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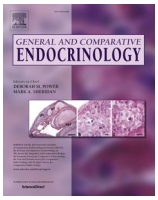

Research paper

Epigenetic modification of the hypothalamic–pituitary–adrenal (HPA) axis during development in the house sparrow (*Passer domesticus*)

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ABSTRACT

Epigenetic modifications such as DNA methylation are important mechanisms for mediating developmental plasticity, where ontogenetic processes and their phenotypic outcomes are shaped by early environments. In particular, changes in DNA methylation of genes within the hypothalamic–pituitary–adrenal (HPA) axis can impact offspring growth and development. This relationship has been well documented in mammals but is less understood in other taxa. Here, we use target-enriched enzymatic methyl sequencing (TEEM-seq) to assess how DNA methylation in a suite of 25 genes changes over development, how these modifications relate to the early environment, and how they predict differential growth trajectories in the house sparrow (*Passer domesticus*). We found that DNA methylation changes dynamically over the postnatal developmental period: genes with initially low DNA methylation tended to decline in methylation over development, whereas genes with initially high DNA methylation tended to increase in methylation. However, sex-specific differentially methylated regions (DMRs) were maintained across the developmental period. We also found significant differences in post-hatching DNA methylation in relation to hatch date, with higher levels of DNA methylation in nestlings hatched earlier in the season. Although these differences were largely absent by the end of development, a number of DMRs in HPA-related genes (*CRH, MC2R, NR3C1, NR3C2, POMC)*—and to a lesser degree HPG-related genes (*GNRHR2)*—predicted nestling growth trajectories over development. These findings provide insight into the mechanisms by which the early environment shapes DNA methylation in the HPA axis, and how these changes subsequently influence growth and potentially mediate developmental plasticity.

1. Introduction

The environment that an individual encounters during a critical period of early life can significantly influence its development, leading to permanent phenotypic changes (reviewed in Forsman 2015). Such an ability to alter developmental trajectories and phenotypic outcomes in response to early life environmental conditions is called developmental plasticity (Moczek et al., 2011). Developmental plasticity can occur in response to a wide variety of environmental cues, including diet and nutrition (Koyama et al., 2013), maternal care (Arsenault et al., 2018), sibling competition (Gil et al., 2008), predation risk (Meuthen et al., 2019), and human disturbance (Watson et al., 2021). One key mechanism that can mediate developmental plasticity is epigenetic modification, or the array of factors that change gene expression and function that are not based in changes in DNA sequence (Champagne 2013b; Richards 2006). DNA methylation, the addition of a methyl group to a cytosine, is one of the most commonly measured epigenetic modifications and can influence gene expression either by inhibiting the binding of transcription factors (Comb and Goodman, 1990; Inamdar et al., 1991) or by recruiting DNA binding proteins that produce chromatin modifications (Nan et al., 1997; Ng and Bird, 1999). DNA methylation can respond not only to changes in the embryonic environment, but also to the postnatal developmental environment, where hormonal, nutritional, social, and ecological conditions have been shown to impact DNA methylation (reviewed in Champagne 2013b). Such epigenetic modifications are therefore dynamic and highly

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responsive to the early environment, potentially facilitating developmental plasticity of numerous phenotypic traits (Champagne, 2013b; Szyf et al., 2008).

Epigenetic modifications set in early life have been shown to have long-lasting effects on the hypothalamic–pituitary–adrenal (HPA) axis (Champagne, 2013a). The HPA axis coordinates the release of glucocorticoid hormones, which are fundamental in regulating energy homeostasis in relation to growth and metabolism through interactions with other physiological pathways (McEwen and Wingfield, 2003; Moisiadis and Matthews, 2014; Mueller et al., 2012). A relationship between HPA axis function and growth has been demonstrated in humans (Ward et al., 2004) and in rats (reviewed in Kapoor et al., 2006), where programming of the HPA axis during development may impact early life growth, with subsequent impacts on adult metabolism. The HPA axis also facilitates physiological and behavioral responses to environmental challenges through the release of stress-induced glucocorticoids (Wingfield et al., 1998). Studies have shown that glucocorticoids and HPA axis functionality are tied not only to variation in physiology, personality, and cognition (reviewed in Schoech et al., 2011), but may also affect reproduction and survival (Bonier et al., 2009; Breuner et al., 2008). DNA methylation of genes involved in the HPA axis, such as the glucocorticoid receptor gene *NR3C1*, can alter HPA axis responsiveness by changing gene expression (Champagne, 2013b), and may subsequently influence numerous aspects of an offspring's development (Cottrell and Seckl, 2009; Seckl, 2004). Changes in DNA methylation of HPA axis genes can also have carryover effects to other life history stages (Turecki and Meaney, 2016), impacting phenotypic outcomes such as stress sensitivity (Murgatroyd et al., 2009), depressive behaviors (Roth et al., 2009), and disease risk (Argentieri et al., 2017) later in life.

The relationship between DNA methylation marks in the HPA axis in response to the early life environment and subsequent changes in development have been well studied in mammals, particularly in captive rodents (Champagne, 2013a; Meaney and Szyf, 2005; Weaver et al., 2004). However, we know much less about how this process works in other vertebrates, particularly in natural settings. Birds are a useful taxonomic group for investigating the role of DNA methylation in development in an independent evolutionary lineage from mammals, but thus far such studies are limited. For example, while it has been well documented in laboratory mammals that DNA methylation changes can occur after birth, particularly during early development (reviewed in Szyf et al., 2008), only a few studies have assessed DNA methylation marks in wild birds during development (Sheldon et al., 2020; von Holdt et al., 2022; Watson et al., 2019). Indeed, there is also limited information on the specific environmental conditions that produce differences in DNA methylation in birds (but see Sepers et al., 2021; Sheldon et al., 2018b; Sheldon et al., 2020). In particular, because variation in conditions that change throughout the season (e.g., incubation temperature, parental condition, and resource availability, can induce plastic changes in embryonic and postnatal avian development, growth, and survival (Arnold et al., 2006; Dawson et al., 2005; Gurney et al., 2012; Yan et al., 2015)), their relationship to early epigenetic changes are critical to explore. Furthermore, only a handful of studies in birds have shown that DNA methylation marks established during early life produce later phenotypic changes, with impacts on the avian stress response (Jimeno et al., 2019), stress resilience (Taff et al., 2019), dispersal (Liebl et al., 2021), and adult sex-specific breeding behavior (Rubenstein et al., 2016). Indeed, there remains a significant gap in our understanding of how DNA methylation of the HPA axis responds to certain early life conditions during avian development, and how these changes impact developmental trajectories to produce alternative phenotypes apparent in later life. Assessing the potential plasticity of DNA methylation in early development and linking early environmentally induced changes in DNA methylation to phenotypic outcomes will be critical for understanding the potential implications of early life epigenetic changes on fitness, adaptation, and evolution in not only avian species, but other taxa as well.

Here, we assess the relationship between developmentally induced changes in DNA methylation of the HPA axis in response to the early environment and changes in growth during development in the house sparrow (*Passer domesticus*). Recent studies have begun to investigate and characterize natural epigenetic variation in this species (Riyahi et al., 2017), with research suggesting that epigenetic variation in house sparrows is strongly influenced by local environmental conditions (Sheldon et al., 2018a) and may be an important facilitator of phenotypic plasticity by providing high epigenetic potential (Hanson et al., 2020). In particular, variation in glucocorticoid regulation in house sparrows has been associated with variation in neural plasticity and epigenetic potential (Kilvitis et al., 2018), making house sparrows an ideal species to investigate the relationship between DNA methylation in the HPA axis and developmental plasticity. In a wild population of house sparrows from North America, we examine the link between natural variation in the early environment based on hatch date and variation in differentially methylated regions (DMRs) throughout the HPA axis, as well as variation in DMRs and differential growth trajectories. Using target enriched enzymatic methyl sequencing (TEEM-seq) (Rubenstein and Solomon, 2023), we analyze DNA methylation in a suite of 25 genes related to the function of the HPA axis, as well as the related hypothalamic-pituitary–gonadal (HPG) axis, or whose expression has otherwise been shown to be impacted by early life conditions. First, we determine how DNA methylation marks change over the developmental period by comparing individuals on days 2 and 10 post-hatching. Next, we assess how DNA methylation marks differ with sex and hatch date, comparing individuals born early and late in the season when environmental conditions markedly differ. Finally, we ask how initial DNA methylation marks predict offspring growth during the first 10 days of life post-hatching. Due to the importance of the HPA axis in mediating both growth and plasticity, we predict that early life changes in DNA methylation of HPA axis genes will correlate with differential nestling growth trajectories. Our findings provide insight into the epigenetic mechanisms by which birds may adapt to early life conditions during development, with potential long-term fitness effects.

