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RESEARCH ARTICLE

Changes in the rearing environment cause reorganization of molecular networks associated with DNA methylation

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Abstract

1. Disentangling the interaction between the genetic basis and environmental context underlying phenotypic variation is critical for understanding organismal evolution. Environmental change, such as increased rates of urbanization, can induce shifts in phenotypic plasticity with some individuals adapting to city life while others are displaced.
2. A key trait that can facilitate adaptation is the degree at which animals respond to stressors. This stress response, which includes elevation of baseline circulating concentrations of glucocorticoids, has a heritable component and exhibits intra- and inter-individual variation. However, the mechanisms behind this variability and whether they might be responsible for adaptation to different environments are not known. Variation in DNA methylation can be a potential mechanism that mediates environmental effects on the stress response, as early-life stressors increase glucocorticoid concentrations and change adult phenotype.
3. We used an inter- and intra-environmental cross-foster experiment to analyse the contribution of DNA methylation to early-life phenotypic variation. We found that at hatching, urban house wren (*Troglodytes aedon*) offspring had higher methylation frequencies compared with their rural counterparts.
4. We also observed age-related patterns in offspring methylation, indicating the developmental effects of the rearing environment on methylation. At fledgling, differential methylation analyses showed that cellular respiration genes were differentially methylated in broods of different origins and behavioural and metabolism genes were differentially methylated in broods of different rearing environments. Lastly, hyper-methylation of a single gene (*CNTNAP2*) is associated with decreased glucocorticoid levels and the rearing environment.
5. These differential methylation patterns linked to a specific physiological phenotype suggest that DNA methylation may be a mechanism by which individuals adjust to novel environments during their lifespan. Characterizing genetic and environmental influences on methylation is critical for understanding the role of epigenetic mechanisms in evolutionary adaptation.

KEYWORDS
cross-foster experiments, DNA methylation, early life, glucocorticoids, house wren

1 | INTRODUCTION

With environments showing rapid and dramatic change, phenotypic traits are increasingly investigated for molecules that contribute towards phenotypic plasticity through modified transcriptional control (e.g. Jeremias et al., 2018; Kilvitis et al., 2017; Ledón-Rettig et al., 2012; Taff et al., 2019; Viitaniemi et al., 2019). While plasticity occurs throughout an organism's life (Pigliucci, 2005), intergenerational plasticity can occur, when offspring phenotypes change under the control of the parental environmental context (Chen et al., 2015; Venney et al., 2021; Verhulst et al., 2016). One main mechanism for the transmission of intergenerational plasticity is through maternal effects (Groothuis & Schwabl, 2008). The underlying heritable basis of variation in phenotypic traits consists of a combination of additive, dominance and maternal effects (Lynch & Walsh, 1998; Marshall & Uller, 2007). Moreover, environmental effects may cause a single genotype to produce different phenotypes, producing so-called reaction norms. When the genotype interacts with the environment (G×E), different genotypes may respond differently to this environmental variation, resulting in variable reaction norms and fitness consequences. Therefore, disentangling the genetic basis, the environmental context and the interaction between genetics and environmental variation is critical for understanding phenotypic variation and organismal evolution.

Epigenetic mechanisms can alter organism function without changes in the DNA sequence, representing a possible mechanism for differences in the contribution of genetic and plastic variation to early-life traits (Bossdorf et al., 2008). The presence of 5-methylcytosine can immediately alter gene expression through a diverse array of mechanisms (e.g., changing splice sites, activating promoters, altering transcript stability), and this epigenetic methylation process commonly underlies environmentally-induced changes in gene expression (e.g., Goerlich et al., 2012; Heard & Martienssen, 2014; Laubach et al., 2018; Lea et al., 2016; Ledón-Rettig et al., 2012; Pedersen et al., 2014; Sasagawa et al., 2017; Suzuki & Bird, 2008). Early-life experiences are one specific external cause that can change methylation within a lifetime with possible intra- and inter-generational stability (e.g. Heard & Martienssen, 2014; Lea et al., 2016; Rubenstein et al., 2016; Sepers et al., 2021). In particular, early-life environments can affect late-life phenotypes with DNA methylation as the mediating mechanism (Rubenstein et al., 2016; Szyf & Bick, 2013). Therefore, DNA methylation may be a potential mechanism for organismal acclimation and adaptation to novel environments (Sepers et al., 2019).

We hypothesize that methylation patterns can indicate the degree to which an individual's early-life environment predicts future phenotype. To investigate this question, we conducted an inter-environment cross-fostering experiment using a free-living population of house wrens (*Troglodytes aedon*) in Nevada, U.S. with subsequent surveillance of DNA methylation variation (Figure 1). Our design included a comparison between an urban site that has increased levels of noise, light and human density compounded by a highly fragmented landscape populated by non-native vegetation to a more natural, rural site (Baldan & Ouyang, 2020; Davies et al., 2017). The anthropogenically altered landscape is characterized by decreased availability of natural high-quality food resources and increased environmental pollutants, such as lead (Baldan & Ouyang, 2020; Mäkinen et al., 2022; White et al., 2022). Urban areas are settled by younger, less-experienced individuals and, consequently, produce smaller-sized offspring than rural or natural habitat counterparts (Marzluff, 2017; Sepp et al., 2017; Sprau et al., 2017). These characteristics reduce reproductive success for house wrens living in urbanized areas, as we have previously reported that their offspring are smaller at fledgling, with higher corticosterone concentrations (a glucocorticoid hormone) ubiquitous across development and individuals (Heppner et al., 2022; Ouyang et al., 2019). The rearing environment was identified as the causative agent as the translocation of rural wrens to urban habitats increased corticosterone levels (Ouyang et al., 2019). Further, exposure of rural adult house wrens to experimental noise resulted in increased corticosterone levels, a trend that was not found in urban wrens, suggesting habituation (Davies et al., 2017). Lastly, a recent study shows that urban and forest great tits (*Parus major*) show differences in methylation patterns in genomic regions enriched for genes with functions related to the stress response (Caizergues et al., 2021). These studies together suggest that there is an underlying mechanism regulating phenotypic response, especially within the glucocorticoid phenotype, to urban environmental challenges.

Methylation in avian genomes follows the classic vertebrate paradigm of decreased transcription with increasing CpG methylation around transcription start sites (Derks et al., 2016; Laine et al., 2016; Luo et al., 2011). To unravel the genetic and environmental determinants of DNA methylation variation, we posed the following questions with respect to CpG methylation in house wrens from the cross-fostering experiment. First, to what degree does the environment (nest location) of hatchlings influence methylation patterns? Second, what role does the underlying genetic variation have on methylation patterns when surveyed at a later life stage (i.e. fledging)? Finally, is there a signal of environmental stressors on methylation at the time of fledging with respect to their fledging location? We further combined DNA methylation sequencing data with surveillance of glucocorticoid levels as a measurement of circulating levels of corticosterone hormones in the blood. This multi-dimensional, four-way, cross-foster experiment allowed us to

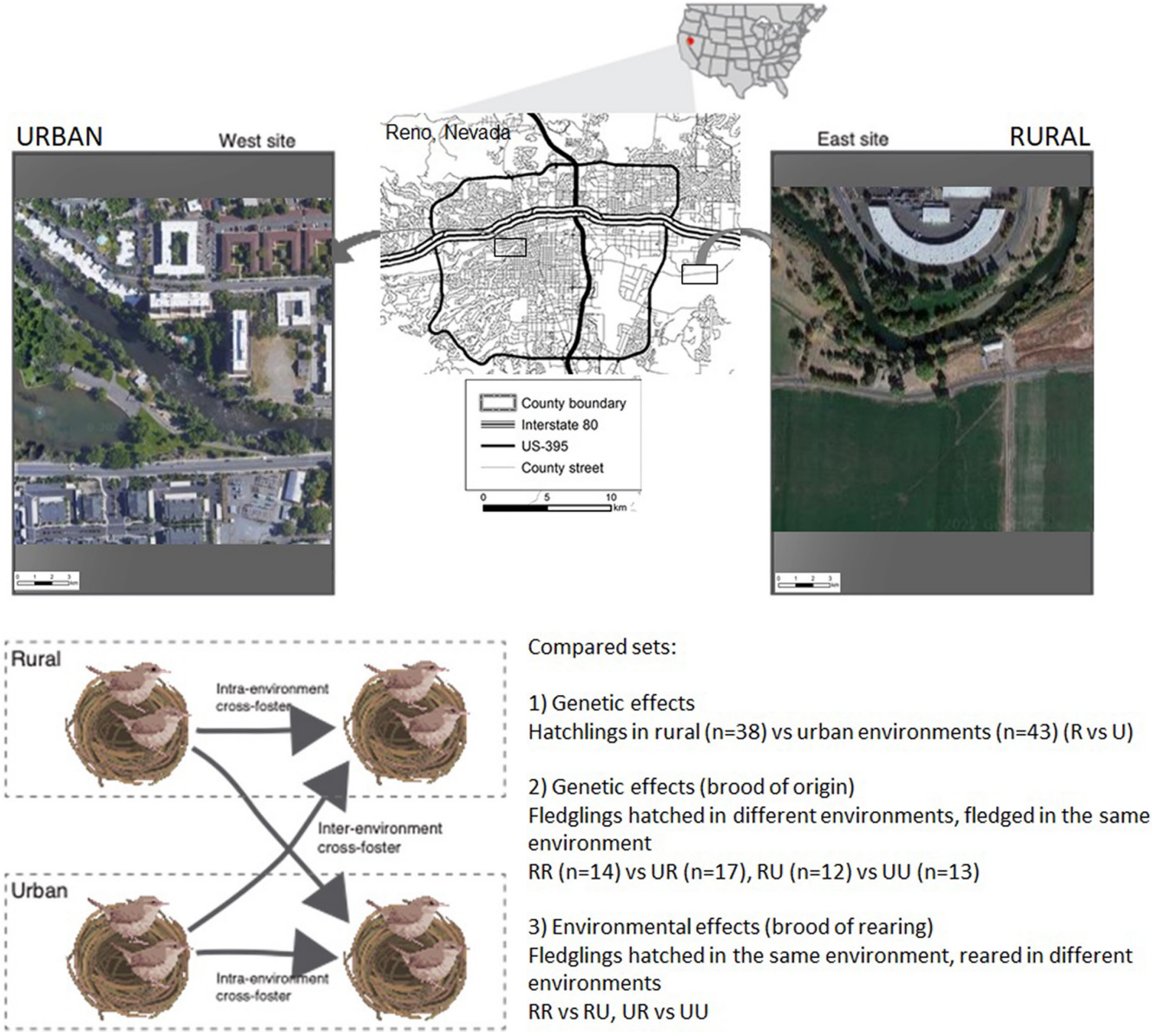

FIGURE 1 Map of geographic area from which source and recipient nests were selected in Reno, Nevada, USA. A schematic of the cross-fostering design depicts the intra- and inter-environmental movement of individual wrens. Hatchlings (on day 0) were translocated for cross-fostering. Sample sizes per cross-fostering treatment and age (hatchling, 0 days old; fledgling, 15 days old) after excluding samples that failed to sequence. Maps built with google earth.

investigate the contributions of genetic and plasticity mechanisms that regulate offspring development.

