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Research article

Temperature predictability and introduction history affect the expression of genes regulating DNA methylation in a globally distributed songbird

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Phenotypic plasticity is a major mechanism whereby organisms adjust their traits within-generations to changes in environmental conditions. In the context of range expansions, plasticity is thought to be especially important, as plastic changes in traits can lead to rapid adaptation. One epigenetic process in particular, DNA methylation, enables organisms to adjust gene expression contingent on the environment, which suggests it may play a role in range expansions. At present, we know little about how methylation is regulated in wildlife, especially expression of the enzymes responsible for altering methyl marks on the genome. In this study, we compared expression of three epigenetic regulator genes (DNA methyltransferase 1, DNMT1; DNA methyltransferase 3, DNMT3; and one ten-eleven translocation methylcytosine dioxygenase, TET2) in three tissues (gut, liver, and spleen) of house sparrows *Passer domesticus* from nine countries. Some countries are in the native range of the species (Israel, the Netherlands, Norway, Spain, and Vietnam) whereas others are sites the species has colonized in the last 150 years (i.e. Australia, Canada, New Zealand, and Senegal).

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In this exploratory study, we asked whether non-native birds and/or birds from sites with comparatively unpredictable climates would express different levels of these genes. We found that all three genes were expressed more in sparrows from the native range and from areas with more stable temperatures. Expression of all three genes was also strongly correlated among-locations and within-individuals, but mean expression was quite different among tissues. Many factors (e.g. urbanization of the capture site, sex of the bird) did not significantly affect gene expression, but others surprisingly did (e.g. latitude). Our results suggest that these enzymes could be important in range expansions or geographic distribution generally, but more detailed investigations will be insightful.

Keywords: epigenetic, house sparrow, introduced species, methylation

Introduction

'The epigenome forms the intersection between the genome and the environment' (Ecker et al. 2018)

Most species have narrow geographic ranges, but a few are distributed across much of the globe. Many cosmopolitan species have maintained their broad ranges for long periods, but others have only recently spread into new areas, oftentimes through accidental or intentional human activities (Jeschke and Strayer 2006). In addition to anthropogenic range expansions, some species have altered their distributions as the environment changed around them (Du et al. 2024). Natural habitats became farms, cities, or other human-modified landscapes, and some species thrived in these new locations (Ducatez et al. 2018, Polaina et al. 2021). Whether range expansion is a natural process or an anthropogenic one, there is an enduring question: how, mechanistically, do individuals endure evolutionarily novel conditions and establish new populations? One favored explanation is phenotypic plasticity (Chown and McGeoch 2023, Usui et al. 2023): organisms that best match their behavioral, morphological, and/or physiological traits to prevailing conditions comprise founder populations (Kilvitis et al. 2017).

Whereas the evidence for a role of plasticity in range expansion is strong (Davidson et al. 2011), the molecular processes whereby it is realized are less known. A promising area of study entails the epigenetic processes that alter how genetic variation is expressed (Marin et al. 2020, Mounger et al. 2021). DNA methylation, histone acetylation, small non-coding RNA activity, and other processes alter the accessibility of the genome to transcription factors (Vogt 2021, Husby 2022). More importantly, the interplay among these elements and genetic sequence variation partly underlies heterogeneity in phenotype among organisms (Vogt 2017b, 2021, Bogan and Yi 2024). Many molecular epigenetic processes are also sensitive to current and past environments, such that some organisms adjust their gene expression contingently, releasing adaptive (or non-adaptive) phenotypes in response to particular environmental signals (Sepers et al. 2019, Zhang et al. 2020).

For practical reasons, DNA methylation has to date been the molecular epigenetic mechanism that has garnered the most research attention (Husby 2022, Laine et al. 2023). DNA methylation tends to reduce gene expression by impeding interactions between transcription factors and regulatory regions of the genome, namely promoters (Vogt 2021), but gene bodies, enhancers, and other genomic regions can be methylated, too. Whereas methyl marks also strongly mitigate transposon activity (Vogt 2021), at the organismal level, DNA methylation plays a critical role during cell differentiation, which underlies many forms of phenotypic plasticity. Indeed, methylation is involved in polyphenisms in honeybees (Lyko et al. 2010), thermal plasticity in zebrafish (Loughland et al. 2021), biorhythms in mammals (Stevenson 2018), and differentiation of various leukocytes across vertebrates (Hong and Medzhitov 2023).

In the context of range expansions, DNA methylation and the genetic substrates on which it acts (i.e. CpG motifs, or cytosines followed by guanines in a DNA sequence) seem to determine which and by what means certain individuals come to found new populations (Kilvitis et al. 2017, Chen et al. 2024). Our focal species, the house sparrow *Passer domesticus*, is one of world's most broadly distributed birds (Hanson et al. 2020b). It is also among the strongest examples of the role of DNA methylation in vertebrate range expansions (Sheldon et al. 2018, Hanson et al. 2020a, 2022). In Kenya, for instance, where the species arrived probably via human shipping activity around 1950, we found a strong inverse correlation between genetic and epigenetic variation among populations (Liebl et al. 2013). In that study, we speculated that the pattern arose because DNA methylation rescued some populations from extinction by enabling some individuals to produce adaptive plasticity or mitigate the effects of lethal alleles or inbreeding depression. A subsequent study revealed more direct support for DNA methylation in the Kenyan range expansion: CpG count in the genomes of individual birds (what we termed *epigenetic potential*) declined from the vanguard population (i.e. the border of Uganda where the species probably arrived no earlier than 2015) to the site of initial introduction (i.e. the city of Mombasa; Hanson et al. 2022). The rationale was that fewer CpG sites would mean fewer opportunities for DNA methylation and hence reduced potential for plasticity. Subsequent analysis revealed that high CpG counts were in fact under directional selection (low Tajima's D) at the range edge but not the core of the Kenyan invasion (Hanson et al. 2022).