2. Methods

2.1. Study site and data collection

We studied free-living house sparrows from April to August 2019 in a nestbox population at North Dakota State University in Fargo, ND (46.9 N, −96.8 W) that has been monitored since 2013. We checked boxes daily for nest building, onset of egg laying, clutch size, brood size, and the number of nestlings that survived to 10 days post-hatching. Nestlings hatched asynchronously over the course of a day and were marked with a colored, non-toxic marker for individual identification. Although parents and nestlings were randomly exposed to experimental stress treatments as part of an ongoing study, these treatments did not impact growth (see Supplementary Data, Table S3), and therefore were not further considered here.

Blood samples were collected on days 2 and 10 post-hatching from the alar vein using a heparinized capillary tube and stored on 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 uL of packed red blood cells using the NucleoSpin Blood kit (Machery Nagel, 740951), and extracts were frozen at −80 °C until further preparation.

2.2. Growth variables and analysis

We analyzed growth measurements for all nestlings hatched between May 9 and August 15 that survived to fledge and had complete measurements through 9 days of age (n = 173 nestlings from 59 nests). Due to within-nest hatching asynchronies, nestlings varied in terms of their

ages of measurement; we therefore only included individuals whose measurements were within a day of the targeted measurement age. Measurements of mass, beak length (BL), beak depth (BD), tarsus length, wing chord, and rectrices were taken on days 2, 6, 8, and 10 of development. Using these growth measurements, we ran a principal component analysis (PCA) based on a correlation matrix using the function *prcomp* in R v4.1.2 (R Core Team 2021) of normality. The first two principal components accounted for 94.8 % of the total variance (PC1 = 89.6 %, PC2 = 5.2 %) (see Supplementary Data, Table S1). Measurements of wing, tarsus, and mass contributed the most to PC1 (Supplementary Table S2). Because principal component 1 (PC1) accounted for most of the variation, we then used the coordinate results of PC1 as a measure of nestling size to assess changes in size (growth) over the developmental period.

To determine the factors that influence nestling growth, we ran a mixed-effects linear regression model with PC1 coordinate as the response variable (Supplementary Table S3). We included linear and quadratic terms for age, nestling sex, nestling rank within the brood (determined by a nestling's relative mass compared to its broodmates at their first measurement), an interaction between age and hatch date (Julian day, mean-centered), and an interaction between parental treatment and nestling treatment as fixed effects, as well as nestling ID, and nest box as random effects, using packages *lme4* (Bates et al., 2015) and *lmerTest* (Kuznetsova et al., 2017) in R. Because our first growth measurement started at day 2, we centered our age variable such that age at day 2 = 0. Since many of the adults in the population are unbanded, we were unable to verify with complete certainty if the nesting pair changed between broods, and therefore did not include brood attempt in our analysis.

We conducted preliminary analyses of nestling growth to inform how to proceed with analysis of growth and methylation (Supplementary Table S3). Nestling size significantly increased with hatch date ($t_{46.5} = 4.61$, $p < 0.001$). There was a positive and significant interaction between nestling age and hatch date ($t_{171.2} = 7.41$, $p < 0.001$), indicating slower growth earlier in the season. Nestling size also varied significantly by nestling rank ($t_{113.1} = -5.22$, $p < 0.001$), with later-ranked nestlings (i.e., nestlings that were relatively lighter than their broodmates at their first measurement) having smaller overall size. Nestling size increased linearly with age ($t_{542.5} = 56.07$, $p < 0.001$), with a significant negative quadratic term indicating a non-linear component to the growth curve ($t_{414.1} = -9.57$, $p < 0.001$). Nestling size was not significantly impacted by sex ($t_{57.5} = -0.32$, $p = 0.749$), parental treatment ($t_{61.8} = 0.41$, $p = 0.685$), nestling treatment ($t_{118.0} = -1.10$, $p = 0.274$), or the interaction between parental and nestling treatments ($t_{114.1} = 0.94$, $p = 0.348$).

Due to the significance of hatch date and the interaction between date and age in these results, we ran a subsequent mixed-effects linear regression model with PC1 coordinate (nestling size) as the response variable (Supplementary Table S4). We included an interaction between age (centered such that age at day 2 = 0) and hatch date (Julian day, mean-centered), and a quadratic term for age, as fixed effects, nestling ID and nest box as random effects, and an age by ID random slope, using packages *lme4* (Bates et al., 2015) and *lmerTest* (Kuznetsova et al., 2017) (Supplementary Fig. S1A). The results guided our analysis of methylation, described below.

2.3. TEEM-seq DNA probe development, library prep and sequencing

To assess DNA methylation, we used the TEEM-seq protocol (Rubenstein and 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 bisulfite sequencing that minimizes DNA damage and reduces GC bias (Hoppers et al., 2020). EM-seq libraries have been shown to perform better than bisulfite-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 bisulfite sequencing (Hoppers et al., 2020).

Using the NCBI house sparrow genome as a reference (Ravinet et al., 2018), we identified target sequences for 25 genes (Table 1). Genes were 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. 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 promoters, as well as exons (excluding introns) for a subset of genes. If the house sparrow genome had multiple gene regions annotated for a target gene, we targeted all candidates. To ensure that we had reliable EM-seq conversion estimates for all samples, we also specifically targeted pUC19 and lambda control sequences in our bait set (Rubenstein and Solomon, 2023). Biotinylated RNA probes were then commercially prepared using myBaits v4.01 Custom 1–20 K 16 Reaction Kits for target enrichment via

Table 1
Targeted genes for house sparrow probes.

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

*Genes targeted both exons in addition to the putative promoter.
^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).

hybridization-based capture (Daicel Arbor BioSciences). We submitted 53 target sequences totaling 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 and 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 130ul 0.1 mM EDTA 1X TE Buffer, with 1–3 ul each of NEB Control DNA CpG methylated pUC19 (0.1 ng/ul) and Control DNA CpG unmethylated Lambda (2 ng/ul). After concentrating DNA down to 80–100 ng in 33ul 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 one-minute extension step at 72 °C. We cleaned the amplified capture pool with AMPure XP beads. Finally, we sequenced blood samples (n = 96) in one partial lane (150G) of a NovaSeq 6000, at Novogene (Sacramento, CA), and we sequenced additional blood samples (n = 37) alongside brain samples (not used in this study; n = 59) at 2x150 bp with 5 percent PhiX in one full lane (110G) of an Illumina HiSeq 4000.

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 bisulfite genome reference generated by Bismark v0.19.0 (Krueger and 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 and 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 and 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 and 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 greater than 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 %. 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 %. Our final dataset for this analysis included blood samples collected on day 10 of development (n = 47), as well as blood samples collected on day 2 of development (n = 44).

2.5. DMR analysis

We used Metilene v0.2-8 (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 forced us to create dichotomous groups for each predictor variable. We first compared nestlings across development by comparing (unpaired) samples collected at day 2 (n = 44) to samples collected at day 10 (n = 47). We then compared nestlings separately at both day 2 and day 10 based on hatch date; we split samples into "early" and "late" based on the mean Julian date of hatching across all nestlings measured throughout the season (day 2: "early" n = 28, "late" n = 16; day 10: "early" n = 30, "late" n = 17). In addition, we also looked at differences at day 2 and day 10 based on sex (day 2: male n = 22, female n = 22; day 10: male n = 26, female n = 21). Finally, we compared nestlings at day 2 based on growth phenotype; we split samples into "low growth" and "high growth" based on their individual slope relative to the mean slope (Supplementary Fig. S1B; "low" n = 18, "high" n = 26). We then performed pairwise comparisons between each group. 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 while it is unknown what percentage of change is needed to affect gene expression, 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 cutoff of MWU-test p-value < 0.01 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 cutoffs to be more inclusive in our analysis and to capture potential DMRs despite our relatively small sample size (Laine et al., 2022). Since we found no DMRs in the exon regions of any genes analyzed in any tissues, we focused our analysis only on putative promoter regions.