2 | MATERIALS AND METHODS

2.1 | Sample collection

We conducted our cross-fostering study from May to July 2017 and 2018 at one urban location in Reno, Nevada, USA, and one rural location in Sparks, Nevada, USA (for a detailed description of the field sites, see Ouyang et al., 2019; Figure 1). Briefly, our urban site was located at Idlewild Park, which is an urban park located near downtown Reno with paved walkaways and artificial ponds that fragment the green spaces in pockets of vegetation. Our rural site was the University of Nevada, Reno, Agricultural Experiment Station, which is a university-owned agricultural farm with ~1000 acres of farmland. The nest-box population at the rural site was set up in a riparian habitat along the Truckee River, in which vegetation is condensed in tree clusters along two lines.

House wrens are second-cavity single-brood nesting songbirds that migrate to the southern United States and Mexico for winter. Long-term studies of house wrens suggest that natal (650 m) and breeding (100 m) dispersal are short (Drilling & Thompsonm, 1988).

We monitored house wren nests regularly to determine the hatch date (day 0). On day 0 or 1, we obtained a blood sample (<10 μl, femoral vein) from all chicks (2.1 ± 0.02 mins from the time we opened the nest box; mean ± S.E.; Romero & Reed, 2005). Chicks sampled on day 0 or 1 did not differ in their corticosterone levels ($p > 0.2$). Any chick that was blood sampled past 3 minutes was not used in the study. After weighing all chicks (mean brood size = 5), we individually marked them with toenail polish and toenail clipping for identification. Hatchlings were then translocated to a new nest (keeping the same brood size) to investigate the mechanistic and molecular basis of phenotypic variation in a 2 × 2 experimental design in a quartet (Figure 1). Internal controls for translation were obtained by fostering a hatchling originating from a rural nest with non-biological rural parents and a hatchling originating from an urban nest with non-biological urban parents (hereinafter referred to as RR and UU, respectively). We also translocated rural hatchlings to urban nests and urban hatchlings to rural nests (hereinafter referred to as RU and UR, respectively). During the cross-foster process, we transported the chicks in a heated nest box to either a rural or an urban nest that hatched on the same day (maximum transport time: 20 mins, average transport time: 10 mins). On day 8, we banded the nestlings with a uniquely numbered tarsal band. On day 15 after hatching (house wrens fledge around day 17 and reach an asymptotic weight at day 15), we obtained another blood sample from the brachial vein of all chicks (1.7 ± 0.02 mins) and weighed them to the nearest 0.1 g.

This study was carried out in accordance with recommendations of the Institutional Animal Care and Use Committee of the University of Nevada, Reno with federal and state permits.

2.2 | Corticosterone hormone collection

We used enzyme-linked immunosorbent assay kits (Enzo Life Sciences) following the manufacturer's instructions. Please see Ouyang et al. (2019) for validation of this assay for house wrens. Based on optimization, we diluted plasma 1:40 in assay buffer with 0.5% steroid displacement reagent. We randomized samples across plates but an individual's day 0 and day 15 plasma were always next to each other on the same plate. We included a standard curve on each plate that ranged from 32 ng/ml to 20,000 ng/ml. The assay sensitivity was 2.1 pg/ml. To calculate intra- and inter-plate variation (CV), we included pooled house wren plasma, assayed in triplicate. The intra-plate CV was 9.8% and the inter-plate CV (5 plates) was 5.6%.

2.3 | Extraction of genomic DNA

We selected to conduct this temporal survey of blood-derived methylation variation as the individuals were not to be sacrificed and blood can be informative for several, but not all, tissue-specific patterns as well as for sites associated with expression (i.e. transcriptional start sites, TSS) (Husby, 2020; Lindner et al., 2021). We extracted genomic DNA from avian whole blood, where both red (~99.5%) and white blood cells (0.5%) are nucleated (Scanes, 2015), as per Qiagen DNeasy's protocol with the following modifications (Qiagen). As we collected typically less than 20 μl of blood, we added 180 μl of 1x phosphate buffered saline (PBS) and 20 μl of proteinase K to each sample. If the sample was represented by >20 μl of whole blood, we added 360 μl of 1x PBS directly into the sample tube, mixed and aliquoted equal volumes into a separate 1.5 ml tube, to which we added 20 μl of proteinase K. The duplicate tubes were combined after the DNA extraction and spin column purification steps.

2.4 | Preparation, sequencing and bioinformatics of bisulfite-converted genomic libraries

We used a reduced representation bisulfite sequencing approach using the *Msp1* restriction enzyme (Boyle et al., 2012) for whole blood–derived, high-molecular-weight genomic DNA. Libraries were prepared following the NEBNext sample preparation kit (New England Biolabs). We added 1 ng of enterobacteria phage lambda DNA to each genomic library as a non-methylated internal control for estimating bisulfite (BS) conversion downstream (e.g. Lea et al., 2015). We purified the genomic libraries and retained fragments between 100 and 400 bp in size using AMPure beads, with subsequent treatment of the fragments with bisulfite to convert unmethylated cytosines with the low DNA input (1 ng-2 μg) protocol in the Qiagen EpiTect Fast Bisulfite Conversion kit (Qiagen). Converted DNA was subjected to 12 cycles of PCR amplification to enrich for adapter-ligated fragments with MyTaq Mix (Bioline Inc.), during which each sample was also barcoded with unique sequence tags. Libraries were randomly pooled with an average of 16 samples per lane for single-end (1 × 100 nt) sequencing on an Illumina Novaseq 6000. We prepared the reference zebra finch genome (TaeGut2, GCA_000151805.2) with *bowtie2* in *BS-Seeker2* for BS-converted reads that were bounded from 50-300 bp (Chen et al., 2010; Langmead, 2010; Langmead & Salzberg, 2012). We demultiplexed sequence pools using perfect sequence matches between expected and observed barcode sequence tags with an in-house python script. FASTQ files were trimmed for low-quality reads ($Q < 20$) and adapter sequences clipped using *cutadapt* 1.8.1 (Martin, 2011), discarding reads that were <20 bp in length. Trimmed reads were mapped to the reference with *bowtie2* and methylation called in *BS-Seeker2*. Cytosines were annotated as being either inter-genic or putatively functional if found within a promoter (within 2Kb of transcriptional start site), gene body (exon methylation), or intron, all of which are potential critical regulatory elements that should be included in features of such analysis (Agirre et al., 2015; Bewick & Schmitz, 2017), using gene annotations obtained from the Genes and Gene Predictions Tracks

using the UCSC Table Browser for TaeGut2. Three TaeGut2 chromosomes (LG2, LG5 and LGE22) were unable to be annotated for genomic context due to a lack of information in the UCSC tracks. A cytosine may have membership in multiple categories; therefore, for any cytosine that is minimally located within a single exon, we considered it as a gene body cytosine. Similarly, cytosines located within an intron but within 2Kb downstream of an exon of a second known gene were annotated as promoter-associated. Cytosines located both in an exon and inferred promoter were considered a unique group. Inference of the regulatory consequences or *cis*-methylation is context-dependent, where promoter methylation has been associated with transcriptional suppression while gene body methylation reduces transcriptional noise in other systems (Huh et al., 2013; Lister et al., 2009; Maunakea et al., 2010; Varley et al., 2013; Yang et al., 2014).

The methylation frequency (MF) per cytosine was calculated as the proportion of methylated cytosines out of the total number of methylated and unmethylated cytosine (sequenced as thymine) reads per site (Chen et al., 2010). We filtered to retain sites with a minimum of 10-fold sequencing coverage. We estimated the conversion efficiency by mapping each genomic library to the 48,502 bp phage lambda linear genome (NC_001416.1) and assessed methylation levels of the converted lambda DNA. Conversion rates were estimated as [*1-average MF across the phage lambda genome*]. We further use the R v3.6.0 program *methylKit* (Akalin et al., 2012; R Core Team, 2019) to apply a coverage filter to retain sites with a minimum of 10-fold and below a maximum read threshold of (95% percentile) that were sequenced in all individuals or samples identified to be analysed. As cytosines found in the CG di-nucleotide motif are the predominant motif in avian erythrocytes (Sepers et al., 2019; Suzuki & Bird, 2008), we analysed CG-motif cytosines only in all subsequent analyses. We also removed cytosines that were not variable ($\sigma^2 = 0$) across individuals. Autosomes and the Z sex chromosome were analysed separately as sex chromosomes show extensive DNA methylation as a chromosomal dosage compensatory mechanism through the epigenetic inactivation of one of the sex chromosomes of the homogametic sex (Graves, 2016). We assessed all samples together for cytosines present in all samples, as well as subsets of the samples for specific analyses (e.g. all rural hatchlings compared with all urban hatchlings, etc.). To assess the samples for quality and help control for false-positives, we used a powerful principal component analysis (PCA) reference-free approach with the *prcomp* function in R v3.6.0 (R Core Team, 2019). This method would identify potential biological, methodological, or batch outliers for exclusion (Horgan & Chua, 2016).

2.5 | Differential methylation analysis

We arranged samples into five different comparisons for identifying differentially methylated sites (Figure 1). These comparisons were grouped into three sets for downstream analysis: (1) hatchlings in rural vs. urban environments (R vs. U); (2) fledglings that were hatched in different environments but fledged in the same environment (RR vs. UR, RU vs. UU), to identify the genetic influences in fledgling methylation patterns; and (3) fledglings that were hatched in the same environment but cross-fostered in different environments (RR vs. RU, UR vs. UU), to identify environmental influences in fledgling methylation patterns. We identified differentially methylated sites with the program *MACAU* which uses a binomial mixed model regression on methylation count data (Lea et al., 2015). PC coordinates were estimated using R's *prcomp* function and the first two PCs were used as covariates in the linear mixed model to account for batch effects, along with the parental nest identity (random effect). We assumed a full-sibling level of relatedness ($r = 0.5$) for hatchlings in the same nest of origin and no inter-nest relatedness ($r = 0$). When no nest information is available, we assumed no relatedness.

To identify genomic sites and regions of differential methylation for further analysis, we used network analysis in the R package IGRAPH (Csardi & Nepusz, 2006). This analysis is based on the assumption that differential methylation (1) that has a functional impact will be found in close physical proximity to other differentially methylated sites (DMSs) within the same treatment comparison and/or (2) is relevant to the cross-fostering design is found in both treatment comparisons within a set (Figure 1). Within a set, DMS lists were pooled and treated as network vertices, with edges that connected physically proximate DMSs (within 40 bp of each other). Clusters of DMSs (independent, maximal subgraphs with at least two vertices) were extracted from the networks for further analysis. We refer to these clusters, which are comprised of DMSs from one or more treatment comparisons, as *differentially methylated clusters*, or DMclusters. We acknowledge that there is an inherent bias in the physical clustering of cytosines given the reduced representation method of fragment selection. Our goal is to capture information at both the single-cytosine and 'neighbourhood' level of methylation.

2.6 | Annotation, enrichment and network analysis

We annotated cytosines in outlier DMclusters using two reference files (transcriptional and coding-sequence annotations) obtained from the Genes and Gene Predictions Tracks using the UCSC Table Browser for TaeGut2 annotated using the *intersect* function of *bedtools v2.28.0* (Quinlan & Hall, 2010). We further assessed if the cytosines annotated as found within the transcriptional or coding sequence were functionally enriched for specific gene ontological (GO) categories with the program *g:Profiler* (Raudvere et al., 2019). We included only reference genes with known annotations and applied the Benjamini-Hochberg FDR adjustment to the signified threshold (FDR < 0.05). We limited the annotation sources for the biological process (BP) GO category, regulatory motifs in DNA as found

in miRTarBase and any information inferred from the human phenotype ontology. Descriptions and child or related GO terms were referenced in *AmiGO 2* version 2.5.12 (last file loaded 2020-03-24). Subcellular localization information on proteins was obtained from the *Compartments* database, and we report the locales with the highest confidence (Binder et al., 2014).