These findings partly motivate the present study: to compare expression of the enzymes that regulate DNA methylation among populations of house sparrows with different introduction histories. Our interest was to explore whether

sparrows from different parts of the species' range would express different levels of the enzymes important to the maintenance, addition and subtraction of methyl marks on the genome (Vogt 2017a, Hanson and Liebl 2022). DNA methylation in vertebrates is coordinated by two methyltransferases, DNA methyltransferase 1 and 3 (DNMT1 and 3), and one ten-eleven translocation methylcytosine dioxygenase, TET2 (Robertson and Wolffe 2000). DNMT1 is largely responsible for the maintenance of methyl marks set down during development; once established, cell identity becomes lost if methyl marks disappear (Law and Jacobsen 2010). The main role of DNMT1 is thus to keep methyl marks intact, imbuing cells with a sort of memory transfer between cell generations, an indispensable trait for species with high cell turnover rates (Regev et al. 1998). DNMT3, by contrast, is important in de novo methylation. DNMT3 sets down the marks during the blastocyst stage (Wu and Zhang 2010), and whereas it can also methylate the genome later in life, these methyl marks are contingent on environmental exposures (Hanson and Liebl 2022). Finally, TET2 is responsible for inactivating methyl marks, functionally eliminating methylation from a previously methylated region (Wang, L. et al. 2018). Historically, methylation was thought to be a quite stable epigenetic mark, but recent work has revealed that methylation can be quite labile (see also Schrey et al. 2025). In some contexts, it is even reversible (Wu and Zhang 2010, Stevenson 2018), especially during early phases of development (Vogt 2017a, 2021).

Here, we compared DNMT/TET gene expression among house sparrows caught from nine different countries (Table 1), choosing specific countries depending on whether birds there: 1) had independent introduction histories, 2) were definitively native or not, and 3) were sufficiently abundant to enable (statistical) comparisons (Hanson et al. 2020b). We also selected capture sites to determine whether the effects of introduction history were smaller or larger than other factors relevant to plasticity, specifically latitude, altitude, and climatic predictability. All three factors should relate to plasticity, as they represent different forms of the rate of environmental change; phenotypic plasticity cannot be adaptive if environmental change happens too fast or too unpredictably for individuals to adjust. High latitude or altitude environments, for instance, are much more dynamic and unpredictable than near-equatorial or low elevation ones (Hau 2001). However, areas of extreme climatic unpredictabililty can exist at various locations across the globe, with such conditions well-described by Colwell's indices (Colwell 1974). Colwell's indices represent day-to-day predictability of temperature and precipitation relative to the local annual cycles. Recently, Colwell's indices were calculated at a 0.5° spatial resolution across the world using climate data from 1901–2012 (Harris et al. 2014, Jiang et al. 2017). These indices can be interpreted as long-term descriptors of the constancy (i.e. day to day similarity in weather) and contingency (i.e. to what extent yesterday's weather predicts today's weather) of the climate at each capture site. We favored this metric over other proxies of climate predictability for two reasons: first, it was

Table 1. Key characteristics of study sites.

Country	City	Lat	Long	Alt	Collected	Native/non^	Genetic group**	TempP#	PrecP#	% Urban$	Birds studied‡
Spain	Turis	39.22	−0.42	12	Oct-22	Native	1	0.80	**0.36**	4	8M:2F
Netherlands	Numansdorp	51.44	4.26	12	Oct-23	Native	1	**0.61**	**0.56**	6	8M:2F
Norway	Uthaug	63	9.8	38.3	Apr-22	Native	1	**0.62**	**0.58**	3	4M:6F
Canada	Edmonton	53.29	−113.32	668	Dec-23	Non	1	**0.70**	**0.59**	70	6M:4F
Australia	Freshwater Creek	−38.15	144.16	35	Feb-23	Non	1	0.78	**0.54**	1	4M:6F
Israel	Avigdor	31.42	34.44	62	Aug-22	Native	2	0.81	**0.56**	24	1M:4F
New	Zealand Cass	−43.02	171.45	560	Feb-23	Non	1	0.76	0.63	0	6M:4F
Vietnam	Vung Tau	10.22	107.03	7.9	May-22	Native	2	0.98	**0.57**	26	4M:6F
Senegal	M'Baling	14.22	−16.9667	11.6	Oct-21	Non	2	1.00	0.72	19	6M:4F

^population is native or non-native (i.e. recently introduced by humans);**genetic groups based on genome sequence data from Ravinet et al., 2018;#indices taken from Jiang et al. (2017) denote climatic predictability (temperature and precipitation) at a capture site. Bold text denotes 'low predictability' sites;$urbanization is fraction of a 10-km radius circle centered on capture site estimated using Google Earth. Grey-shaded cells denote low urbanization sites;‡M = male and F = female.

quantified in the same way over a long period (i.e. several generations of house sparrows) across the entire globe, and second, we expected climate predictability to favor plasticity, which would then relate to DNMT/TET expression because of the roles of these enzymes in methylation.