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

Table 2

DMRs over the developmental period. Differentially methylated regions (DMRs) comparing nestlings at day 2 to day 10. The base pair start and stop location is provided, as well as number of CpG sites within the DMR, and the mean and standard deviation (SD) DNA methylation for each group. The subscript number after the gene name indicates the specific probe used to capture that distinct region of the gene if multiple probes were used either due to: length of the gene, the inclusion of exons, or multiple gene region candidates found in the genome during probe development. Direction indicates which sample mean (day 2 or day 10) has higher DNA methylation.

Gene	Start	Stop	CpG	Day 2		Day 10		Mean methylation difference	Direction	p-value	q-value
			#	mean	SD	mean	SD				
AVPR1B	3822	3964	6	48.33	10.17	53.94	6.17	−5.61	day 10	<0.001	0.005
	3972	4061	11	60.32	7.51	64.87	4.30	−4.55	day 10	<0.001	<0.001
	4157	4183	7	71.71	6.86	75.3	4.51	−3.59	day 10	<0.001	<0.001
	4200	4458	31	62.91	7.14	69.43	2.91	−6.51	day 10	<0.001	<0.001
	4458	4668	41	72.96	6.51	76.44	2.54	−3.49	day 10	<0.001	<0.001
	5956	6007	4	37.86	6.65	43.6	6.22	−5.75	day 10	<0.001	0.1
	6082	6100	3	49.55	18.20	58.87	12.86	−9.32	day 10	0.001	0.044
CRH	2130	2279	35	17.48	5.55	14.2	4.61	3.28	day 2	<0.001	<0.001
CRHR1	3496	3514	3	63.98	9.38	70.37	4.53	−6.39	day 10	<0.001	0.003
	5986	6087	3	56.78	5.98	61.16	4.57	−4.38	day 10	<0.001	0.03
DNMT3a	412	486	8	80.65	12.77	85.55	2.67	−4.89	day 10	<0.001	0.022
	664	778	6	67.36	7.46	71.42	4.78	−4.06	day 10	<0.001	0.04
	947	1264	24	74.91	6.93	77.1	3.05	−2.19	day 10	0.032	0.09
	2236	2247	3	82.37	5.60	74.9	5.56	7.47	day 2	<0.001	<0.001
	2264	2328	3	71.7	8.61	57.94	7.08	13.76	day 2	<0.001	<0.001
	2334	2387	5	52.24	8.87	31.53	7.55	20.71	day 2	<0.001	<0.001
DNMT3b	460	704	6	23.9	6.31	13.03	3.96	10.87	day 2	<0.001	<0.001
	848	1214	8	23.61	4.54	14.84	2.92	8.77	day 2	<0.001	<0.001
	1580	1816	25	32.52	5.60	18.26	3.12	14.26	day 2	<0.001	<0.001
	1848	2181	33	8.56	2.54	3.87	1.68	4.68	day 2	<0.001	<0.001
	8050	8098	3	68.75	10.56	64.99	6.42	3.77	day 2	<0.001	0.015
	8357	8540	11	74.88	7.23	71.41	4.32	3.47	day 2	<0.001	0.047
EGR1	1887	1912	3	15.71	3.08	12.5	2.53	3.22	day 2	0.003	0.069
FKBP5	2165	2274	3	43.16	12.82	35.6	11.13	7.56	day 2	<0.001	0.016
	3056	3466	9	5.42	2.41	2.27	1.20	3.15	day 2	<0.001	<0.001
	3705	3731	3	30.97	4.30	25.7	3.81	5.27	day 2	<0.001	<0.001
	3893	3907	3	57.47	15.91	50.65	12.88	6.82	day 2	<0.001	0.06
GNRHR2$_1$	1103	1279	24	86.78	8.95	90.05	1.90	−3.27	day 10	<0.001	<0.001
	2180	2265	3	69.77	6.73	73.79	3.84	−4.02	day 10	<0.001	0.024
	2618	2736	5	53.77	8.34	61.01	4.46	−7.24	day 10	<0.001	<0.001
GNRHR2$_2$	226	254	3	55.6	10.04	63.66	7.79	−8.06	day 10	<0.001	0.001
	294	336	18	84.31	8.18	89.51	1.76	−5.2	day 10	<0.001	<0.001
	344	721	21	50.64	5.56	57.9	3.32	−7.26	day 10	<0.001	<0.001
	962	1011	3	38.27	4.29	45.03	5.22	−6.77	day 10	<0.001	0.005
	1129	1332	15	38.77	11.20	47.33	13.92	−8.56	day 10	<0.001	<0.001
	1339	1350	3	15.84	5.71	19.48	6.09	−3.64	day 10	<0.001	0.097
	1385	1420	18	58.46	7.12	69	5.79	−10.54	day 10	<0.001	<0.001
	1443	1488	16	26.21	6.33	31.12	7.58	−4.91	day 10	<0.001	0.001
	1501	1535	10	46.95	8.83	54.48	11.01	−7.53	day 10	<0.001	<0.001
HSD11B1$_4$	370	380	3	62.38	9.35	51.49	10.97	10.89	day 2	<0.001	<0.001
	565	605	3	57.35	8.74	48.45	8.13	8.9	day 2	<0.001	<0.001
	692	808	3	59.33	7.17	51.69	6.01	7.64	day 2	<0.001	0.009
	905	1225	11	28.44	5.00	23.04	5.88	5.4	day 2	<0.001	<0.001
HSD11B1$_5$	480	498	3	73.62	8.10	69.03	6.96	4.59	day 2	<0.001	0.049
	1120	1141	3	21.51	7.61	12.65	4.09	8.86	day 2	<0.001	<0.001
HSD11B1$_8$	48	492	27	6.08	4.82	9.77	8.96	−3.69	day 10	<0.001	<0.001
HSD11B2	1803	2991	32	72.35	5.92	75.29	2.64	−2.94	day 10	<0.001	0.013
	3047	3422	18	58.94	7.03	67.74	4.27	−8.8	day 10	<0.001	<0.001
	3441	3549	10	42.01	7.95	56.18	5.00	−14.17	day 10	<0.001	<0.001
	3568	3589	3	40.7	7.80	52.65	6.92	−11.94	day 10	<0.001	<0.001
	3622	3698	3	69.66	7.14	77.01	3.66	−7.35	day 10	<0.001	<0.001
	4771	5645	33	65.38	6.82	68.51	3.98	−3.12	day 10	<0.001	0.002
MC2R	510	518	3	75.56	8.15	81.62	3.59	−6.05	day 10	<0.001	0.001
NLRC5	993	2251	24	24.8	8.84	12.92	4.28	11.88	day 2	<0.001	<0.001
NR3C1$_4$	834	917	3	72.36	7.08	79.55	4.49	−7.19	day 10	<0.001	<0.001
NR3C1$_7$	301	686	31	80.35	11.19	84.51	4.19	−4.15	day 10	<0.001	<0.001
	2294	2347	8	66.95	7.11	71.57	2.05	−4.62	day 10	<0.001	0.035
NR3C1$_8$	70	246	11	77.55	12.51	75.48	13.13	2.07	day 2	<0.001	0.029
	260	291	5	80.91	6.30	85.41	4.32	−4.5	day 10	<0.001	0.074
	451	536	20	35.46	10.12	25.95	4.38	9.51	day 2	<0.001	<0.001
	539	549	4	41.97	7.36	36.75	7.30	5.22	day 2	<0.001	<0.001
	567	577	5	73.54	10.08	67.86	8.09	5.68	day 2	<0.001	<0.001
	668	687	10	89.55	6.65	92.81	7.04	−3.26	day 10	0.086	0.101
	1152	1238	3	24.02	10.71	19.45	3.45	4.57	day 2	<0.001	0.003
	1272	1280	3	20.99	6.30	12.36	4.89	8.63	day 2	<0.001	<0.001
	1280	1307	3	23.17	8.42	16	5.20	7.17	day 2	<0.001	0.002
	1313	1465	12	5.36	8.99	2.37	6.46	2.99	day 2	<0.001	<0.001