Using *StringApp* in *Cytoscape* v3.8.0 (Assenov et al., 2008), we queried human protein databases to generate a network rooted by each candidate gene name that was differentially methylated and associated with a set of enriched GO term(s). We used a confidence cutoff of 0.4 and viewed up to 50 interactors in the network. To explore if the network themselves were enriched for any functional terms, we then retrieved the functional enrichment information for the nodes contained with these networks that included our candidate gene previously identified from differential methylation analysis.

2.7 | Genetic variant discovery from methylation sequence data

To maximize single-nucleotide polymorphism (SNP) variation discovery, we merged the paired sequence from hatchlings and fledglings per individual house wren. We then discovered SNPs from the merged converted DNA after mapping it to the TaeGut2 reference genome using *BS-SNPer* (Gao et al., 2015), a program that explicitly discovers genetic variation from bisulfite-treated sequence data using a dynamic matrix. We used default parameters with respect to frequencies of alleles to confidently identify heterozygous or homozygous sites (minimum of 10% or 85%, respectively), minimum of 15 base quality, minimum and maximum sequence coverage (10–1000), a minimum of two reads per mutation observed, a minimum mutation rate of 0.02 and mapping value of 20. We used the *intersect* function of *bedtools v2.28.0* to determine if SNPs were annotated in any genomic interval of interest.

2.8 | Differential methylation analysis and corticosterone data

Following from above, we also used *MACAU*'s binomial mixed model regression analysis to identify differentially methylated sites with corticosterone as the quantitative and continuous predictor phenotype for either the hatchlings with corticosterone measured at day 0 or for fledglings measured at day 15 (Table S1). We included the first two PC coordinates to represent possible batch effects and parental nest identity as covariates, with the relatedness matrix constructed as described above. We followed the annotation, enrichment and network analysis methods as described above. However, we limited our outliers to cytosines with *p*-values in the lowest first-percentile of the distribution that also had beta (*ß*) values in the first-percentile of either end of the *ß* distribution. While this threshold is relatively relaxed, we focused on the enrichment analysis to identify significant cytosines that would establish a candidate gene list. A binomial linear regression model with nests of origin and nests of rearing as random effects was conducted in R (version 3.6) to examine the relationship between outlier cytosine methylation frequencies and corticosterone levels.

2.9 | Analyses of average methylation frequency (generalized linear mixed models)

We used R (version 3.6) to perform all generalized linear mixed models (GLMMs) with the *glmer* function in the LME4 package (Bates et al., 2015) to compare average methylation frequencies between treatment groups. We implemented Tukey post-hoc tests with Bonferroni correction using the *emmeans* function in the EMMEANS package (Lenth, 2016). All final models met assumptions and significance was defined as $\alpha = 0.05$. We used a GLMM with repeated measures to test if average MF at day 0 and day 15 were different within individuals before and after cross-fostering. We included the interaction of treatment (rural to rural, urban to urban, urban to rural and rural to urban) and time (day 0 or day 15) as fixed effects. Date of capture and brood size were initially included in all models, but due to lack of variation and significance, they were removed from the final models. Individual ID, chromosome number and nest ID of the genetic and foster parents were included as random effects. We calculated the variance explained by random effects using the package SJPLOT and model estimates (Lüdecke, 2016). We used the Tukey post-hoc multiple-comparison tests for the interaction to test whether each treatment group was different from others.

3 | RESULTS

3.1 | Samples and corticosterone summary

We collected 168 blood samples from 86 individual house wrens, resulting in a total of 85 wrens in the study (Table S1). These 85 wrens provided us with 81 hatchlings (R = 38, U = 43) and 60 fledglings (R = 27, U = 33) (Figure 1). There were fewer fledglings compared with hatchlings sequenced, since we could not extract enough DNA from a random subset of fledgling blood samples. We constructed three datasets to investigate the impact of genetic and environmental contributions to corticosterone levels and methylation levels in (1) 80 hatchlings from either rural (R) or urban (U) nests, (2) 56 fledglings grouped based on their hatching (i.e. nest) location of rural or urban to reflect the "genetic" influence on corticosterone and methylation and (3) the same 56 fledglings grouped based on their fledgling location of rural or urban, regardless of their hatching location, to reflect the influence of their fledgling location on corticosterone and methylation.

Our phenotype analysis of corticosterone was for a subsample of that previously published (Ouyang et al., 2019). As such, we briefly present a survey of corticosterone to ensure the overall trend was consistent with a smaller sample size (Figure S1). Similar to the previous study, we found no significant difference in corticosterone levels between fledglings at day 15 with respect to their hatching locations (fledglings that hatched from rural = 16.7 ng/ml, urban = 17.8 ng/ml, $t = -0.4$, $df = 48.1$, $p = 0.6633$); rather, their fostered environment post-translocation significantly increased corticosterone in urban fledglings (fledged in rural = 12.5 ng/ml, urban = 24.2 ng/ml, $t = -5.2$, $df = 46.3$, $p < 0.001$; Figure S1). These results support the further investigation of the genetic versus environmental influence on methylation patterns as it pertains to the nest environment and corticosterone levels.

3.2 | Methylation summary and outlier sample screen

We obtained an average of 27,891,898 raw reads per individual (total of 141 samples) after filtering for a minimum of 10-fold sequence coverage, with an average of 9,880,751 reads that uniquely mapped to the reference zebra finch genome (Table S1). Post filtering, we obtained 17.9-fold average sequence depth across cytosines (SD = 7.3×) and all bisulfite conversion rates were high (range = 0.981–0.997). We estimated MF values for 141 samples at a minimum of 10-fold cytosine sequence coverage for 41,184 cytosines across CG di-nucleotide motifs (n, autosomes = 39,692; Z chromosome = 1492). We analysed three datasets (Figure 1) limited to cytosines in the CG motif with a minimum of 10× coverage. The PCA of hatchlings did not reveal clear clustering of samples by hatching environment (Figure S2). Methylation data from fledglings, however, revealed a tighter clustering of urban hatched and fledged wrens, spatially distinct from rural hatchlings raised in urban nests (Figure S2).

3.3 | Urban hatchlings are hypermethylated, with decreased methylation when environments changed

The interaction of time (day 0 or day 15) and treatment on methylation percentage was significant (Table 1). Day 15 MF in general was lower than methylation at day 0. At day 0, rural-hatched nestlings had lower average methylation frequencies than urban-hatched nestlings (Figure 2; Tables 1 and 2). Specifically, within-individual changes from day 0 to day 15 showed that average methylation frequencies increased in chicks that stayed in the same parental or foster environment, that is, moved from rural nests to rural nests (RR coef = 0.05, S.E. = 0.001, $z = 81.3$, $p < 0.001$) or moved from urban to urban nests (UU coef = 0.1, S.E. = 0.001, $z = 120.4$, $p < 0.001$). The increase in autosomal MF from day 0 to day 15 was smaller in RU or absent in UR when hatchlings were moved to foster nests across environments, that is, moved from rural nests to urban nests (RU coef = −0.005, S.E. = 0.001, $z = -6.14$, $p < 0.001$) or moved from urban nests to rural nests (UR coef = −0.01, S.E. = 0.001, $z = -22.3$, $p < 0.001$). Individual ID, chromosome number and parental and foster nest ID explained very little variation in average methylation frequencies (Table 1).

TABLE 1 Model estimates for the effect of cross-foster treatment on average MF of nestling house wrens. Individual estimates are given from summary statistics of the GLMM (binomial for frequency). Random effects include chromosome number, individual ID and parental and natal nest identity. Time is either day 0 or day 15 for blood sampling. (abbreviations: R, rural; RR, rural hatchling that fledged from rural nest; RU, rural hatchling that fledged from urban nest; U, urban; UR, urban hatchling that fledged from rural nest; UU, rural hatchling that fledged from urban nest)

Variable	Estimate	SE	*z*	*p*-value
GLMM for corticosterone levels				
(Intercept)	−2.29	0.01	−183.018	<0.001
Cross-foster group RU (*reference group RR*)	0.001	0.01	0.081	0.935
Cross-foster group UR	0.05	0.01	3.96	<0.001
Cross-foster group UU	0.08	0.01	5.89	<0.001
Time (day 15)	−0.05	0.00	−81.32	<0.001
Cross-foster group RU × Time (day 15)	0.06	0.001	56.77	<0.001
Cross-foster group UR × Time (day 15)	0.07	0.001	75.05	<0.001
Cross-foster group UU × Time (day 15)	−0.04	0.001	41.12	<0.001
Random Effects			**Variance**	**SD**
Chick ID			<0.001	0.089
Chromosome ID			0.15	0.38
Foster nest ID			<0.001	0.01
Parental nest ID			<0.001	<0.001

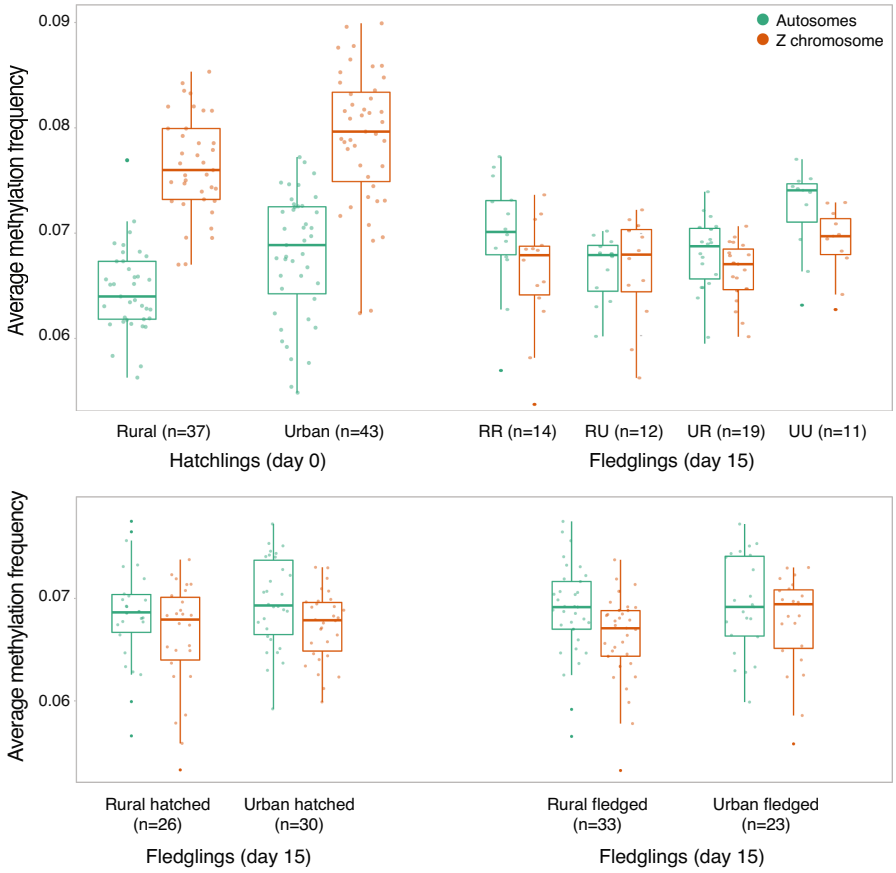

FIGURE 2 Box-and-jitter plots of methylation frequency of CG-motif cytosines across autosomes and Z chromosomes for hatchlings and fledglings. For significant treatment pairwise comparisons, please see Table 2. Briefly, urban-hatched young had higher average methylation frequencies than rural-hatched young, and cross-fostered young had decreased methylation at day 15 when environments switched (RU, UR) compared with staying within the same environment (RR, UU). (abbreviations: n, sample size; RR, rural hatchling that fledged from the rural nest; RU, rural hatchling that fledged from the urban nest; UR, urban hatchling that fledged from the rural nest; UU, rural hatchling that fledged from the urban nest).