As with climate, we expected that human-habitat modification might also influence the expression of DNMT/TET2. Urbanization is evolutionarily novel, so birds dwelling in or near cities should express more DNMTs or TET2 to help them adjust their phenotypes to unnatural conditions. Finally, individual-level factors such as sex, body mass, and genetic ancestry could also affect DNMT/TET expression. In the case of sex, methylation is a major means by which heterogametic genes are differentially silenced or enhanced (Vogt 2017a, 2021). Body mass can often be a proxy of health, and healthier birds might express more of a given gene. In the case of genetic ancestry, similarity in gene expression could come from shared evolutionary history. There appears to be two major lineages of house sparrows from which all extant populations derive (Ravinet et al. 2018). Overall, we predicted that introduction history and climatic predictability would be the strongest predictors of variation in enzyme expression (Coyle et al. 2020, Mishra et al. 2020). We expected that the effects of other factors such as individual sex, body mass, genetic ancestry, urbanization, latitude, altitude, and tissue type would be detectable but comparatively weaker than the above forces. We did not make directional predictions for each gene; despite their distinct effects on methylation, it would be possible to achieve phenotypic plasticity at the organismal, physiological, or system level differently depending on which genes are methylated and over what time scale. Exactly what cocktail of enzymes would maximize phenotypic flexibility is too hard to predict much less expect to capture by measuring expression of these genes in just three tissues at one point in time.

Material and methods

Bird capture, husbandry and tissue collection

We captured adult house sparrows using mist nets from sunrise until ~ 11:00 h at each location (see Table 1 for site details and sex ratios captured at each site). Upon capture, we measured wing chord (to the nearest 1 mm), tarsus length (to the nearest 1 mm), and body mass (to the nearest 0.1 g). We also collected approximately 50 µl of blood from the brachial vein of each bird, which was then stored in 300 µl of DNA/RNA Shield (Zymo R1100-50). Immediately after, each bird was injected subcutaneously with 100 µl of 1 mg ml^{-1} LPS (from *E. coli* 055; Fisher L4005) in sterile saline over the breast muscle (for a different study). Birds were housed individually in wire songbird cages (approx. 35.6 × 40.6 × 44.5 cm) with food and water provided ad libitum, while maintaining visual and vocal contact. Forty-eight hours post-injection, between 07:00 and 10:00 h, birds were euthanized via isoflurane overdose followed by rapid decapitation. We then collected liver, spleen, and gut samples in 500 µl of DNA/RNA Shield, and all samples were stored at −80°C until further analysis. We chose these tissues because the goal of the larger project for which we collected sparrows involves epigenetic regulation of immune gene expression; these tissues are among the most active lymphoid tissues in the body. All of these tissues should express all of our genes of interest, as DNMTs and TET2 are important to cell differentiation and gene expression in most cell types. All animal procedures complied with local ethical guidelines and were approved by the USF IACUC (IS00011653) and relevant authorities in the countries of capture. Export and import of animal tissues followed all relevant U.S. regulations, including USDA-APHIS permission.

RNA extraction and gene expression analyses:

We extracted RNA from liver, gut, and spleen samples of each sparrow using a standard phenol:chloroform protocol (Sambrook and Russell 2006). Reverse transcription was carried out using the iScript cDNA Synthesis kit (Bio-Rad 1708891) according to the manufacturer's instructions. We then quantified the absolute copy numbers of DNMT1, DNMT3, and TET2 using droplet digital PCR (ddPCR). Each ddPCR reaction contained 5 µl ddPCR Multiplex Supermix (12005909, Bio-Rad), 2.25 µl of forward and reverse primers (10 µM), 0.63 µl of probe FAM, 0.63 µl of probe HEX, and 0.63 µl of FAM + HEX probe mixture (for 50% FAM + HEX, 0.31 µl of each), and 1 µl of cDNA sample (3500 ng $µl^{-1}$; see the Supporting information for details). The reactions were run on a C1000 Touch™ Thermal Cycler with a 96–Deep Well Reaction Module (1851197, Bio-Rad). After amplification, droplets were separated and analyzed as positive (containing the target sequence) or negative (without the target sequence) using the QXDx Droplet Reader (12008020, Bio-Rad). Expression data were analyzed using QuantaSoft™ Analysis Pro software (ver. 1.05).

Data analysis

Table 1 presents key characteristics of all capture sites. Most site characteristics (e.g. latitude, longitude, altitude) were obtained from Google Earth based on coordinates determined on-site at the time of capture. Other factors were known a priori (e.g. native versus non-native status) or were obtained from peer-reviewed literature (e.g. Colwell's indices). Yet other factors (e.g. genetic group membership) were determined from other work (Ravinet et al. 2018), and one factor, urbanization at the capture site, was quantified by us using Google Earth. For this factor, we first found capture sites in Google Earth using latitude and longitude data. A screen shot of the site was then taken such that a 10-km radius transect from the capture site was identifiable. ImageJ was then used to quantify the area within this 10-km circle that was urbanized including obvious human-built structures such as buildings, parking lots, or other paved surfaces, which were confirmed by higher-resolution images assessable in Google Earth. We did not include landscape modified for agriculture such as crop fields and orchards in our assessments of urbanization. As in Table 1, urbanization was split

into high and low categories. High urbanization included all sites where the habitat was greater than or equal to 19% modified; low urbanization included all sites where very little urbanization was detectable (< 10% total area).

Table 1 also lists dates of birds' capture from each location and provides sample sizes and sex ratios for all sites. Whereas time of year (i.e. phase of the breeding season) could have affected gene expression, we could not design our study to exclude any seasonal effects on gene expression for two reasons. First, field work had to coincide with the availability of our collaborators at each site; alternative timing of sampling was not possible. Second, breeding phenology in many countries is unknown (e.g. Senegal, Vietnam). We did our best to sample birds outside what we expected to be their breeding season, and except for Israel, rarely did we capture obviously immature individuals.

