential biomarker for DNA methylation of specific genes in target central nervous system regions.

In conclusion, using a full factorial experimental design, we were able to compare DNA methylation in nestlings exposed to stress during different early life-history stages. In addition, by examining a larger suite of genes in multiple tissue types, we were able to gain a more comprehensive understanding of the role that DNA methylation modifications play in shaping multiple components of the HPA axis in response to early life stress. Overall, early life stress led to differentially methylated regions in the putative promoters of three key genes related to HPA axis functioning. In addition, the life-history stage in which early life stress was encountered impacted genes differently, with the glucocorticoid receptor gene (*NR3C1*) responding more to parental stress and the mineralocorticoid receptor gene (*NR3C2*) responding more to post-natal developmental stress. We also found similar DMRs in both of these receptor genes across tissues, suggesting that blood may be able to function as an effective biomarker for stress in the brain, at least for some genes. Finally, we found evidence that post-natal stress may mitigate parental stress via DNA methylation, specifically in the genes *HSD11B1* and *NR3C2*.

However, to fully understand the adaptive potential of these changes, we need to consider additional phenotypic and fitness measures alongside these epigenetic modifications. Our results provide a crucial first step in understanding the mechanisms by which early life stress in different life-history periods contributes to changes in the epigenome of the HPA axis.

AUTHOR CONTRIBUTIONS

Stefanie J. Siller Wilks: Conceptualization, Investigation, Data curation, Formal analysis, Original draft preparation. **Britt J. Heidinger**: Conceptualization, Methodology, Resources, Writing—Reviewing and Editing. **David F. Westneat**: Conceptualization, Methodology, Writing—Reviewing and Editing. **Joseph Solomon:** Methodology, Validation. **Dustin R. Rubenstein**: Supervision, Conceptualization, Resources, Writing—Reviewing and Editing.

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CONFLICT OF INTERST STATEMENT

The authors report no financial interests or potential conflict of interest.

DATA AVAILABILITY STATEMENT

Raw genetic data are available at the NCBI Sequence Read Archive (SRA) (BioProject PRJNA1018102). Metadata are also stored in the SRA (BioProject PRJNA1018102). Code is available at https://doi.org/10.6084/m9.figshare.24164100.

ANIMAL STUDIES

This study was performed under the approval of North Dakota State University IACUC (protocol A17035).

BENEFIT-SHARING STATEMENT

A research collaboration was developed with scientists from laboratories at Columbia University, University of Kentucky and North Dakota State University, which provided genetic samples. All collaborators are included as co-authors, and contributions outlined (see below). The results of this research have been shared with the collaborators' academic communities and the broader scientific community via biological databases (see above).

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