TABLE 2 Estimates from Tukey post-hoc multiple-comparisons to test differences among cross-foster groups. Individual estimates are given from post-hoc test on the interaction between time (day 0 or day 15) and cross-foster group. (abbreviations: R, rural; RR, rural hatchling that fledged from rural nest; RU, rural hatchling that fledged from urban nest; U, urban; UR, urban hatchling that fledged from rural nest; UU, rural hatchling that fledged from urban nest)

Cross-foster group	Estimate	SE	*z*	*p*-value
Post-hoc comparisons of average methylation frequencies at day 0 (hatch date)				
RR-RU	−0.001	0.01	−0.08	1.00
RR-UR	−0.05	0.01	−3.96	0.002
RR-UU	−0.08	0.01	−5.89	<0.001
RU-UR	−0.05	0.02	−3.01	0.05
RU-UU	−0.08	0.02	−4.19	<0.001
UR-UU	−0.03	0.02	−1.51	0.80
Post-hoc comparisons of average methylation frequencies at day 15 (~2 days before fledge)				
RR-RU	−0.06	0.01	−4.56	<0.001
RR-UR	−0.12	0.01	−9.25	<0.001
RR-UU	−0.04	0.01	−2.62	0.15
RU-UR	−0.06	0.02	−3.54	0.01
RU-UU	0.03	0.02	1.39	0.86
UR-UU	0.08	0.02	4.67	<0.001

3.4 | Differentially methylated clusters identify environment-associated patterns

With the physical definition that a cluster of differentially methylated cytosines (referred to as DMclusters) had an inter-cytosine distance ≤40 bp, we identified DMclusters with respect to nest location and age or environment of house wrens (Figure S3), to determine the relative importance of environment and genetics in shaping methylation patterns across the genome. We outlined the results from each analysis of differential methylation here.

3.5 | Mechanosensory behaviour genes are differentially methylated in hatchlings from different 'origin broods'; set 1 (genetic effects, R vs. U)

We identified 7472 differentially methylated cytosines residing in 2491 DMclusters in 80 hatchlings with respect to nest locations in rural or urban landscapes. We found 3190 cytosines that were hyper-methylated in rural hatchlings (average MF, rural = 0.14, urban = 0.10) and included cytosines in 42 exons (rural = 0.15, urban = 0.13), 67 introns (rural = 0.16, urban = 0.11), 112 promoters (rural = 0.13, urban = 0.10) and 2969 cytosines were intergenic (rural = 0.14, urban = 0.10). We found 4282 cytosines hyper-methylated in urban hatchlings (average MF, rural = 0.17,

urban = 0.22) across 39 exons (rural = 0.12, urban = 0.16), 70 introns (rural = 0.18, urban = 0.23), 143 promoters (rural = 0.15, urban = 0.19) and 4030 cytosines were intergenic (rural = 0.17, urban = 0.22).

We found that 127 DMclusters contained transcribed or coding sequences of 34 known or 65 suspected gene regions. An analysis for gene enrichment identified 70 ontological terms significantly enriched (FDR adjusted $p < 0.05$) for BP, composed of unique genes populating three broad functional categories: behaviour/learning; metabolism and cellular response; and detoxification (Tables S2A and S8A). The most significantly enriched term was mechanosensory behaviour (GO:0007638, $p = 0.0028$), supported by differential methylation in clusters that contained the genes *CNTNAP2*, *FOXP2* and *STRBP* (Table 3a). Both *CNTNAP2* and *FOXP2* were associated with hyper-methylated DMclusters in rural hatchlings (average MF: rural = 0.08 and 0.30, urban = 0.06 and 0.19, respectively), while the DMcluster associated with *STRBP* was hyper-methylated in urban hatchlings (rural = 0.01, urban = 0.04). A network analysis of these three genes revealed two networks with a single-edge connection between them. The largest network was centered by *CNTNAP2* and *FOXP2* (40 nodes and 181 edges), with 401 significantly enriched terms that included the nervous system (GO:0007399, FDR $p = 1.95\times10^{-12}$), generation of neurons (GO:0048699, FDR $p = 2.62\times10^{-12}$) and vocalization behaviour (GO:0071625, FDR $p = 8.29\times10^{-12}$; Table S3). The smaller network contained *STRBP* (13 nodes and 28 edges), with a single connection to the *CNTNAP2*/*FOPX2* network (Figure 3a). This network contained 56 enriched terms, dominated by mRNA binding (GO:0003729, $p = 0.0037$), cytoplasm (KW-0963, $p = 0.0041$) and RNA processing (GO: 0006396, $p = 0.0043$; Table S4). Finally, we examined if underlying genetic variation could explain differences in methylation within these DMclusters. We found no segregating SNPs across groups of hatchlings (Table S9A).

3.6 | Cellular respiration genes are differentially methylated in fledglings of different origins (brood of origin); set 2 (genetic effects: RR vs. UR, RU vs. UU)

We identified 16,431 cytosines residing in 4745 DMclusters with respect to the hatchling location of 60 fledglings. We found 7571 cytosines that were hyper-methylated in fledglings that hatched from rural nests (average MF, rural = 0.19, urban = 0.14) and included cytosines in 75 exons (rural = 0.28, urban = 0.24), 129 introns (rural = 0.25, urban = 0.19), 222 promoters (rural = 0.19, urban = 0.15) and 7078 cytosines were intergenic (rural = 0.19, urban = 0.14). We found 8860 cytosines hyper-methylated in fledglings that hatched from urban nests (average MF, rural = 0.16, urban = 0.21) across 117 exons (rural = 0.11, urban = 0.15), 147 introns (rural = 0.19, urban = 0.24), 225 promoters (rural = 0.14, urban = 0.18) and 8311 cytosines were intergenic (rural = 0.16, urban = 0.21).

We found that 175 DMclusters contained transcribed or coding sequence of 45 known or 91 suspected gene regions. An analysis for gene enrichment identified 113 ontological terms significantly enriched (FDR adjusted $p < 0.05$) for BP, made up of cytosines associated with 22 unique genes that populated five broad functional categories: behaviour/learning; metabolism and cellular response; cellular respiration; detoxification and biosynthetic process (Tables S2B and S8B). As found in the hatchlings, the GO term mechanosensory behaviour (GO:0007638) was still significant, albeit now ranking as the 5th most significant term ($p = 0.0015$). The top four GO terms involved the electron transport chain: mitochondrial ATP synthesis coupled electron transport (GO:0042775, $p = 0.0006$); ATP synthesis coupled electron transport (GO:0042773, $p = 0.0006$); electron transport chain (GO:0022900, $p = 0.0008$) and respiratory electron transport chain (GO:0022904, $p = 0.0010$), supported by differential methylation in clusters that contained the genes *COQ9*, *NDUFA12*, *PARK7*, *TXNRD3* and *UQCRH* (Table 3b). A network analysis of these five genes revealed one large network containing *COQ9*, *NDUFA12*, *PARK7* and *UQCRH* (55 nodes and 1301 edges) while *TXNRD3* was unconnected as a singleton (Figure 3b). The network contained genes enriched in 151 terms, with the most significant including respiratory electron transport (HSA-163200, $p = 1.06\times10^{-101}$), Parkinson's disease (hsa05012, $p = 3.07\times10^{-99}$), oxidative phosphorylation (GO:0006119, $p = 5.03\times10^{-98}$) and the citric acid cycle (HSA-1428517, $p = 1.51\times10^{-95}$; Table S5). Additionally, we found no segregating SNPs across groups of fledglings and, thus, cannot explain patterns in methylation driven by underlying *cis*-genetic variation (Table S9B).

3.7 | Behaviour and metabolism genes are differentially methylated in fledglings from different rearing environments ('brood of rearing'); set 3 (environmental effects: RR vs. RU, UR vs. UU)

We identified 9283 cytosines residing in 2882 DMclusters in 60 fledglings assessing environmental influence with respect to nest locations in rural or urban landscapes. We found 4327 cytosines that were hyper-methylated in rural fledglings regardless of their hatching locations (average MF, rural = 0.20, urban = 0.17) and included cytosines in 44 exons (rural = 0.14, urban = 0.11), 71 introns (rural = 0.22, urban = 0.19), 97 promoters (rural = 0.22, urban = 0.19) and 4116 cytosines were intergenic (rural = 0.20, urban = 0.17). We found 4956 cytosines hyper-methylated in urban fledglings (average MF, rural = 0.23, urban = 0.27) across 67 exons (rural = 0.20, urban = 0.23), 103 introns (rural = 0.23, urban = 0.27), 141 promoters (rural = 0.25, urban = 0.28) and 4645 cytosines were intergenic (rural = 0.23, urban = 0.27).

We found that 109 DMclusters contained transcribed or coding sequences of 33 known or 59 suspected gene regions. An analysis for gene enrichment identified 33 ontological terms significantly enriched (FDR adjusted $p < 0.05$) for BP, composed of 14 unique genes across the same five broad functional categories: behaviour/

TABLE 3 Details for the most significantly enriched gene ontology (GO) term for significantly differentially methylated clusters (DMcluster) with respect to environment (nest location) in (a) 80 hatchlings (rural versus urban nest locations), (b) 60 fledglings based on their hatching (not fledgling) nest location and (c) 60 fledglings based on their fledgling (not hatching) nest location. All genomic coordinates are provided as chromosome and position mapped to the zebra finch reference genome (TaeGut2). Bolded values indicate the group with hyper-methylation. (abbreviations: Chr, chromosome; MF, methylation frequency; N, number; R, rural; U, urban)