(*continued on next page*)

Table 2 (*continued*)

Gene	Start	Stop	CpG	Day 2		Day 10		Mean methylation difference	Direction	p-value	q-value
			#	mean	SD	mean	SD				
NR3C1$_{10}$	291	400	3	19.8	2.93	13.79	1.78	6.01	day 2	<0.001	<0.001
NR3C2$_2$	724	761	4	67.82	6.63	79.97	3.70	−12.14	day 10	<0.001	<0.001
NR3C2$_3$	1245	1350	3	67.75	11.89	74.1	7.96	−6.35	day 10	0.004	0.032
	1606	1631	3	69.32	16.92	76.61	14.91	−7.3	day 10	0.001	0.046
	1693	1778	13	86.58	5.88	89.14	3.74	−2.56	day 10	<0.001	0.014
	2083	2212	23	86.56	9.88	89.88	2.17	−3.32	day 10	<0.001	<0.001
NR4A1$_2$	899	908	5	20.32	5.34	15.83	4.31	4.49	day 2	<0.001	0.005
	941	946	3	17.27	5.09	11	3.81	6.27	day 2	<0.001	<0.001
	1044	1059	3	40.29	9.63	50.3	8.57	−10.01	day 10	<0.001	<0.001
NR4A1$_3$	169	981	38	38.01	6.33	44.66	5.68	−6.64	day 10	<0.001	<0.001
	998	1176	14	53.25	8.81	61.95	7.63	−8.71	day 10	<0.001	<0.001
	3369	3623	20	76.3	10.33	81.36	5.55	−5.06	day 10	<0.001	<0.001
	3646	4019	39	54.48	8.98	62.92	7.82	−8.44	day 10	<0.001	<0.001
POMC	29	381	38	78.41	8.13	82.24	4.05	−3.83	day 10	<0.001	<0.001
	493	535	5	80.77	9.00	85.07	6.19	−4.29	day 10	<0.001	0.097
SIK2	1559	1603	5	22.77	6.83	19.61	6.69	3.17	day 2	<0.001	0.008
	1833	2115	12	73.73	7.73	77.95	2.83	−4.22	day 10	<0.001	<0.001

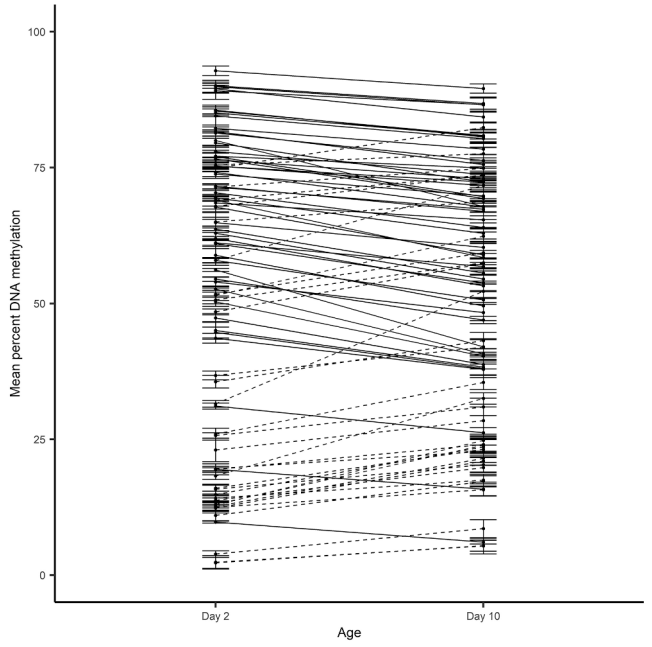

Fig. 1. Changes in DNA methylation across development. We found 84 differentially methylated regions (DMRs), across 17 genes, between day 2 and day 10 of development. Each point represents the mean percent DNA methylation of a DMR at day 2 or 10. DMRs at each day are connected by a line, which indicates if percent DNA methylation increased (solid line) or decreased (dashed line) over time. Bars indicate ± 1 standard error from the mean. There were 34 DMRs with higher DNA methylation at day 2 compared to day 10, and 50 DMRs with higher DNA methylation at day 10 compared to day 2.

checks, and had over 28x deduplication coverage, sequence depth ranged from 1,041,387 to 5,627,204 reads.

3.2. DNA methylation changes across development

To assess how DNA methylation marks change over development, we first compared DNA methylation in the blood at day 2 to day 10. We found 84 differentially methylated regions (DMRs) across 17 genes (Table 2); 34 of these DMRs (41 %) had higher DNA methylation at day 2, while 50 of these DMRs (59 %) had higher DNA methylation at day 10. In general, DMRs that decreased in methylation over development had lower average DNA methylation (mean = 35.23, SD = 23.64), while DMRs that increased in methylation over development had higher average DNA methylation (mean = 64.36, SD = 18.51) (Fig. 1). In other words, differentially methylated regions of the genome that had high DNA methylation on day 2 tended to increase over time, whereas those that had low DNA methylation day 2 tended to decrease over time.

3.3. DNA methylation marks differ between sexes

We assessed differences in DNA methylation based on sex in blood collected on days 2 and 10. On day 2, we found four DMRs that differed between males and females in the putative promoter regions of *HSD11b2, HTR1A, NLRC5*, and *NR4A1* (Fig. 2A; Table 3). DMRs in the putative promoter regions of *HSD11b2, HTR1A* and *NR4A1* had higher DNA methylation in males, while the DMR in the putative promoter region of *NLRC5* had higher DNA methylation in females. On day 10, we found two DMRs in the putative promoter regions of *HTR1A* and *NR4A1*, both with higher DNA methylation in males (Fig. 2B; Table 3).

3.4. DNA methylation marks correspond to hatch date

Next, we used hatch date to assess DNA methylation in blood collected on days 2 and 10. On day 2, we found 45 DMRs across 14 genes between nestlings born earlier in the season versus later in the season (Fig. 3A; Table 4). DNA methylation was higher in nestlings born earlier in the season in every DMR. On day 10, we found one DMR between nestlings born earlier in the season versus later in the season in the putative promoter region of *AR*, with higher DNA methylation in nestlings born later in the season (Fig. 3B; Table 4).

3.5. Initial DMRs predict growth differences

To determine if initial DNA methylation marks were predictive of growth differences over development, we compared DNA methylation in the blood at day 2 between nestlings characterized as “low growth” versus “high growth” based on their random slopes from the growth model (Supplementary Fig. S1). We found 19 DMRs in the putative promoter regions of 9 genes that differed between nestlings in the two groups (Fig. 4; Table 5). “High growth” nestlings had higher DNA methylation in each DMR of 6 genes (*CRH, DNMT3a, GNRHR2, MC2R, NR3C2*, and *POMC*), while “low growth” nestlings had higher DNA methylation in each DMR of 2 genes (*NR4A1* and *VIP*). In contrast, two DMRs in *NR3C1* had higher DNA methylation in “high growth” nestlings, while three DMRs in *NR3C1* had higher DNA methylation in “low growth” nestlings. With the exception of *CRH*, DMRs with higher initial levels of DNA methylation were also higher in “high growth” nestlings,