As gene expression data were non-normal, all were $\log_{10}$ transformed before analyses. Our first directive was to evaluate whether there were interactions between gene and tissue, which would determine whether we should analyze gene expression for each tissue separately. First, we constructed a univariate model using the *lmer* function from the 'lme4' package in R (Bates 2014) with $\log_{10}$ gene expression as a response variable, and gene (factor, 3 levels), tissue (factor, 3 levels), and their interaction as fixed effects. Individual and country were included as random effects to account for non-independence of tissue and gene samples from the same individuals and individuals sampled in the same countries, respectively. We used the variance components estimates from this model to calculate the adjusted repeatability for individual and country following published methods (Nakagawa and Schielzeth 2010). In addition to providing more complete model output (e.g. as opposed to only reporting fixed effects output), estimating among-individual and among-country repeatability in gene expression was necessary to inform whether we could reasonably explore patterns of (co-)variation between gene expression at these two levels. We then used the *sim* function from the 'arm' package (Gelman et al. 2007) to generate a posterior distribution of estimates and report the posterior mode and 95% confidence intervals (CI) for repeatability estimates. These analyses revealed significant gene:tissue interactions, therefore, we analyzed gene expression separately in subsequent models to simplify interpretation.

Next, we determined the set of variables that best-explained among-country variation in gene expression by model selection using the *dredge* function from the 'MuMIn' package in R (Barton and Barton 2015). First, we constructed three separate global models (i.e. one for each gene: DNMT1, DNMT3 and TET2) with gene expression ($\log_{10}$ transformed) as the response variable. All global models included the same explanatory variables: tissue (gut, spleen, or liver), population type (native or non-native), temperature predictability (high or low), precipitation predictability (high or low), genetic group (1 or 2), urbanization, latitude, altitude, sex, and body mass at capture. Individual was fit as a random effect to account for repeated measures (i.e. tissues) within the same individual. We checked for correlations between environmental predictor variables, and all pairwise correlations were $r < |0.57|$, with the exception of the correlation between latitude and temperature predictability, which was $r = -0.76$. Although this exceeds the commonly used threshold of $r = |0.7|$ for collinearity, we followed the recommendation of Dormann et al. (2013) and retained both these predictors in our global model based on the ecological rationale for their inclusion. We did not include country as a random effect since this led to model singularity given that the combination of values for fixed effects was unique to each country. The *dredge* function runs all subsets of the global model. We then determined the best-fit models based on AICc values. When alternative models were within Δ2AICc of the top model, we retained these models and used the *model.ave* function from the 'MuMIn' package and report the average model results here.

Finally, given that our initial models revealed significant among-country and among individual repeatability, we assessed correlations in gene expression across different levels (i.e. within individuals, among individuals, among countries). Expression levels for each gene were treated as separate response variables, and we included tissue as a fixed effect to account for tissue specific differences in gene expression. Individual ID (band) and country were also included as random effects, which allowed us to estimate covariation between response variables at the among-country, among-individual, and within-individual (i.e. residual) levels. Models were constructed using the *MCMCglmm* function in R (Hadfield 2010). Models were run for 106 000 iterations with a burn-in of 6000 and thinning of 100. Thus, 1000 estimates were retained for estimating the posterior distributions. We extracted among-country, among-individual, and within-individual correlations between each pairwise combination of genes following published methods (Houslay and Wilson 2017). Results presented here used a non-informative inverse-Wishart prior, however, we verified that results were robust across different prior specifications, which they were (results not shown). Correlations presented are posterior modes and 95% CI. We interpret estimates that were non-zero and with 95% CIs that did not overlap zero statistically significant, as this corresponds to a $p < 0.05$ for a two-sided test (Cumming and Finch 2005).

Results

First, we evaluated whether there were two-way interactions between gene and tissue and between gene and country to determine whether subsequent analyses should be carried out separately for each gene. There was a significant interaction between gene and tissue ($F_{4,660} = 5.69$, $p < 0.001$). Inclusion of individual ID and country as random effects, when appropriate, also revealed significant repeatability of among-individual ($r = 0.26$, 95% CI = 0.23, 0.28) and among-country ($r = 0.42$, 95% CI = 0.28, 0.59) variation in gene expression.

Next, we were interested in determining which covariates best explained the among-country variation observed. Our model selection results (Table 2) revealed that the same set of factors were consistently retained in top models of predictors of expression for all three genes: tissue, population type, temperature predictability, genetic group, and latitude. Although urbanization was retained in models for both DNMT3 and TET2, urbanization was not a statistically significant predictor of variation in expression for either. Sex, body mass, altitude, and precipitation predictability were excluded from all models. Moreover, the best-fit models were quite effective at explaining variation in expression of all three genes (conditional R^2 range: 0.36–0.48; Table 2).

Finally, given that our first analysis revealed significant among-individual and among-country repeatability, and our model selection revealed that similar sets of factors explained variation in gene expression of all three genes, we evaluated correlations in gene expression among-countries, among-individuals, and within-individuals. We found strong support for correlations in gene expression among-countries and within-individuals, but not among-individuals (Fig. 1). Controlling for tissue type in countries where house sparrows exhibited high average expression of DNMT1, we found that birds also had average expression of DNMT3 (r = 0.15, 95% CI = 0.11, 0.35) and TET2 (r = 0.16, 95% CI = 0.11, 0.36), and similarly high expression of DNMT3 was associated with high expression of TET2 (r = 0.18, 95% CI = 0.11, 0.36). However, when controlling for these among-country correlations, there was no correlation between an individual's average expression of DNMT1 and DNMT3 (r = 0.000, 95% CI = −0.018, 0.059), between DNMT1 and TET2 (r = 0.000, 95% CI = −0.024, 0.031), or between DNMT3 and TET2 (r = 0.00, 95% CI = −0.02, 0.06). There were significant within-individual correlations in gene expression. Higher residual expression of DNMT1 was positively correlated with expression of DNMT3 (r = 0.23, 95% CI = 0.20, 0.26) and TET2 (r = 0.16, 95% CI = 0.13, 0.19), and higher expression of DNMT3 was correlated with higher expression of TET2 (r = 0.20, 95% CI = 0.17, 0.23), controlling for tissue type.