DMcluster ID (N cytosines)	Annotation	Coordinates	Hatchling MF in R, U
(a)			
Chr2_62 (2)	Intron of *CNTNAP2*	Chr 2: 30,213,394	**0.113**, 0.052
	Intron of *CNTNAP2*	Chr 2: 30,213,385	0.040, **0.068**
Chr1A_27 (8)	Transcriptional start of *FOXP2*	Chr 1A: 25,574,528	**0.222**, 0.116
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,534	**0.224**, 0.149
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,521	**0.237**, 0.170
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,545	**0.250**, 0.140
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,546	**0.332**, 0.238
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,522	**0.344**, 0.143
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,539	**0.380**, 0.318
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,540	**0.413**, 0.244
Chr17_46 (3)	Transcriptional start of *STRBP*	Chr 17: 10,382,806	0.005, **0.026**
	Transcriptional start of *STRBP*	Chr 17: 10,382,802	0.008, **0.043**
	Transcriptional start of *STRBP*	Chr 17: 10,382,811	0.010, **0.040**
DMcluster ID (N cytosines)	**Annotation**	**Coordinates**	**Fledgling MF that hatched in R, U**
(b)			
Chr11_23 (3)	Intron of *COQ9*	Chr 11: 4,340,734	**0.046**,0.025
	Intron of *COQ9*	Chr 11: 4,340,736	**0.144**,0.108
	Exon of *COQ9*	Chr 11: 4,340,757	0.238, **0.310**
Chr1A_78 (3)	Exon of *NDUFA12*	Chr 1A: 44,626,272	0.255, **0.270**
	Exon of *NDUFA12*	Chr 1A: 44,626,275	**0.010**, 0.004
	Exon of *NDUFA12*	Chr 1A: 44,626,281	0.262, **0.290**
Chr21_20 (3)	Transcriptional start of *PARK7*	Chr 21: 1,830,294	**0.038**, 0.024
	Transcriptional start of *PARK7*	Chr 21: 1,830,297	**0.031**, 0.018
	Transcriptional start of *PARK7*	Chr 21: 1,830,300	**0.021**, 0.005
Chr12_62 (4)	Intron of *TXNRD3*	Chr 12: 10,818,436	0.070, **0.104**
	Intron of *TXNRD3*	Chr 12: 10,818,439	0.066, **0.104**
	Intron of *TXNRD3*	Chr 12: 10,818,442	0.063, **0.103**
	Intron of *TXNRD3*	Chr 12: 10,818,445	0.063, **0.109**
Chr8_72 (4)	Exon of *UQCRH*	Chr 8: 18,754,511	0.029, **0.049**
	Intron of *UQCRH*	Chr 8: 18,754,521	0.049, **0.080**
	Intron of *UQCRH*	Chr 8: 18,754,529	0.133, **0.195**
	Intron of *UQCRH*	Chr 8: 18,754,534	**0.035**, 0.015
DMcluster ID (N cytosines)	**Annotation**	**Coordinates**	**Fledgling MF that fledged in R, U**
(c)			
Chr2_57 (3)	Intron of *CNTNAP2*	Chr 2: 30,213,382	0.010, **0.011**
	Intron of *CNTNAP2*	Chr 2: 30,213,399	0.227, **0.256**
	Intron of *CNTNAP2*	Chr 2: 30,213,399	0.227, **0.256**
Chr1A_98 (8)	Transcriptional start of *FOXP2*	Chr 1A: 25,574,521	**0.226**, 0.192
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,522	**0.275**, 0.244

TABLE 3 (Continued)

DMcluster ID (N cytosines)	Annotation	Coordinates	Fledgling MF that fledged in R, U
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,527	**0.286**, 0.238
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,528	**0.195**, 0.167
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,533	**0.359**, 0.288
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,534	**0.210**, 0.180
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,539	**0.395**, 0.309
	Transcriptional start of *FOXP2*	Chr 1A: 25,574,545	**0.219**, 0.184
Chr17_73 (2)	Transcriptional start of *STRBP*	Chr 17: 10,225,696	**0.108**, 0.077
	Transcriptional start of *STRBP*	Chr 17: 10,225,699	**0.101**, 0.087

learning; metabolism and cellular response; cellular respiration; detoxification and biosynthetic process (Tables S2C and S8C). As found in the hatchlings, the GO term mechanosensory behaviour (GO:0007638) was the most significant term ($p = 0.0009$), represented by the same three previously described genes *CNTNAP2*, *FOXP2* and *STRBP* (Table 3c). The difference from hatchling patterns was that rural fledglings were hyper-methylated at *STRBP* (rural = 0.10, urban = 0.08). Furthermore, *FOXP2* was entirely hyper-methylated in fledglings that fledged from rural nests, regardless of their hatchling location (rural = 0.27, urban = 0.23). *CNTNAP2* was the only DMcluster hyper-methylated in urban fledglings (rural = 0.15, urban = 0.17). The same network interactors and composition were found as in the hatchlings, with a concentration of protein members with functions in behaviour and neurodevelopment (Figure 3c; Tables S4–S6). We found no segregating SNPs across groups of fledglings and, thus, cannot explain patterns in methylation driven by underlying genetic variation (Table S9B).

3.8 | Hyper-methylation at a single gene is associated with decreased corticosterone levels

A final effort was motivated by the significant corticosterone differences observed between rural and urban environments (Figure S1). We used the quantitative corticosterone phenotypes to conduct two independent genome-wide association analyses across (1) 80 hatchlings with methylation data at 57,723 cytosines and corticosterone levels measured at day 0; and (2) 56 fledglings (i.e. not partitioned into subgroups based on cross-fostering) and corticosterone on day 15 for 63,876 cytosines. We found 277 cytosines with significant methylation differences associated with corticosterone levels in 80 hatchlings, with only 10 sites annotated within transcriptional starts or coding sequences (Table S7A). A single gene, *CNTNAP2*, was enriched for functional ontology, with this gene a member of 36 different GO terms (Table S8D). The terms were heavily populated by behaviour, brain and auditory functions (e.g. mechanosensory, observed learning, imitative learning, vocal learning, etc.). A network analysis of *CNTNAP2* revealed that one large network (51 nodes and 306 edges) contained 438 significantly enriched functional terms, with the most significant involved in neurexins/neuroligins (HSA-6794361, $p = 1.17\times10^{-17}$), neuron parts (GO:0097458, $p = 1.86\times10^{-17}$) and neuronal systems (HSA-112316, $p = 5.86\times10^{-17}$; Figure 3c; Table S8). MF (methylated counts vs total counts) was analysed in a GLMM with binomial errors and the logit link function with corticosterone and site as fixed effects and parental and genetic nests as random effects. We confirmed a significant negative logarithmic trend of corticosterone and methylation at *CNTNAP2* (cytosine at Chr2.30213392) across 80 hatchlings (Coef = −0.04, S.E. = 0.01, $z = 2.1$, $p = 0.047$; Figure 4). We found only two wrens (hatching location, rural = 1, urban = 1) with SNPs annotated and thus do not explain patterns in methylation at gene *CNTNAP2* (Table S9A). Overall, we found significant differences in methylation at 290 cytosines in 56 fledglings, with only 13 sites annotated within transcriptional starts or coding sequences (Table S9B).

4 | DISCUSSION

We used an inter- and intra-environmental cross-foster experiment to analyse the molecular and genetic mechanisms that contribute to early-life phenotypic variation. We found that at hatching, urban offspring had increased average methylation compared with their rural counterparts. Differential methylation analyses showed that cellular respiration genes were differentially expressed at hatching and behavioural and metabolism genes were differentially expressed at fledgling. Lastly, hyper-methylation of a single gene is associated with decreased corticosterone levels.

4.1 | Innate DNA methylation differences between urban and rural birds

We found strong differentiation in methylation at hatching with signals of the environment into which an offspring was born, whether in an urban or rural environment. Most of these signals were involved in metabolism, for example, respiratory electron transport, electron transport chain, mitochondrial respirasome, oxidative phosphorylation and cellular respiration. A recent study also found that differentially methylated CpG sites between urban and

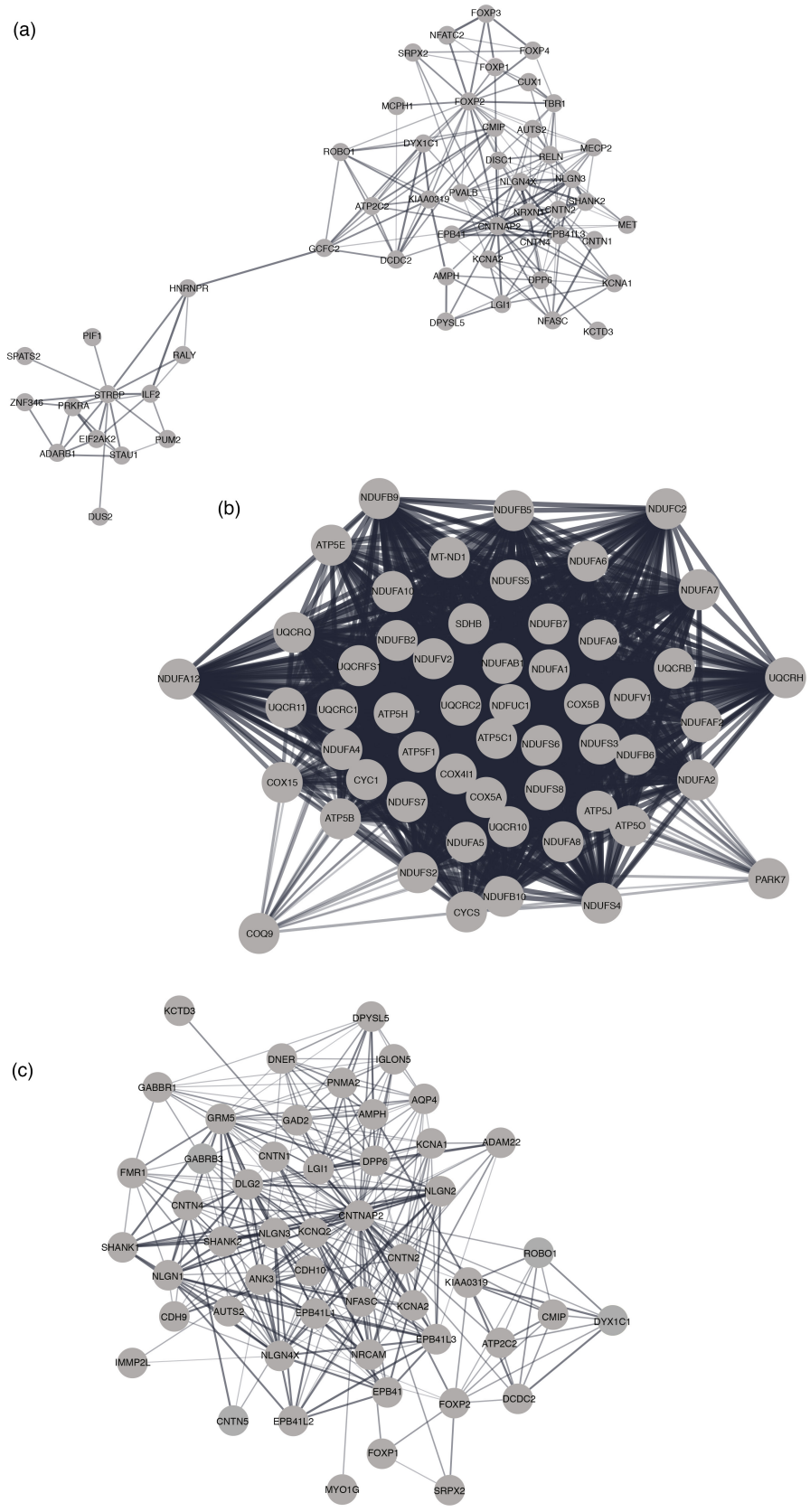

FIGURE 3 Protein networks for (a) *STRBP* (lower left) and *CNTNAP2*/*FOXP2* (upper right); comparing hatchlings in rural vs urban environments (R vs. U) showed two major gene networks were differentially methylated (*STRBP* and *CNTNAP2*/*FOXP2*), which include the nervous system, neuron generation and vocalization behaviour. (b) Protein network containing *COQ9*, *NDUFA12*, *PARK7* and *UQCRH*, with the single unconnected protein of *TXNRD3*; comparing fledglings hatched in the same environment but reared in different environments showed differential methylation in cellular respiration gene networks and (c) protein network of *CNTNAP2*, an enriched gene ontology term with differential methylation associated with corticosterone levels in 80 hatchlings.