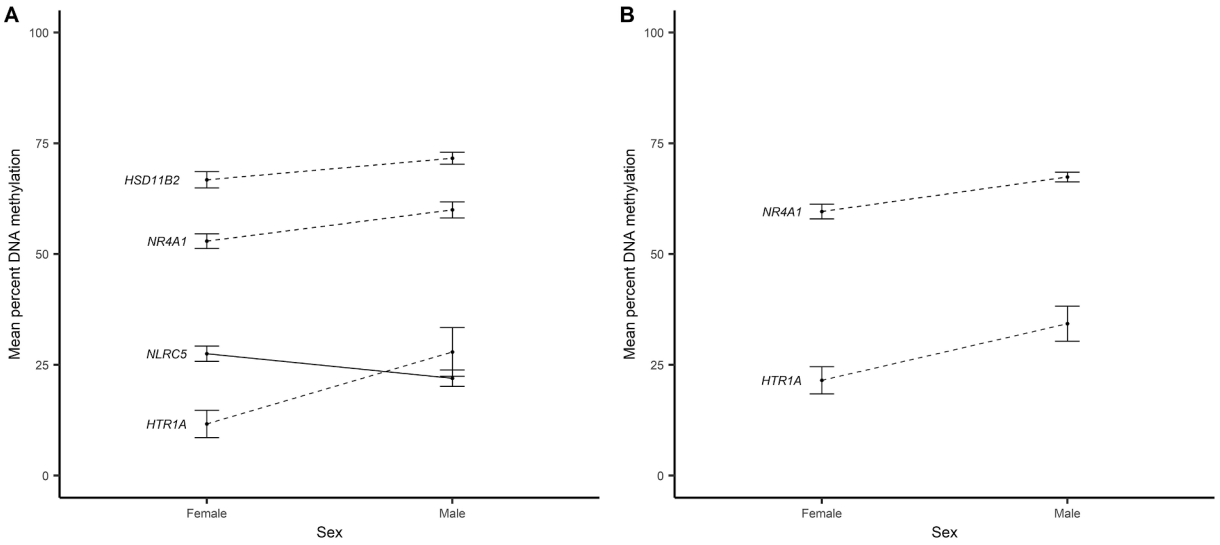

Fig. 2. DMRs based on sex at day 2 (A) and day 10 (B). Each point represents the mean percent DNA methylation of a DMR at day 2 (A) and day 10 (B) that differed between male and female nestlings. DMRs are labeled by the name of the gene in which they occur. DMRs in each group (male or female) are connected by a line to indicate which group (solid line = female, dashed line = male) had higher percent DNA methylation in that DMR. Bars indicate ± 1 standard error from the mean.

Table 3
DMRs based on sex. Differentially methylated regions (DMRs) comparing nestlings based on sex at day 2 and day 10. The base pair start and stop location is provided, as well as number of CpG sites within the DMR, and the mean and standard deviation (SD) DNA methylation for each group. The subscript number after the gene name indicates the specific probe used to capture that distinct region of the gene if multiple probes were used either due to: length of the gene, the inclusion of exons, or multiple gene region candidates found in the genome during probe development. Direction indicates which sample mean (male or female) has higher DNA methylation.

Age	Gene	Start	Stop	CpG	Male		Female		Mean methylation difference	Direction	p-value	q-value
				#	mean	SD	mean	SD				
day 2	*HSD11B2*	5337	5627	8	71.63	6.34	66.76	8.78	4.86	male	<0.001	0.009
	$HTR1A_2$	859	928	4	27.91	25.79	11.66	14.72	16.25	male	<0.001	0.08
	$NLRC5_5$	993	2251	24	21.98	8.70	27.50	8.28	−5.53	female	<0.001	0.03
	$NR4A1_3$	3575	4019	45	59.97	8.59	52.91	7.94	7.06	male	<0.001	<0.001
day 10	$HTR1A_2$	889	1446	11	34.29	20.56	21.52	14.06	12.77	male	<0.001	0.061
	$NR4A1_3$	3616	4061	44	67.40	5.75	59.59	7.66	7.81	male	<0.001	<0.001

whereas DMRs with lower initial levels of DNA methylation were higher in “low growth” nestlings.

4. Discussion

We investigated the potential role of DNA methylation in facilitating developmental plasticity of a wild avian species, the house sparrow, by assessing the relationship between changes in DNA methylation in a suite of HPA-related genes in the blood and various environmental and phenotypic factors. We found a suite of genes that had differentially methylated regions (DMRs) appearing within 8 days of nestling development, between the sexes, from early to late in the breeding season, and between slow-growing and fast-growing individuals. The specific genes involved in each case and the overall pattern have an array of potential implications for understanding the role of methylation in early development and the potential for developmental plasticity to shape later phenotypes.

We note at the outset that while DNA methylation can be tissue specific, studies have shown that blood may be used as a biomarker in general for DNA methylation of genes in target central nervous system regions (Palma-Gudiel et al., 2015). In great tits, for example, CpG methylation in both whole brain and whole blood have been shown to similarly decrease within CpG islands and near the transcription start site (Laine et al., 2016), and changes in DNA methylation over time are positively correlated in red blood cells and liver (Lindner et al., 2021), suggesting that changes in DNA methylation in one tissue type may be detectable in a peripheral tissue in at least some species of birds (Husby, 2020). That said, determining whether methylation patterns of specific genes differ between blood and regions of the brain will be an important next step for ascertaining the impact these patterns have on HPA function in house sparrows. Furthermore, as we were unable to assess gene expression in this study, these findings should be taken as preliminary results that suggest potential functional changes, though further research is needed to determine the consequences of these methylation changes. For now, we assume that our results obtained from blood samples are likely to reflect changes in other tissues as well.

One important result is that we found changes in methylation from day 2 of nestling development to day 10 in over half of the genes studied, and often in multiple DMRs in the same genomic regions. Genomic regions with initially low levels of DNA methylation tended to become further demethylated, whereas those with initially high levels of DNA methylation tended to become further methylated over the period of development. In addition, we found that for many regions, all (or most) DMRs within that genomic region changed in the same direction: each DMR in the HPA-related genes *AVPR1B, CRHR1, HSD11b2, NR3C2*, and *POMC,* as well as the HPG-related gene *GNRHR2,* increased in methylation across development, whereas each DMR in the methyltransferase gene *DNMT3b,* the HPA-related gene *FKBP5,* and all but one in the HPA-related gene *HSD11b1* decreased in methylation across development. The exceptions were DMRs within the HPA-related gene *NR3C1,* the

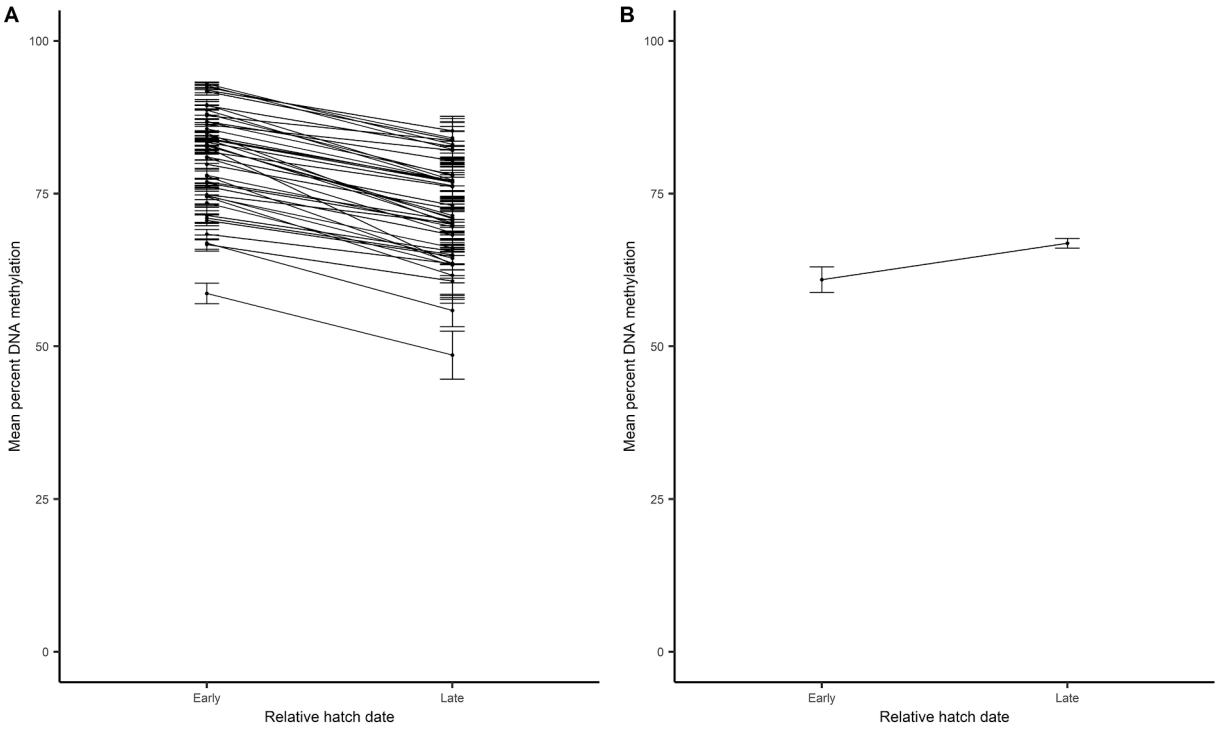

Fig. 3. DMRs based on relative hatch date at day 2 (A) and day 10 (B). Each point represents the mean percent DNA methylation of a DMR at day 2 (A) and day 10 (B) that differed between nestlings that hatched relatively early and late in the season. DMRs in each group (early or late relative hatch date) are connected by a line to indicate which group had higher percent DNA methylation in that DMR. Bars indicate ± 1 standard error from the mean.