Discussion

To our knowledge, ours is one of the first studies to consider how DNMT/TET2 expression might affect phenotypic plasticity in a free-living vertebrate (Cardoso-Júnior et al. 2018, Sharma et al. 2018), and only one other study (besides our own, see below) to our knowledge considered these genes in the context of range expansions (Fu et al. 2021). Given the strength of the patterns we found, it seems quite likely that these enzymes played some role in the geographic distribution of this species; introduction history, latitude and temperature predictability had strong effects on the expression of all three genes. Many other conspicuous factors were minimally predictive (e.g. sex, precipitation predictability), but for yet other factors, strong variation was also observed, namely tissue differences.

Variation in enzyme gene expression among tissues is not that surprising (Feng et al. 2010, Noguchi et al. 2015, Rasmussen et al. 2015), but as we had no specific expectations about tissue differences in the context of range expansion, we avoid extensive speculation here. One reason tissue differences might be of particularly interest to organismal biologists is that in many taxa, liver, spleen, gut or other organ samples will be unavailable for analysis. This limitation means that researchers must use blood samples to describe methylation and/or the actions of enzymes and hope

Table 2. Conditional averaged coefficients for top models explaining variation in DNMT1, DNMT3, and TET2 expression. Top models were selected using the *get.models* function in the 'MuMIn' package in R to select all models within 2AICc of the top model. Results presented below are the output using *model.avg* function on the top selected models.

	DNMT1			DNMT3			TET2		
	Estimate (SE)	z-value	p-value	Estimate (SE)	z-value	p-value	Estimate (SE)	z-value	p-value
Intercept[1]	−0.49 (0.50)	0.91	0.32	−0.16 (0.54)	0.29	0.77	−1.05 (0.49)	2.11	0.065
Genetic group[2]	0.72 (0.24)	3.02	0.03	0.99 (0.29)	3.43	< 0.01	1.17 (0.29)	3.96	< 0.01
Temperature[3]	0.85 (0.17)	5.02	< 0.01	0.66 (0.20	3.36	< 0.01	0.53 (0.19)	2.74	< 0.01
Tissue[4]									
Liver	−0.72 (0.09)	8.31	< 0.01	−0.26 (0.09)	2.81	< 0.01	−0.38 (0.08)	4.92	< 0.01
Spleen	−0.23 (0.09)	2.68	0.01	0.06 (0.09)	0.71	0.48	0.19 (0.08)	2.46	0.014
Latitude[5]	0.05 (0.01)	5.52	< 0.01	0.05 (0.01)	5.21	< 0.01	0.05 (0.01)	5.42	< 0.01
Pop type[6]	−0.22 (0.09)	2.31	0.02	−0.20 (0.10)	1.99	0.047	−0.16 (0.10)	1.58	0.11
Sex[7]	−0.08 (0.09)	0.88	0.38	NA	NA	NA	0.06 (0.09)	0.64	0.52
Urban[8]	NA	NA	NA	−0.25 (0.18)	1.41	0.16	−0.30 (0.17)	1.78	0.074
Body mass[9]	NA	NA	NA	NA	NA	NA	−0.02 (0.02)	1.14	0.25
Conditional r^2 [10]		0.45			0.35			0.47	

[1]Intercept estimated for gut tissue, genetic group 1, low temperature predictability, native, non-urban populations.[2]Reference category is 'Genetic group 1'. Estimate is difference for 'Genetic group 2'.[3]Reference category is Temperature predictability is 'low'. Estimate here is difference associated with 'high' predictability.[4]Reference category is 'gut tissue'. Estimates are differences in 'liver' and 'spleen'.[5]Latitude is a continuous variable, expressed in absolute degrees.[6]Reference category is 'native'. Estimated effect is difference associated with being 'non-native'.[7]Reference category is 'male'. Estimated effect is the difference associated with being 'female'.[8]Reference category is 'urban'. Estimated effect is the difference associated with being 'non-urban'.[9]Body mass is a continuous variable, expressed in grams.[10]Conditional r^2 calculated from top model.