rural birds were over-represented in metabolic pathways (Watson et al., 2021). We also found this gene to be differentially methylated, and part of the GO-enriched terms, explaining differences in the genetic influence of fledgling methylation. Urbanization is often associated with increased exposure to pollutants, resulting in increased oxidative damage (Isaksson, 2010; Salmon et al., 2018) and increased thermal temperatures due to large areas of concrete pavement (Arnfield, 2003). Exposure to mercury or methylmercury

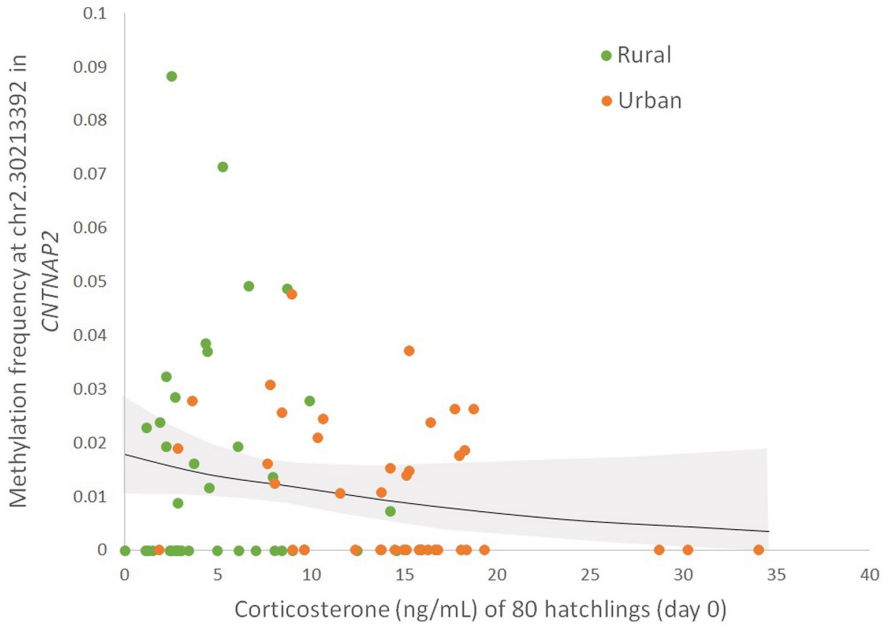

FIGURE 4 General linear regression with logit link binomial function of 80 hatchlings and corticosterone levels at day 0 as a function of methylation frequency at the cytosine (chr2.30213392) in the functionally enriched gene *CNTNAP2*. Logarithmic trendline and 95% CI (shaded) are provided.

contaminants resulted in methylation changes at the global and gene level in several species (e.g. bears, mink, rats; Basu et al., 2014; Bossdorf et al., 2008; Crews & McLachlan, 2006; Vandegehuchte & Janssen, 2011). Airborne particulate matter is also linked with changes in genome-wide methylation patterns and negative health consequences (Rider & Carlsten, 2019; Sun et al., 2018). Along with differences in nutrient availability where urban areas have less available high-quality food items, it is likely to result in changes in cellular metabolism (Isaksson et al., 2017). There are no studies that we are aware of that have measured endothermic metabolic rates across an urban gradient, but our study points to a promising avenue of hypothesis-driven research, given that some studies have found increased glucocorticoid levels, a hormone that drives glucose metabolism, in cities (Bonier, 2012). Additionally, we cannot rule out maternal effects, such as incubation behavioural differences or deposition of yolk hormones (Heppner et al., 2022; Heppner & Ouyang, 2021; Love & Williams, 2008).

4.2 | Effects of urban and rural environments on DNA methylation plasticity

Increased methylation frequencies based on environmental differences have been noted for several taxa (Heard & Martienssen, 2014; Laubach et al., 2018; Lea et al., 2016; Pedersen et al., 2014; Sasagawa et al., 2017). We found that methylation frequencies also change as young develop (Taff et al., 2019; Venney et al., 2021). In the same environment, offspring increased MF but when moved to a different environment, offspring decreased MF. This type of genetic and environment impact on DNA methylation has recently been shown in Chinook salmon in which significant parental, age and environmental variables explained methylation differences (Venney et al., 2021). We do not know if these changes in MF are adaptive, but we speculate that when environmental changes happen during development, the young may benefit from the ability to effectively modify gene transcriptional activity (Herman & Sultan, 2016; O'Dea et al., 2016). To test if these methylation changes are adaptive, we need recruitment rates to estimate offspring survival and transgenerational differences between individuals from control and cross-fostered treatments.

In fledglings regardless of hatching location, we found strong environmental signatures related to their fledged environment. The most significantly enriched GO subterm was mechanosensory behaviour (GO:0007638), which includes auditory behaviour (GO:0031223) or a behavioural response to sound. In urban hatchlings, cytosines in the transcriptional element of *STRBP* were hypermethylated. We hypothesize that urban hatchlings need to have reduced receptivity to an excess of stimulus in the urban environment; thus, increased methylation in the transcriptional start site of this gene could possibly damp the gene's reactivity. Urban environments are often associated with increased avian song frequency due to increases in anthropogenic noise (Halfwerk et al., 2011). We previously found that our population of urban house-wren adults do not respond physiologically when traffic noise was played, which suggests a level of habituation or perhaps reduced sensitivity to sound stimulus in urban environments (Davies et al., 2017). Two additional genes intersected this GO subterm, *CNTNAP2* and *FoxP2*, with hyper-methylation patterns found in rural hatchlings. *CNTNAP2* is a direct *FoxP2* target gene in songbirds, likely affecting synaptic function relevant for song learning and song maintenance (Adam et al., 2017). During periods of enhanced plasticities, such as during development, *FoxP2* influences *CNTNAP2* expression in a linear manner (Adam et al., 2017). Therefore, urban offspring may increase the expression of both genes during development as an adaptation to the noisy rearing environment. Other studies have reported regulatory changes in neural-related genes. For example, *EGR1* (also referred to as *ZENK*) is a neural activity-dependent intermediate early gene reported to have decreased expressed in zebra finches that

experienced acute social isolation that can be rescued through the addition of a partner bird (George et al., 2020). This gene is known to regulate song during singing and in central auditory pathways during hearing (Clayton, 2013; Jarvis & Nottebohm, 1997; Mello, 2002; Reiner et al., 2004). Furthermore, exposure to novel acoustics increases *EGR1* levels in song sparrows and zebra finch, regardless of the social context (Jarvis et al., 1997; Vignal et al., 2005).

4.3 | Modification of corticosterone concentrations through DNA methylation of CNTNAP2

As we have found significant and persistent physiological differences in corticosterone expression between urban and rural offspring and adults, we also found a single gene (*CNTNAP2*) that was enriched for functional ontology in urban birds. In other words, hypermethylation of *CNTNAP2* is associated with decreased corticosterone levels. If *CNTNAP2* is involved in corticosterone regulation and/or expression and DNA hypermethylation occurs in a promoter of the gene in rural hatchlings, this may provide a mechanism to down-regulate baseline corticosterone levels when hatchings are in more challenging environments. GO terms were heavily populated by behaviour, brain and auditory functions, for example, observed learning, imitative learning and vocal learning. Urban areas are associated with explorative individuals that exhibit less neophobic responses (Grunst et al., 2019). Stress-reactivity is related to explorative behaviour and recently has been shown to be related to differentially methylated regions across the genome (Baugh et al., 2012; Taff et al., 2019). To illustrate, females with higher stress resilience, and thus lower feedback in the stress response, had lower overall methylation at a set of differentially methylated regions (Taff et al., 2019). Increased levels of corticosterone have been associated with decreased learning and memory acquisition (Monaghan & Spencer, 2014; Spencer & Verhulst, 2007). These results combined indicate a relationship between corticosterone levels and learning abilities that may be related to environmental context, although we note that the relationship is weak. Therefore, we hypothesize that decreased *CNTNAP2* expression may result in increased corticosterone levels or vice versa (Cottrell & Seckl, 2009; Ing, 2005), especially in urban offspring.

5 | CONCLUSIONS

We show that DNA methylation exhibits phenotypic plasticity in response to environmental change, such that both parentage and the rearing environment influence the methylation status of specific genes. Furthermore, we found molecular signatures related to the glucocorticoid phenotype. Our findings are suggestive that DNA methylation can shape the physiological phenotype and is empirical evidence for a mechanism by which individuals thrive in challenging environments. This study provides novel insights into avian epigenetic variation and the effects of urbanization, which can generate future empirical studies on specific gene networks and phenotypic traits under selection. We focus on one urban and rural site as cross-fostering at a finer scale, that is, across an urbanization gradient, would be logistically challenging for matching hatch-date; therefore, our results could be attributed to site differences rather than urbanization itself. Nevertheless, our study is experimental, and the correlation between methylation patterns and glucocorticoid levels is continuous within and among sites. Future studies disentangling genetic and environmental processes across different sizes and locations of cities are necessary for urban evolutionary ecology (Alaasam & Ouyang, 2021). With the rapid development of sequencing technologies, studying urban evolution has entered the genomic era, expanding upon candidate gene approaches (Caizergues et al., 2021; Riyahi et al., 2015). Future studies will need to genotype more loci and more individuals to disentangle polygenic adaption in urban environments (Szulkin et al., 2020). Understanding the genetic and environmental basis of local adaptation will be important in predicting species' responses to urbanization and establishing epigenetic processes as a mechanism for novel environmental acclimation.

AUTHOR CONTRIBUTIONS

Jenny Q. Ouyang, Kees van Oers, Koen J. F. Verhoeven and Bridgett M. von Holdt conceived the project design; Bridgett M. von Holdt and Jenny Q. Ouyang collected the data; Bridgett M. von Holdt, Rebecca Y. Kartzinel and Jenny Q. Ouyang performed the analyses; all co-authors contributed towards manuscript preparation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Sorted BAM files were deposited on NCBI's Short Read Archive (PRJNA703476).

ETHICS STATEMENT

Our methods were approved by the University of Nevada Reno's Institutional Animal Care and Use Committee (#2016-00677) and conducted under relevant state (NDOW-5042946) and federal permits (permit number 24034).

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REFERENCES

Adam, I., Mendoza, E., Kobalz, U., Wohlgemuth, S., & Scharff, C. (2017). CNTNAP2 is a direct FoxP2 target in vitro and in vivo in zebra finches: Complex regulation by age and activity. *Genes, Brain and Behavior*, *16*, 635–642.

Agirre, X., Castellano, G., Pascual, M., Heath, S., Kulis, M., Segura, V., Bergmann, A., Esteve, A., Merkel, A., Raineri, E., Agueda, L., Blanc, J., Richardson, D., Clarke, L., Datta, A., Russiñol, N., Queirós, A. C., Beekman, R., Rodríguez-Madoz, J. R., … Martín-Subero, J. I. (2015). Whole-epigenome analysis in multiple myeloma reveals DNA hypermethylation of B cell-specific enhancers. *Genome Research*, *25*(4), 478–487.