methyltransferase gene *DNMT3a,* and the nuclear hormone receptor gene *NR4A1,* which were more variable in their direction of change. In general, regions in genes encoding receptors tended to increase in DNA methylation over development (with the exception of *NR3C1*). Interestingly, we observed contrasting trends in *HSD11b1* and *HSD11b2*, which activate and inactivate glucocorticoids (respectively), suggesting that glucocorticoids may be activated over this period of early development. Although studies that have measured whole-genome DNA methylation patterns in birds have shown a general increase in methylation in the blood across the early developmental period (Sheldon et al., 2020; Watson et al., 2019), studies in mammals have shown that changes in developmental methylation are more dynamic, with tissue-specific patterns of demethylation and methylation (Simmons et al., 2013; Song et al., 2009). By using a targeted approach, we show that DNA methylation in birds also changes dynamically over the developmental period, and in different ways in different genomic regions. This is important because it indicates that not only do epigenetic changes continue to occur in the postnatal developmental period in birds (Sheldon et al., 2020), but also that these changes differ across genes, suggesting that there is a wide window in which early life conditions can potentially influence specific phenotypic outcomes.

Another intriguing result is that the sexes differed in DNA methylation patterns. At day 2, we found three DMRs with higher DNA methylation in males than females, in the putative promoter regions of *HSD11b2, HTR1A*, and *NR4A1*. *HSD11b2* encodes an enzyme that converts active glucocorticoids into an inactive form, preventing activation of glucocorticoid receptors (Benediktsson et al., 1997). DNA methylation of this gene has primarily been studied in mammals for its role in protecting developing fetuses from maternal glucocorticoids (Marsit et al., 2012; Peña et al., 2012), as well as mediating the development of sex differences in adrenal function of offspring (Chen et al., 2021). Its role in birds, however, is unclear, though the presence of this DMR immediately after hatching suggests that it may play a related embryonic role. *HTR1A* encodes the serotonin receptor, and is important for both neuronal development and regulation of behavior (Holmes, 2008; Lanfumey et al., 2008). In particular, the serotonin pathway is important in mediating the stress response by enhancing glucocorticoid receptor expression (Meaney and Szyf, 2005; Mitchell et al., 1990). This pathway is impacted by early life stress and environmental conditions in both mammals (Bodden et al., 2017; Harris and Seckl, 2011; Smythe et al., 1994) and birds (Ahmed et al., 2014). Consistent with our findings, studies in rats (Ngun et al., 2011; Zhang et al., 1999) have shown evidence for region-specific sex differences in both transcription and concentration of *HTR1A*, which encodes a nuclear hormone receptor in the brain. Notably, *HTR1A* also showed high levels of variation in DNA methylation in our analysis (Table 3). *NR4A1* has primarily been studied in male mice, in which early life stress and maternal diet have been shown to alter DNA methylation in this gene (Kasch et al., 2018; Kember et al., 2012). However, recent studies also show differences in *NR4A1* expression between male and female mice (Wahlang et al., 2019). We also found a DMR in the putative promoter of *NLRC5*, with higher DNA methylation in females than in males. *NLRC5* is an important mediator of immune responses (Kobayashi and van den Elsen, 2012), but other studies in birds have not shown sex-specific differences in the expression of this gene (Diaz-Real et al., 2017). While DMRs in *HSD11b2* and *NLRC5* were no longer present by day 10, we found overlapping DMRs in the putative promoter regions of *HTR1A* and *NR4A1* at the end of development, with slightly higher levels of DNA methylation and the same directional difference (higher methylation in males) as at day 2. Overall, our findings indicate that in birds, there is sex-specific differential DNA methylation of a number of stress response and immune-related genes after hatching, some of which are maintained throughout development. This highlights the importance of studying these genes in both males and females, and suggests that sex-specific DMRs in *HTR1A* and *NR4A1* in particular may be stably maintained over time.

We also found that patterns of DNA methylation are environmentally sensitive, as methylation marks differed by hatch date. Hatch date, a proxy for a suite of seasonally changing aspects of the environment, affects numerous aspects of chick development and phenotype in house

Table 4

DMRs based on hatch date. Differentially methylated regions (DMRs) comparing nestlings based on hatch date at day 2 and day 10. The base pair start and stop location is provided, as well as number of CpG sites within the DMR, and the mean and standard deviation (SD) DNA methylation for each group. The subscript number after the gene name indicates the specific probe used to capture that distinct region of the gene if multiple probes were used either due to: length of the gene, the inclusion of exons, or multiple gene region candidates found in the genome during probe development. Direction indicates which sample mean (early or late hatch date) has higher DNA methylation.

Age	Gene	Start	Stop	CpG	Early date		Late date		Mean methylation difference	Direction	p-value	q-value
				#	mean	SD	mean	SD				
day 2	*AVPR1B*	4168	4316	16	70.99	3.64	64.99	10.12	6	early	<0.001	0.031
		4414	4482	16	79.80	3.54	72.45	12.28	7.35	early	<0.001	0.003
		4606	4631	9	92.99	1.43	83.06	15.26	9.93	early	<0.001	<0.001
		4692	4878	36	66.67	4.10	60.68	11.13	6	early	<0.001	0.001
		5917	5940	5	74.80	7.76	70.16	7.87	4.64	early	0.001	0.101
	CRHR1	2834	2885	6	89.40	3.58	82.69	10.65	6.71	early	<0.001	0.03
		3197	3432	5	82.83	5.90	71.40	20.11	11.42	early	<0.001	0.019
		3587	3605	5	84.90	5.95	69.92	16.50	14.98	early	<0.001	<0.001
	DNMT3a	200	665	23	78.02	3.61	69.60	11.96	8.42	early	<0.001	<0.001
		940	1264	28	76.19	2.41	68.23	8.65	7.96	early	<0.001	<0.001
		2001	2040	10	82.63	5.01	63.41	26.09	19.22	early	<0.001	<0.001
		2060	2170	12	81.02	2.52	73.06	9.44	7.96	early	<0.001	0.006
	DNMT3b	8121	8266	3	82.88	4.01	71.07	18.30	11.81	early	<0.001	0.02
	FKBP5	1687	1702	6	91.71	3.02	83.84	11.69	7.87	early	<0.001	0.034
		2564	2939	8	74.71	3.72	64.41	13.36	10.3	early	<0.001	0.025
		3893	3963	6	58.66	8.93	48.57	16.20	10.09	early	<0.001	0.062
		5337	5352	5	88.04	7.32	77.93	17.45	10.11	early	<0.001	0.101
	$GNRHR2_1$	806	990	10	73.49	6.83	63.31	11.97	10.18	early	<0.001	<0.001
		1103	1185	15	92.70	2.45	82.24	15.48	10.46	early	<0.001	<0.001
		1198	1311	12	86.80	2.43	80.39	9.48	6.4	early	<0.001	0.001
	$GNRHR2_2$	253	326	16	85.62	2.16	77.10	12.30	8.52	early	<0.001	<0.001
	$HSD11B1_4$	201	439	19	68.37	4.04	63.52	7.95	4.85	early	<0.001	0.033
	HSD11B2	2017	2634	20	84.07	2.32	77.13	10.86	6.94	early	<0.001	<0.001
		2871	3307	13	70.63	4.69	64.74	9.17	5.89	early	<0.001	0.035
		4874	5000	12	66.91	6.97	55.87	10.99	11.04	early	<0.001	0.002
	MC2R	51	896	27	76.94	3.10	70.62	8.46	6.32	early	<0.001	0.001
	$NR3C1_3$	992	997	3	74.58	7.32	66.14	10.82	8.44	early	0.006	0.054
	$NR3C1_4$	916	928	3	77.88	5.94	63.61	21.73	14.27	early	<0.001	0.062
	$NR3C1_7$	301	719	33	83.41	2.53	76.29	10.51	7.13	early	<0.001	<0.001
		1343	1451	5	74.48	7.78	61.63	16.16	12.84	early	<0.001	0.032
	$NR3C1_8$	668	764	31	92.45	2.27	84.11	13.14	8.34	early	<0.001	<0.001
		773	890	15	83.94	2.58	76.85	12.58	7.09	early	<0.001	0.029
	$NR3C2_3$	1343	1493	3	80.88	4.94	68.56	15.26	12.31	early	<0.001	0.014
		1581	1798	31	86.28	5.18	82.09	6.14	4.18	early	<0.001	<0.001
		1883	2211	35	86.74	2.16	78.12	10.90	8.62	early	<0.001	<0.001
		2211	2471	21	87.76	5.99	83.70	6.11	4.07	early	<0.001	<0.001
		2474	2915	19	81.88	2.23	76.13	6.43	5.75	early	<0.001	<0.001
		3027	3068	11	88.68	4.51	76.84	14.63	11.84	early	<0.001	<0.001
	$NR4A1_3$	3369	3449	8	83.08	5.49	70.20	18.07	12.88	early	<0.001	<0.001
		3499	3743	20	71.44	6.87	65.58	12.52	5.86	early	<0.001	0.05
	POMC	29	98	10	89.52	4.63	77.21	18.28	12.31	early	<0.001	<0.001
		109	296	19	84.49	2.68	76.93	10.67	7.56	early	<0.001	<0.001
		394	540	31	91.93	2.33	85.28	9.81	6.65	early	<0.001	<0.001
		544	548	3	83.92	6.00	70.89	13.92	13.03	early	<0.001	0.008
	SIK2	1877	2115	11	76.74	3.52	69.98	10.80	6.77	early	<0.001	0.035
day 10	*AR*	1652	2252	30	60.92	11.65	66.87	3.30	−5.95	late	<0.001	0.06