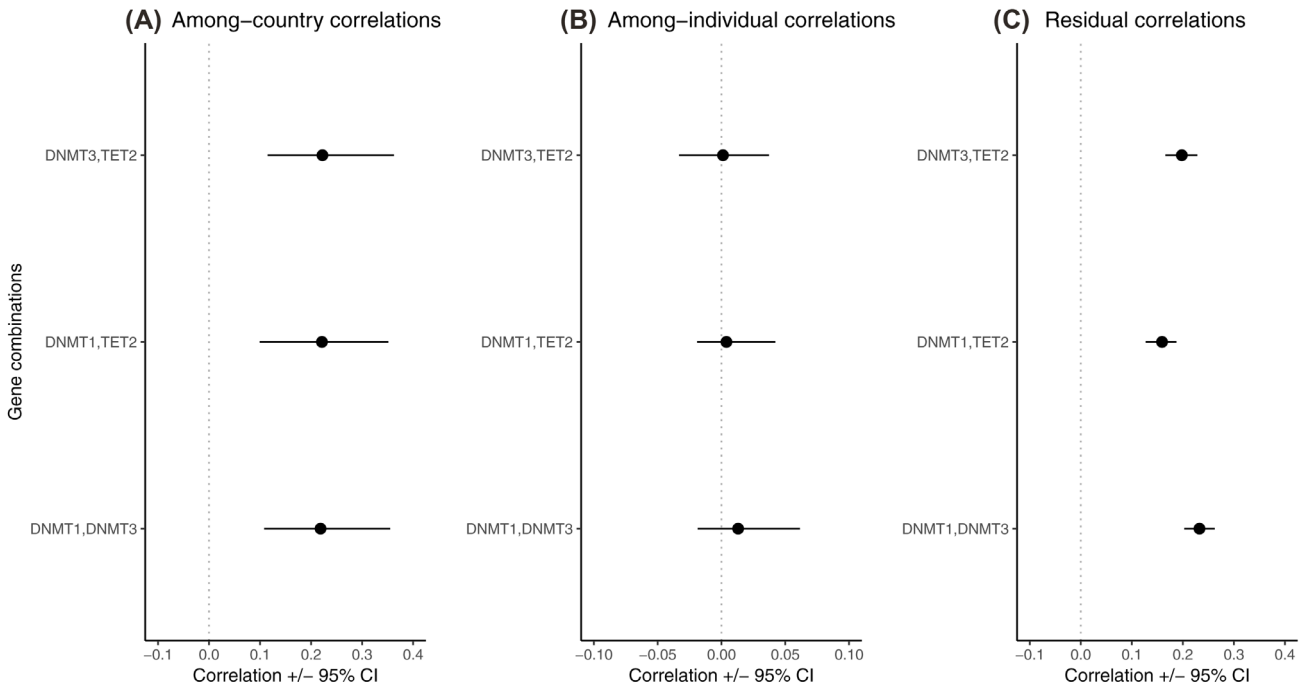

Figure 1. Correlations in gene expression levels (log_{10} transformed) for DNMT1, DNMT3 and TET2: (A) among-countries, (B) among-individuals, and (C) within-individuals (i.e. residual correlations). The dotted vertical line denotes a correlation of zero (i.e. no correlation). Points are estimates derived from the *MCMCglmm* model output, and whiskers denote 95% CI. 95% CI that overlap zero are interpreted as not significantly different from zero.

blood-borne levels are representative of other tissues. In some cases, methylation in the blood will be correlated to methylation in other regions of the body, but in some cases it will not (Siller and Rubenstein 2019). Some such discrepancies could be explained by differences in DNMT and TET activity, but many other factors are plausible, too. Here, we cannot really address this issue, as we did not measure DNMT and TET in blood. Future research should investigate the roles of these enzymes in tissue and cellular heterogeneity in methylation. Moreover, it would be intriguing to investigate *why* tissues express different levels of these enzymes including whether variation changes over development or generally across the lifetime of individuals. All such work would inform how we can use DNMT and TET expression (as well as its product, methylation) in free-living animals.

Differences in expression among countries where birds were captured, however, were strong and consistent with another study we conducted of house sparrows invading Senegal. There, we detected a similar pattern among house sparrows caught from different cities in Senegal (Kilvitis et al. 2018). In that study, we asked whether hippocampal DNMT1 and DNMT3 expression were higher in birds at the vanguard (city of Richard Toll) relative to birds from an intermediately-aged population (Saint Louis) and the site of introduction of the species in ~ 1980 (Dakar). We chose the hippocampus, as methylation in that specific brain region was expected to be an important mechanism whereby neurogenesis was regulated via glucocorticoid hormones (Liebl and Martin 2012, 2014, Martin et al. 2017b). Whereas we detected a main effect of expected population age on DNMT1 expression (and an interesting relationship with glucocorticoid regulation), small sample sizes prevented us from determining whether expression increased or decreased towards the vanguard. The trend, however, was that older (native) populations expressed *more* DNMT1 and DNMT3 than the youngest one.

Why were differences in methylating enzyme expression opposite of our hypothesis?

In the present study, we detected a similar but significant effect of native versus non-native status on the expression of all three genes with more expression being observed in older populations. What are reasonable explanations for this pattern, though? We think there is some insight to gain by comparing the most important *other* drivers of gene expression among the factors we considered to native status (Fig. 2). After tissue identity, temperature predictability, latitude, and genetic group were the next strongest predictors of expression with native/non-native status significant but a fraction as informative. We cannot explain the effect of genetic group here because we had no a priori reason to expect this factor to be so important. This predictor captures the evolutionary history of populations and thus could represent more of a phylogenetic than functional difference (Ravinet et al. 2018).

The very strong effect of temperature predictability, by contrast, is intriguing because it resembles the directionality of the difference we saw for native/non-native status here