Akalin, A., Kormaksson, M., Li, S., Garrett-Bakelman, F. E., Figueroa, M. E., Melnick, A., & Mason, C. E. (2012). methylKit: A comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biology*, *13*(10), R87.

Alaasam, V. J., & Ouyang, J. Q. (2021). The power of large-scale community science in addressing anthropogenic change. *Global Change Biology*, *27*, 3953–3955. https://doi.org/10.1111/gcb.15741

Arnfield, A. J. (2003). Two decades of urban climate research: A review of turbulence, exchanges of energy and water, and the urban heat Island. *International Journal of Climatology*, *23*(1), 1–26.

Assenov, Y., Ramirez, F., Schelhorn, S. E., Lengauer, T., & Albrecht, M. (2008). Computing topological parameters of biological networks. *Bioinformatics*, *24*(2), 282–284.

Baldan, D., & Ouyang, J. Q. (2020). Urban resources limit pair coordination over offspring provisioning. *Scientific Reports*, *10*(1), 15888.

Basu, N., Goodrich, J. M., & Head, J. A. (2014). Ecogenetics of mercury: From genetic polymorphisms and epigenetics to risk assessment and decision-making. *Environmental Toxicology and Chemistry*, *33*, 1248–1258.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*, 48.

Baugh, A. T., Schaper, S. V., Hau, M., Cockrem, J. F., de Goede, P., & Oers, K. V. (2012). Corticosterone responses differ between lines of great tits (*Parus major*) selected for divergent personalities. *General and Comparative Endocrinology*, *175*, 488–494.

Bewick, A. J., & Schmitz, R. J. (2017). Gene body DNA methylation in plants. *Current Opinion in Plant Biology*, *36*, 103–110.

Binder, J. X., Pletscher-Frankild, S., Tsafou, K., Stolte, C., O'Donoghue, S. I., Schneider, R., & Jensen, L. J. (2014). COMPARTMENTS: Unification and visualization of protein subcellular localization evidence. *Database*, *2014*, bau012.

Bonier, F. (2012). Hormones in the city: Endocrine ecology of urban birds. *Hormones and Behavior*, *61*, 763–772.

Bossdorf, O., Richards, C. L., & Pigliucci, M. (2008). Epigenetics for ecologists. *Ecology Letters*, *11*, 106–115.

Boyle, P., Clement, K., Guy, H. C., Smith, Z. D., Ziller, M., Fostel, J. L., Holmes, L., Meldrim, J., Kelley, F., Gnirke, A., & Meissner, A. (2012). Gel-free multiplexed reduced representation bisulfite sequencing for large-scale DNA methylation profiling. *Genome Biology*, *13*, R92.

Caizergues, A. E., Le Luyer, J., Grégoire, A., Szulkin, M., Senar, J.-C., Charmantier, A., & Perrier, C. (2021). Epigenetics and the city: Non-parallel DNA methylation modifications across pairs of urban-forest Great tit populations. *Evolutionary Applications*, *15*(1), 149–165.

Chen, P.-Y., Cokus, S. J., & Pellegrini, M. (2010). BS seeker: Precise mapping for bisulfite sequencing. *BMC Bioinformatics*, *11*, 203.

Chen, Q., Yan, M., Cao, Z., Li, X., Zhang, Y., Shi, J., Feng, G. H., Peng, H., Zhang, X., Zhang, Y., Qian, J., Duan, E., Zhai, Q., & Zhou, Q. (2015). Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. *Science*, *351*, 397–400.

Clayton, D. F. (2013). The genomics of memory and learning in songbirds. *Annual Review of Genomics and Human Genetics*, *14*, 45–65.

Cottrell, E. C., & Seckl, J. R. (2009). Prenatal stress, glucocorticoids and the programming of adult disease. *Frontiers in Behavioral Neuroscience*, *3*(19), 1–9.

Crews, D., & McLachlan, J. A. (2006). Epigenetics, evolution, endocrine disruption, health, and disease. *Endocrinology*, *147*, s4–s10.

Csardi, G., & Nepusz, T. (2006). The igraph software package for complex network research. *InterJournal*, *Complex Systems*, *1695*. https://igraph.org

Davies, S., Haddad, N., & Ouyang, J. Q. (2017). Stressful city sounds: Glucocorticoid responses to experimental traffic noise are environmentally dependent. *Biology Letters*, *13*(10), 20170276.

Derks, M. F. L., Schachtschneider, K. M., Madsen, O., Schijlen, E., Verhoeven, K. J. F., & van Oers, K. (2016). Gene and transposable element methylation in great tit (*Parus major*) brain and blood. *BMC Genomics*, *17*, 332.

Drilling, N. E., & Thompsonm, C. F. (1988). Natal and breeding dispersal in house wrens (*Troglodytes aedon*). *The Auk*, *105*(3), 480–491.

Gao, S., Zou, D., Mao, L., Liu, H., Song, P., Chen, Y., Zhao, S., Gao, C., Li, X., Gao, Z., Fang, X., Yang, H., Orntoft, T. F., Sorensen, K. D., & Bolund, L. (2015). BS-SNPer: SNP calling in bisulfite-seq data. *Bioinformatics*, *31*, 4006–4008.

George, J. A., Bell, Z. W., Condliffe, D., Dohrer, K., Abaurrea, T., Spencer, K., Leitão, A., Gahr, M., Hurd, P. J., & Clayton, D. F. (2020). Acute social isolation alters neurogenomic state in songbird forebrain. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(38), 23311–23316.

Goerlich, V. C., Nätt, D., Elfwing, M., Mcdonald, B., & Jensen, P. (2012). Transgenerational effects of early experience on behavioral, hormonal and gene expression responses to acute stress in the precocial chicken. *Hormones and Behavior*, *61*, 711–718.

Graves, J. A. (2016). Evolution of vertebrate sex chromosomes and dosage compensation. *Nature Reviews. Genetics*, *17*, 33–46.

Groothuis, T. G. G., & Schwabl, H. (2008). Hormone-mediated maternal effects in birds: Mechanisms matter but what do we know of them? *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1497), 1647–1661.

Grunst, A. S., Grunst, M. L., Pinxten, R., & Eens, M. (2019). Personality and plasticity in neophobia levels vary with anthropogenic disturbance but not toxic metal exposure in urban great tits: Urban disturbance, metal pollution and neophobia. *Science of the Total Environment*, *656*, 997–1009.

Halfwerk, W., Bot, S., Buikx, J., van der Velde, M., Komdeur, J., ten Cate, C., & Slabbekoorn, H. (2011). Low-frequency songs lose their potency in noisy urban conditions. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(35), 14549–14554.

Heard, E., & Martienssen, R. A. (2014). Transgenerational epigenetic inheritance: Myths and mechanisms. *Cell*, *157*, 95–109.

Heppner, J. J., Krause, J. S., & Ouyang, J. Q. (2022). Urbanization and maternal hormone transfer: Endocrine and morphological phenotypes across ontogenetic stages. *General and Comparative Endocrinology*, 114166.

Heppner, J. J., & Ouyang, J. Q. (2021). Incubation behavior differences in urban and rural house wrens, *Troglodytes aedon*. *Frontiers in Ecology and Evolution*, *9*, 590069.

Herman, J. J., & Sultan, S. E. (2016). DNA methylation mediates genetic variation for adaptive transgenerational plasticity. *Proceedings of the Royal Society B: Biological Sciences*, *283*, 20160988.

Horgan, G. Q., & Chua, S.-P. (2016). Statistical methods for methylation data. In P. Haggarty & K. Harrison (Eds.), *Population epigenetics. Methods in molecular biology* (Vol. *1589*). Humana Press. https://doi.org/10.1007/7651_2015_316

Huh, I., Zeng, J., Park, T., & Yi, S. V. (2013). DNA methylation and transcriptional noise. *Epigenteics Chromatin*, *6*, 9.

Husby, A. (2020). On the use of blood samples for measuring DNA methylation in ecological epigenetic studies. *Integrative and Comparative Biology*, *60*(6), 1558–1566.

Ing, N. H. (2005). Steroid hormones regulate gene expression posttranscriptionally by altering the stabilities of messenger RNAs. *Biology of Reproduction*, *72*(6), 1290–1296.

Isaksson, C. (2010). Pollution and its impact on wild animals: A meta-analysis on oxidative stress. *EcoHealth*, *7*(3), 342–350.

Isaksson, C., Andersson, M. N., Nord, A., von Post, M., & Wang, H.-L. (2017). Species-dependent effects of the urban environment on fatty acid composition and oxidative stress in birds. *Frontiers in Ecology and Evolution*, *5*, 44.

Jarvis, E. D., & Nottebohm, F. (1997). Motor-driven gene expression. *Proceedings of the National Academy of Sciences of the United States of America*, *94*, 4097–4102.

Jarvis, E. D., Schwabl, H., Ribeiro, S., & Mello, C. V. (1997). Brain gene regulation by territorial singing behavior in freely ranging songbrds. *Neuroreport*, *8*, 2073–2077.

Jeremias, G., Barbosa, J., Marques, S. M., Asselman, J., Goncalves, F. J. M., & Pereira, J. L. (2018). Synthesizing the role of epigenetics in the response and adaptation of species to climate change in freshwater ecosystems. *Molecular Ecology*, *27*(13), 2790–2806.

Kilvitis, H. J., Hanson, H., Schrey, A. W., & Martin, L. B. (2017). Epigenetic potential as a mechanism of phenotypic plasticity in vertebrate range expansions. *Integrative and Comparative Biology*, *57*(2), 385–395.

Laine, V. N., Gossmann, T. I., Schachtschneider, K. M., Garroway, C. J., Madsen, O., Verhoeven, K. J. F., de Jager, V., Megens, H. J., Warren, W. C., Minx, P., Crooijmans, R. P., Corcoran, P., Great Tit HapMap Consortium, Sheldon, B. C., Slate, J., Zeng, K., van Oers, K., Visser, M. E., & Groenen, M. A. M. (2016). Evolutionary signals of selection on cognition from great tit genome and methylation. *Nature Communications*, *7*, 10474

Langmead, B. (2010). Aligning short sequencing reads with bowtie. *Current Protocols in Bioinformatics*, *32*(1), 11.7.1–11.7.14.

Langmead, B., & Salzberg, S. (2012). Fast gapped-read alignment with Bowtie2. *Nature Methods*, *9*, 357–359.

Laubach, Z. M., Perng, W., Dolinoy, D. C., Faulk, C. D., Holekamp, K. E., & Getty, T. (2018). Epigenetics and the maintenance of developmental plasticity: Extending the signaling theory framework. *Biological Reviews*, *93*, 1323–1338.

Lea, A. J., Altmann, J., Alberts, S. C., & Tung, J. (2016). Resource base influences genome-wide DNA methylation levels in wild baboons (*Papio cynocephalus*). *Molecular Ecology*, *25*, 1681–1696.

Lea, A. J., Tung, J., & Zhou, X. (2015). A flexible, efficient binomial mixed model for identifying differential DNA methylation in bisulfite sequencing data. *PLoS Genetics*, *11*, 1–31.

Ledón-Rettig, C. C., Richards, C. L., & Martin, L. B. (2012). Epigenetics for behavioral ecologists. *Behavioral Ecology*, *24*(2), 311–324.

Lenth, R. V. (2016). Least-squares means: The R package lsmeans. *Journal of Statistical Software*, *69*, 33.