sparrows including survival and fledgling body condition (Kinnard and Westneat, 2009; Moreno-Rueda, 2004; Ringsby et al., 1998). Indeed, in our analysis of nestling growth, the only significant variables that impacted nestling size were hatch date and rank in the nest (Supplementary Table S3). Across our gene suite, we found a widespread pattern of higher initial DNA methylation in nestlings born earlier in the season compared to those born later in the season, with multiple DMRs in both HPA- and HPG-related genes. However, these differences were no longer present by day 10 of development. We also found that nestlings born later in the season tended to have higher variation in DNA methylation (Table 4).

The effect of hatch date may be indicative of temporal environmental factors, such as temperature, which is positively correlated with Julian date at our study site (Supplementary Fig. S2). Temperature has been shown to significantly impact development in numerous bird species (Eastwood et al., 2022; Griffith et al., 2016; Sheldon et al., 2018b; Wada et al., 2015). In house sparrows, temperature impacts variation in clutch and egg size (Aslan and Yavuz, 2010), and extreme temperatures experienced during incubation have been shown to impact hatching success and nestling body mass (Pipoly et al., 2013). In wild zebra finches in Australia, nestlings that developed in hotter temperatures had higher genome-wide DNA methylation than those developing in colder temperatures (Sheldon et al., 2020). This is the opposite pattern to our results, however, as earlier dates in our breeding season were associated with cooler temperatures. The effect of hatch date could also be indicative of parental body condition or investment, as well as food supply. Studies have shown that breeding success declines in birds later in the season due to deteriorating environmental conditions and/or poorer condition of parents who decrease their energetic investment in later broods (Mock et al., 2009; Moreno-Rueda, 2004; Robinson et al., 2010). Nestlings born later in the season are more likely to be from a second or third nesting attempts, while nestlings born earlier in the season are more likely to be from a first nesting attempt (Westneat et al., 2014). Furthermore, female body condition can influence both clutch size and egg size in birds (Christians, 2002), which in turn is an important factor in nestling growth and survival (Williams, 1994). These and other

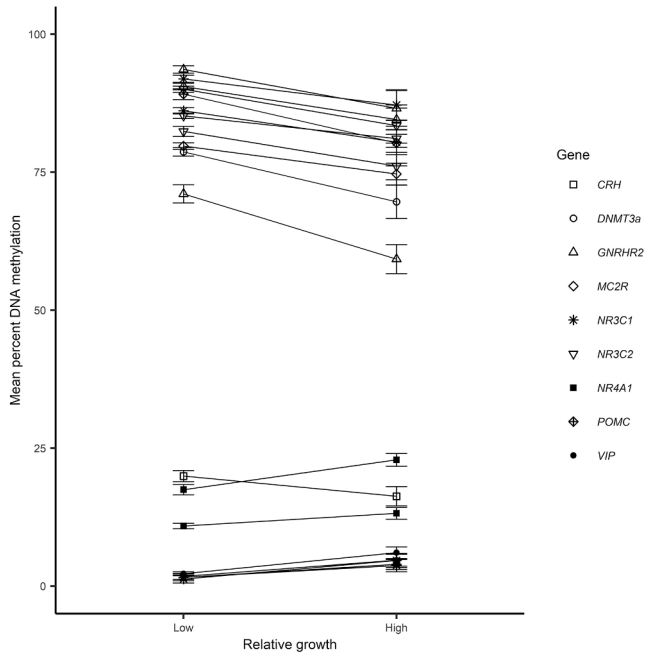

Fig. 4. DMRs in genes predicting nestling growth. Each point represents the mean percent DNA methylation of a DMR at day 2 that differed between nestlings characterizes as "low growth" and "high growth". DMRs are labeled with a symbol indicating the name of the gene in which they occur. Open shapes indicate genes with DMRs that all had higher DNA methylation in nestlings with high relative growth; closed shapes indicate genes with DMRs that all had higher DNA methylation in nestlings with low relative growth. The one exception is *NR3C1*, indicated by a star, which had DMRs in both directions. DMRs in each group (low or high growth) are connected by a line to indicate which group had higher percent DNA methylation in that DMR. Bars indicate ± 1 standard error from the mean.

potential factors that may correlate with date will be useful to identify, as they may illuminate not only the proximate mechanisms leading to shifting DNA methylation patterns but will help determine the phenotypic consequences and the potential selective forces favoring them. Although these differentially methylated regions were mostly no longer present at the end of development, as we describe below, these initial marks may still impact aspects of phenotype, including metabolic rates, HPA axis function, and growth trajectories (Jimeno et al., 2019; Lea et al., 2016; Sepers et al., 2021) that can have carryover effects into later life (Bonier et al., 2009; Schoech et al., 2011; Weber et al., 2018).

Perhaps most importantly, we found initial support for the idea that DNA methylation very early in development alters the pattern of development. In order to assess whether initial DNA methylation marks at day 2 were predictive of offspring growth trajectories, we first modeled nestling size, and used this analysis to split nestlings into "low growth" and "high growth" categories based on their random slopes in the model (see Supplementary Fig. S1B). By including date as a variable in the model, we ensured that these differences in growth among nestlings were independent of hatch day (which are confounded with age in their effects, see Supplementary Fig. S1A). As with other DNA methylation analysis approaches, our analysis method requires categorical comparisons, which limits our ability to assess more fine-scale differences between nestlings. Nonetheless, our results provide insight into DNA methylation differences between individuals of broadly different growth patterns.