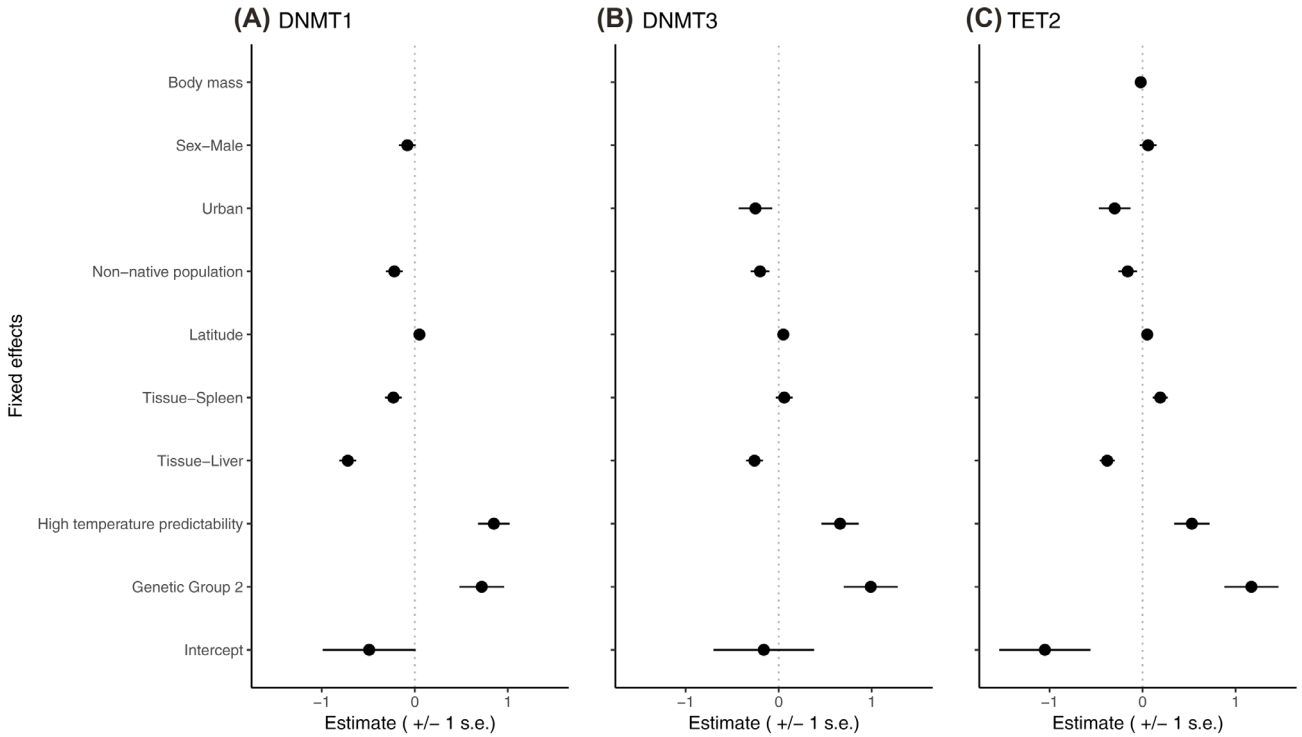

Figure 2. Factors that predict DNMT1 (A), DNMT3 (B) and TET2 (C) gene expression in house sparrows from across the globe. Visual summary of intercepts and fixed-effects estimates from model selection results presented in Table 2.

and in Senegal: birds from sites where plasticity should be more favorable (i.e. the youngest populations) expressed *less* of all three enzymes that regulate methylation. Our initial thought was that if DNMT1/3 and TET2 expression were mediators of reversible plasticity (Wu and Zhang 2010, Bogan and Yi 2024), expression of these genes should be highest where conditions are least predictable so as to turn on or off methylation in the appropriate context (McCaw et al. 2020). This result was not the case, though; the results were reverse of this expectation. However, there are a few caveats to consider. Temperature predictability was strongly negatively correlated with latitude in our dataset, which may result in mis-attribution of effects of latitude to temperature predictability (or vice versa). Our current country set also prevents us from disentangling genetic history and temperature predictability. Despite our ambitious sampling effort, we only collected tissues from nine countries. More importantly, sparrows from Israel, Senegal and Vietnam are also members of genetic group 2, the most climatically predictable sites we studied (Table 1). In other words, the genetic group and latitude effects could be echoes of temperature predictability, but we lack data to test this possibility directly. Fully disentangling temperature predictability from latitude and/or genetic group would require sampling at multiple sites at similar latitudes that vary in both temperature predictability and genetic group. Nonetheless, we suspect that temperature predictability is the driving force for gene expression here.

Another potentially useful way of interpreting our results involves the *kinds* of plasticity that might be fostered by DNA methylation. Our results may reflect the dispositions of birds from different locations to realize canalized, developmental plasticity to predictable cues over development. Our hypotheses focused on plasticity activated and suppressed rapidly and reversibly to match current conditions, as might also be expected as a successful invasion process (Vogt 2017a, 2017b). Developmental plasticity might be more relevant, though. In other words, latitudinal effects might reflect a tendency for DNMT/TET expression to track photoperiod-related seasonality (Stevenson 2018), which increases towards the poles. Seasonality as a predictable form of environmental variation is quite distinct from Colwell's indices, which better captures climatic *unpredictability*. Perhaps DNMT/TET expression is more important for plastic responses to predictable than unpredictable environmental change (Lynch et al. 2016, McCaw et al. 2020).

There could also be an upper limit on the rate at which environmental variation can be transduced into DNMT expression, then methylation, and ultimately organismal plasticity (Snell-Rood et al. 2018). DNA methylation plays a role in the regulation of several seasonal plasticities (Fishman and Tauber 2024) from the neuroendocrine coordination of biorhythms to the recrudescence of reproductive tissues (Sharma et al. 2018). We expected that DNMT expression differences to affect reversible plasticity, too, but

the consistent effects of latitude on expression could suggest that DNMT1/3 and TET2 expression levels might instead be set permanently in early development (i.e. epigenetically programmed). By measuring gene expression in only adult birds, we could be capturing the roles these enzymes played in coordinating what would become rhythmic changes in phenotypes in consistently periodic environments (Friston 2010). Measurements in immature animals could have produced very different patterns.