Lindner, M., Verhagen, I., Viitaniemi, H. M., Laine, V. N., Visser, M. E., Husby, A., & van Oers, K. (2021). Temporal changes in DNA methylation and RNA expression in a small song bird: Within- and between-tissue comparisons. *BMC Genomics*, *22*, 36.

Lister, R., Pelizzola, M., Dowen, R. H., Hawkins, R. D., Hon, G., Tonti-Filippini, J., Nery, J. R., Lee, L., Ye, Z., Ngo, Q. M., Edsall, L., Antosiewicz-Bourget, J., Stewart, R., Ruotti, V., Millar, A. H., Thomson, J. A., Ren, B., & Ecker, J. R. (2009). Human DNA methylomes at base resolution show widespread epigenomics differences. *Nature*, *462*, 315–322.

Love, O. P., & Williams, T. D. (2008). The adaptive value of stress-induced phenotypes: Effects of maternally derived corticosterone on sex-biased investment, cost of reproduction, and maternal fitness. *The American Naturalist*, *172*(4), e135–e149.

Lüdecke, D. (2016). SjPlot: Data visualization for statistics in social science. R 1062 package version, 2(0).

Luo, J., Yu, Y., Zhang, H., Tian, F., Chang, S., Cheng, H., & Song, J. (2011). Down-regulation of promoter methylation level of CD4 gene after MDV infection in MD-susceptible chicken line. *BMC Proceedings*, *5*(Suppl 14), S7.

Lynch, M., & Walsh, B. (1998). *Genetics and analysis of quantitative traits*. Sinauer Associates, Inc.

Mäkinen, H., van Oers, K., Eeva, T., & Ruuskanen, S. (2022). The effect of experimental lead pollution on DNA methylation in a wild bird population. *Epigenetics*, *17*(6), 625–641.

Marshall, D. J., & Uller, T. (2007). When is a maternal effect adaptive? *Oikos*, *116*, 1957–1963.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet Journal*, *17*(1). https://doi.org/10.14806/ej.17.1.200

Marzluff, J. M. (2017). A decadal review of urban ornithology and a prospectus for the future. *Ibis*, *159*(1), 1–13.

Maunakea, A. K., Nagarajan, R. P., Bilenky, M., Ballinger, T. J., D'Souza, C., Fouse, S. D., Johnson, B. E., Hong, C., Nielsen, C., Zhao, Y., Turecki, T., Delaney, A., Varhol, R., Thiessen, N., Shchors, K., Heine, V. M., Rowitch, D. H., Xing, X., Fiore, C., … Costello, J. F. (2010). Conserved role of intragenic DNA methylation in regulating alternative promoters. *Nature*, *466*, 253–257.

Mello, C. V. (2002). Mapping vocal communication pathways in birds with inducible gene expression. *Journal of Comparative Physiology A*, *188*, 943–959.

Monaghan, P., & Spencer, K. A. (2014). Stress and life history. *Current Biology*, *24*, R408–R412.

O'Dea, R. E., Noble, D. W. A., Johnson, S. L., Hesselson, D., & Nakagawa, S. (2016). The role of non-genetic inheritance in evolutionary rescue: Epigenetic buffering, heritable bet hedging and epigenetic traps. *Environ Epigenet*, *2*, dvv014.

Ouyang, J. Q., Baldan, D., Munguia, C., & Davies, S. (2019). Genetic inheritance and environment determine endocrine plasticity to urban living. *Proceedings of the Royal Society B Biological Sciences*, *286*(1908), 20191215.

Pedersen, J. S., Valen, E., Vargas Velazquez, A. M., Parker, B. J., Rasmussen, M., Lindgreen, S., Lilje, B., Tobin, D. J., Kelly, T. K., Vang, S., Andersson, R., Jones, P. A., Hoover, C. A., Tikhonov, A., Prokhortchouk, E., Rubin, E. M., Sandelin, A., Gilbert, M. T. P., Krogh, A., … Orlando, L. (2014). Genome-wide nucleosome map and cytosine methylation levels of an ancient human genome. *Genome Research*, *24*, 454–466.

Pigliucci, M. (2005). Evolution of phenotypic plasticity: Where are we going now? *Trends in Ecology & Evolution*, *20*, 481–486.

Quinlan, A. R., & Hall, I. M. (2010). BEDTools: A flexible suite of utilities for comparing genomic features. *Bioinformatics*, *26*(6), 841–842.

R Core Team. (2019). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Raudvere, U., Kolberg, L., Kuzmin, I., Arak, T., Adler, P., Peterson, H., & Vilo, J. (2019). g:Profiler: A web server for functional enrichment analysis and conversions of gene lists. *Nucleic Acids Research*, *47*, W191–W198. https://doi.org/10.1093/nar/gkz369

Reiner, A., Perkel, D. J., Bruce, L., Butler, A. B., Csillag, A., Kuenzel, W., Medina, L., Paxinos, G., Shimizu, T., Wild, M., Ball, G. F., Durand, S., Gunturkun, O., Lee, D. W., Mello, C. V., Powers, A., White, S. A., Hough, G., Kubikova, L., … Jarvis, E. D. (2004). Revised nomenclature for avian telencephalon and some related brainstem nuclei. *Journal of Comparative Neurology*, *473*(3), 377–414.

Rider, C. F., & Carlsten, C. (2019). Air pollution and DNA methylation: Effects of exposure in humans. *Clinical Epigenetics*, *11*, 131.

Riyahi, S., Sánchez-Delgado, M., Calafell, F., Monk, D., & Senar, J. C. (2015). Combined epigenetic and intraspecific variation of the *DRD4* and *SERT* genes influence novelty seeking behavior in great tit *Parus major*. *Epigenetics*, *10*(6), 516–525.

Romero, L. M., & Reed, J. M. (2005). Collecting baseline corticosterone samples in the field: Is under 3 min good enough? *Comparative*

Biochemistry and Physiology Part A: Molecular & Integrative Physiology, *140*, 73–79.
Rubenstein, D. R., Skolnik, H., Berrio, A., Champagne, F. A., Phelps, S., & Solomon, J. (2016). Sex-specific fitness effects of unpredictable early life conditions are associated with DNA methylation in the avian glucocorticoid receptor. *Molecular Ecology*, *25*(8), 1714–1728.
Salmon, P., Stroh, E., Herrera-Duenas, A., von Post, M., & Isaksson, C. (2018). Oxidative stress in birds along a NOx and urbanisation gradient: An interspecific approach. *Science of the Total Environment*, *622-623*, 635–643.
Sasagawa, T., Horii-Hayashi, N., Okuda, A., Hashimoto, T., Azuma, C., & Nishi, M. (2017). Long-term effects of maternal separation coupled with social isolation on reward seeking and changes in dopamine D1 receptor expression in the nucleus accumbens via DNA methylation in mice. *Neuroscience Letters*, *641*, 33–39.
Scanes, C. G. (2015). *Sturkie's avian physiology* (6th ed., Eds. C.G. Scanes, pp. 167–191). Academic Press.
Sepers, B., Erven, J. A. M., Gawehns, F., Laine, V. N., & van Oers, K. (2021). Epigenetics and early life stress: Experimental brood size affects DNA methylation in great tits (*Parus major*). *Frontiers in Ecology and Evolution*, *9*. https://doi.org/10.3389/fevo.2021.609061
Sepers, B., van den Heuvel, K., Lindner, M., Viitaniemi, H., Husby, A., & van Oers, K. (2019). Avian ecological epigenetics: Pitfalls and promises. *Journal of Ornithology*, *160*, 1183–1203.
Sepp, T., McGraw, K. J., Kaasik, A., & Giraudeau, M. (2017). A review of urban impacts on avian life-history evolution: Does city living lead to slower pace of life? *Global Change Biology*, *24*(4), 1452–1469.
Spencer, K. A., & Verhulst, S. (2007). Delayed behavioral effects of postnatal exposure to corticosterone in the zebra finch (*Taeniopygia guttata*). *Hormones and Behavior*, *51*, 273–280.
Sprau, P., Mouchet, A., & Dingemanse, N. J. (2017). Multidimensional environmental predictors of variation in avian forest and city life histories. *Behavioral Ecology*, *28*(1), 59–68.
Sun, B., Shi, Y., Yang, X., Zhao, T., Duan, J., & Sun, Z. (2018). DNA methylation: A critical epigenetic mechanism underlying the detrimental effects of airborne particulate matter. *Ecotoxicology and Environmental Safety*, *161*, 173–183.
Suzuki, M. M., & Bird, A. (2008). DNA methylation landscapes: Provocative insights from epigenomics. *Nature Reviews Genetics*, *9*, 465–476.
Szulkin, M., Munshi-South, J., & Charmantier, A. (2020). *Urban evolutionary biology*. Oxford University Press.
Szyf, M., & Bick, J. (2013). DNA methylation: A mechanism for embedding early life experiences in the genome. *Child Development*, *84*, 49–57.
Taff, C. C., Campagna, L., & Vitousek, M. N. (2019). Genome-wide variation in DNA methylation is associated with stress resilience and plumage brightness in a wild bird. *Molecular Ecology*, *28*(16), 3722–3737.
Vandegehuchte, M. B., & Janssen, C. R. (2011). Epigenetics and its implications for ecotoxicology. *Ecotoxicology*, *20*, 607–624.
Varley, K. E., Gertz, J., Bowling, K. M., Parker, S. L., Reddy, T. E., Pauli-Behn, F., Cross, M. K., Williams, B. A., Stamatoyannopoulos, J. A., Crawford, G. E., Absher, D. M., Wold, B. J., & Myers, R. M. (2013). Dynamic DNA methylation across diverse human cell lines and tissues. *Genome Research*, *23*, 555–567.
Venney, C. J., Wellband, K. W., & Heath, D. D. (2021). Rearing environment affects the genetic architecture and plasticity of DNA methylation in Chinook salmon. *Heredity*, *126*(1), 38–49.
Verhulst, E. C., Mateman, A. C., Zwier, M. V., Caro, S. P., Verhoeven, K. J. F., & Van Oers, K. (2016). Natural variation in animal personality is associated with transgenerationally consistent DNA methylation. *Molecular Ecology*, *25*, 1801–1811.
Vignal, C., Andru, J., & Mathevon, N. (2005). Social context modulates behavioural and brain immediate early gene responses to sound in male songbird. *European Journal of Neuroscience*, *22*(4), 949–955.
Viitaniemi, H. M., Verhagen, I., Visser, M. E., Honkela, A., van Oers, K., & Husby, A. (2019). Seasonal variation in genome-wide DNA methylation patterns and the onset of seasonal timing of reproduction in Great tits. *Genome Biology and Evolution*, *11*(3), 970–983.
Watson, H., Powell, D., Salmón, P., Jacobs, A., & Isaksson, C. (2021). Urbanization is associated with modifications in DNA methylation in a small passerine bird. *Evolutionary Applications*, *14*(1), 85–98.
White, J. H., Heppner, J. J., & Ouyang, J. Q. (2022). Increased lead and glucocorticoid concentrations reduce reproductive success in house sparrows along an urban gradient. *Ecological Applications*, *32*(8), e2688.
Yang, X., Han, H., De Carvalho, D. D., Lay, F. D., Jones, P. A., & Liang, G. (2014). Gene body methylation can alter gene expression and is a therapeutic target in cancer. *Cancer Cell*, *26*(4), 577–590.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1.

Data S1.

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