We found that DMRs in a number of HPA and HPG-related genes predicted subsequent nestling growth. Specifically, offspring with "high growth", which tended to grow faster and be of larger size at the end of development (Supplementary Fig. S1A), had higher initial DNA methylation in DMRs in genes related to HPA axis functioning (*CRH, MC2R, NR3C2,* and *POMC*), as well as in genes related to reproduction (*GNRHR2*) and DNA methyltransferase (*DNMT3a*). In addition, "low growth" offspring had generally higher variation in DNA methylation (Table 5). Specifically, *GNRHR2* encodes for the receptor that binds gonadotropin releasing hormone, an essential vertebrate reproductive hormone (McGuire et al., 2013); *DNMT3a* encodes a DNA methyltransferase enzyme responsible for de novo methylation, with a particularly critical role for embryonic development (Okano et al., 1999). The genes implicated in HPA axis functioning relate directly to both the stress response and negative feedback regulation: *CRH* encodes a preprohormone that stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary, which is itself cleaved from the protein produced by *POMC* and which binds to melanocortin 2 receptors encoded by *MC2R; NR3C2* encodes the mineralocorticoid receptor, which regulates circulating levels of glucocorticoids alongside *NR3C1* and is

Table 5

DMRs predicting growth trajectories. Differentially methylated regions (DMRs) comparing nestlings with "low growth" and "high growth" at day 2. The base pair start and stop location is provided, as well as number of CpG sites within the DMR, and the mean and standard deviation (SD) DNA methylation for each group. The subscript number after the gene name indicates the specific probe used to capture that distinct region of the gene if multiple probes were used either due to: length of the gene, the inclusion of exons, or multiple gene region candidates found in the genome during probe development. Direction indicates which sample mean ("low growth" or "high growth") has higher DNA methylation.

Gene	Start	Stop	CpG	"Low growth"		"High growth"		Mean methylation difference	Direction	p-value	q-value
			#	mean	SD	mean	SD				
CRH	2219	2256	16	16.28	7.43	19.93	5.17	−3.66	high	<0.001	0.013
DNMT3a	448	665	14	69.62	12.77	78.69	3.96	−9.07	high	<0.001	0.066
$GNRHR2_1$	806	944	7	59.24	11.18	71.06	8.41	−11.83	high	<0.001	0.012
	1150	1185	9	86.56	13.75	93.61	3.37	−7.06	high	<0.001	0.001
$GNRHR2_2$	295	311	9	84.53	11.35	90.51	3.06	−5.98	high	<0.001	0.013
$MC2R_1$	51	640	24	74.66	8.30	79.73	3.01	−5.06	high	<0.001	0.066
$NR3C1_7$	301	550	24	80.44	9.66	86.12	2.92	−5.68	high	<0.001	0.034
$NR3C1_8$	674	764	28	87.15	11.77	91.90	3.32	−4.74	high	<0.001	0.002
$NR3C1_9$	35	115	19	4.65	5.45	1.27	3.63	3.38	low	<0.001	0.001
	116	254	26	3.72	4.70	1.59	3.23	2.13	low	<0.001	<0.001
	256	338	34	3.95	3.97	1.57	1.93	2.37	low	<0.001	0.001
$NR3C2_3$	2083	2203	20	83.44	13.43	90.02	2.82	−6.58	high	<0.001	<0.001
	2203	3029	44	81.08	6.76	85.21	2.41	−4.13	high	<0.001	<0.001
	3041	3142	18	76.09	10.52	82.38	4.73	−6.29	high	<0.001	0.046
$NR4A1_2$	904	963	19	13.19	4.53	10.88	2.46	2.31	low	<0.001	0.002
	1018	1023	3	22.87	4.96	17.47	4.74	5.4	low	0.001	0.085
POMC	29	98	10	80.30	17.57	89.13	5.02	−8.82	high	<0.001	0.043
VIP_2	471	525	17	6.05	4.46	2.25	1.86	3.8	low	<0.001	0.013
	552	613	23	4.70	4.60	1.76	2.79	2.94	low	<0.001	0.002

particularly critical for mediating baseline levels of this hormone (Vazquez et al., 1996). Similarly, two DMRs in *NR3C1* also had higher DNA methylation in nestlings with high growth. Interestingly, both of the DMRs in *NR3C1*, as well as the DMRs in *DNMT3a, GNRHR2, MC2R, NR3C2,* and *POMC*, directly overlapped DMRs found in relation to hatch date, with higher DNA methylation in nestlings hatched earlier in the season. This suggests that early season environmental conditions may produce higher levels of DNA methylation in these genes, which in turn drive higher offspring growth. While this relationship is purely correlational, it is consistent with the hypothesis that environmentally-driven changes in DNA methylation mediate phenotypic plasticity (Champagne, 2013a; von Holdt et al., 2022); further research is needed to determine the functional impact of differences in DNA methylation in these genes. Furthermore, these findings are consistent with previous research in house sparrows that found that *DNMT* expression, an important regulator of epigenetic potential, covaried with stress-induced glucocorticoid levels (Kilvitis et al., 2018), suggesting that epigenetic changes in methyltransferase and HPA-axis genes are important mediators of plasticity.

We also found that nestlings with "low growth" had higher initial DNA methylation in a DMR in *NR4A1*, which encodes a nuclear hormone receptor and has been shown to be an important growth regulator of skeletal muscle as well as metabolism (Kasch et al., 2018), and in *VIP*, an important circadian regulator of the HPA axis (Loh et al., 2008). In addition, three DMRs in *NR3C1* also had higher initial DNA methylation in "low growth" nestlings. However, none of these DMRs overlapped with any found in our hatch date analysis, suggesting that these predictive growth DMRs are not driven solely by hatch date, and thus other early life factors should be considered. Numerous studies have shown that stressful early environmental and social conditions increase DNA methylation in the putative promoter region of *NR3C1* (reviewed in Turecki and Meaney 2016), and methylation of *NR3C1* is associated with several phenotypic and behavioral outcomes (Conradt et al., 2013; Cottrell and Seckl, 2009), including reduced birth weight in humans (Mulligan et al., 2012). Our results are consistent with these findings in other taxa, and suggest that DNA methylation changes in other genes may also play a role in mediating this relationship between HPA axis function and growth. Determining the origin of these DMRs, and confirming whether they directly cause changes in HPA axis function and growth, will be an important next step in linking DNA methylation with developmental plasticity. In particular, because the relationship between DNA methylation and growth differed among various DMRs in *NR3C1*, it will be critical to further investigate how methylation of specific gene regions of the *NR3C1* promoter, especially those that have previously been associated with the early-life environment (e.g., exon 1_7; Turecki and Meaney, 2016) relate to these changes in developmental growth.

5. Conclusion

Our results provide initial support for the hypothesis that early differences in DNA methylation, induced by the early environment, may shape growth trajectories and thus facilitate developmental plasticity in wild birds. We showed that DNA methylation in wild house sparrows is dynamic, not only changing across the developmental period and over the breeding season, but also providing an important postnatal window in which environmental factors can potentially alter DNA methylation of specific genes. In particular, we found that DNA methylation immediately after hatching was related to hatching date, as nestlings born earlier in the season had higher initial DNA methylation in many of the target genes. Further investigation should identify the potential temporal early life and environmental factors (e.g., variation in temperature or parental quality) that are driving these mechanistic changes. Although initial DNA methylation marks based on date of hatching were no longer apparent by the end of development, we showed that post-hatching methylation in genes related to the HPA and HPG axes predicted differential nestling growth throughout development. Some of these DMRs overlapped those found based on hatch date, suggesting that changes in DNA methylation may mediate the relationship between the early environment and changes in growth. Thus, early post-hatching modifications may still influence a nestling's phenotype, with potential long-term fitness effects past the initial developmental period. Future studies should look beyond this period to understand the potential impacts these epigenetic modifications may have on adult phenotype and fitness. Finally, we found that sex-specific DMRs were maintained across development. Although beyond the scope of this study here, this raises an intriguing question of how different factors such as hatch date and sex may interact to influence early life development and growth. Together, these findings further our understanding of the mechanisms by which the early environment shapes development, demonstrating the potential role of DNA methylation in mediating developmental plasticity.

CRediT authorship contribution statement

Stefanie J. Siller Wilks: Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft. **David F. Westneat:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Britt J. Heidinger:** Conceptualization, Methodology, Resources, Writing – review & editing. **Joseph Solomon:** Methodology, Validation. **Dustin R. Rubenstein:** Supervision, Conceptualization, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2023.114336.

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