We likewise measured expression after exposure to LPS exposure. LPS was delivered to birds to induce a transient but systemic inflammatory response (Owen-Ashley et al. 2006, Owen-Ashley and Wingfield 2007, Coon et al. 2011) integral to the project that funded the current study. LPS quite likely induced some amount of DNMT and/or TET activity (Wang, J. et al. 2018), however. Whether any such effects would have differed among populations, especially native and non-native groups, which would have confounded our comparison, is hard to say. On the one hand, we exposed birds to LPS because we expected immune responses of native and non-native populations to differ for reasons having to do with pathogen surveillance (Martin et al. 2014), enemy release (Marzal et al. 2011, Coon and Martin 2014), and the cost-benefit ratio of inflammation to enduring infection (Martin et al. 2010, 2017a). We do expect DNA methylation to have played a role in shaping the immune systems of the different groups, but we do not expect that LPS treatment would have masked those effects. On the other hand, some DNA methylation and demethylation can occur quite rapidly (Luo et al. 2012), but also quite variably among cell types. In human macrophages, for instance, LPS can hypermethylate (via DNMT1) critical negative regulators of inflammation (e.g. SOCS1, suppressor of cytokine signalling-1), which amplifies pro-inflammatory cytokine activity. In other cell types (e.g. bovine endometrial epithelial cells (Wang, J. et al. 2018) and human microglia (Carrillo-Jimenez et al. 2019)), LPS can elevate DNMT1 and TET2 expression, with effects on inflammation being enhancive or reductive depending on time scale and context. Over long periods (i.e. at least several days), LPS can even induce a form of endotoxin (LPS) tolerance in some leukocytes via widespread DNA hypermethylation (Abhimanyu et al. 2024).

The only thing that is clear from these and other results is that the interplay among LPS, DNMTs and TET2, methylation and the regulation of inflammation are very complex. We therefore hope that future studies will exclude LPS and other potent experimental factors that could alter DNMT and TET expression, or better, more deliberately use LPS to reveal changes to DNMTs and TET and hence methylation and ultimately immune gene expression and responses among and within sparrow populations. Indeed, DNMT1/3 and TET2 expression (Lynch et al. 2016) and resultant methylation (Sheldon et al. 2020) can be rapid and quite dynamic in many vertebrates (see also Schrey et al. 2025). Expression of all three enzymes can change over months, weeks, days, and even hours (Alvarado et al. 2015, Stevenson 2017). To our knowledge, no one has yet evaluated how quickly expression of these enzymes can change in adult house sparrows, but there is no reason to believe that this species would be unlike others. DNMTs surely play roles over the timescales and contexts that underpin our interest in plasticity and immunity (Luo et al. 2012). For instance, in domesticated chickens, strain differences in resistance to Marek's disease virus were related to DNMT expression; exposure to a novel and lethal environmental stimulus (i.e. the virus) led different individuals to distinctly respond to and cope with the stimulus via plasticity over a fairly short time scale. If broadly applicable, these results suggest that appreciable within-individual variation in the expression of these DNMT/TET is achievable. Indeed, inter-cell type variation in expression of these genes is well-known, and it is partly for this reason we measured expression in three tissues.

A final surprising result that warrants discussion is the strong within-individual and among-country correlations in gene expression (Fig. 1). We did not expect such strong relationships among genes given their unique functions, yet these results suggest that the persistence, creation and erasure rates of methyl marks across tissues and within individuals are probably similar among birds and across sites. Such strong covariation suggests that these genes might evolve and/or operate as a unit. In several human and domesticated rodent studies, correlations have been found in the amount of measurable methylation in tissue and blood samples. These relationships have enabled researchers to use blood samples as proxies for methylation in hard to measure tissues such as the brain (Siller and Rubenstein 2019). Less attention has been directed to similarity in expression of DNMT/TET across tissues, but this topic nevertheless warrants future attention. As with the methylation correlations above, the existence of similar expression patterns in blood and other tissues of the same individual animal would be very valuable in research contexts (e.g. conservation biology) where destructive sampling is impossible.

Conclusion

Clearly, our study raises as many questions as it answers, yet despite our suboptimal study design, several strong drivers of DNMT and TET2 expression were identified. There are a few next steps that could be particularly enlightening to understand the role of DNMTs and TET2 in this or related systems. First, experiments could be conducted that involve transcriptional repression of DNMT expression or exclusion of DNMTs from nuclei, allowing methyl marks to naturally degrade (Law and Jacobsen 2010). This work could reveal both the role of DNMTs in phenotypic plasticity in various cells of house sparrows but also the success of individual birds in new contexts (Luo et al. 2012). Relatedly, DNMT/TET expression could be studied in embryonic and nestling birds (Wilks et al. 2023, Siller Wilks et al. 2024). Our focus on adults probably missed important among and within population variation, but the study of adults is justified from other work (Sun et al. 2021). It will also be important

to consider alternative, functional roles for DNMTs besides plasticity. In *Daphnia magna* (Agrelius et al. 2023), DNMT3 expression was higher in calorically-restricted individuals, altering the life history trajectories faced by individuals depending on the environments in which they were reared (Nguyen et al. 2021).

A final lens through which to view DNMT/TET expression relates to the concept of epigenetic potential (Kilvitis et al. 2017), genetic variation among individuals in the propensity for their genomes to be methylated. Originally, we proposed genetic polymorphisms in DNMTs as a possible, ecologically relevant form of epigenetic potential, but neither we nor others have yet to search for DNMT genetic variants in invaders. One recent study (Sepers et al. 2023) revealed nine SNPs in DNMT3a, two of which were associated with methylation of two distant CpGs in great tits *Parus major*. One of these CpGs occurred in an exon of the gene, SELPLG, and another intronically in CTNNA3. Whether those SNPs affect expression of their associated genes, the expression of DNMT3 itself, or the physiological functions of these genes was not considered. Nevertheless, in future work, it would be valuable to determine whether different DNMT forms are directionally selected, just as the CpG content of gene promoters was in the Kenyan house sparrow range expansion (Kilvitis et al. 2017). Indeed, it is quite possible that population differentiation has occurred such that even plasticity differences among populations are underpinned by genetic variation.

It is an exciting time for ecological epigenetics, as the technical toolkit it requires is expanding rapidly (Loughland et al. 2021). We are also becoming better able to 'iteratively measure plastic traits' (Dupont et al. 2024) and distinguish plasticity via epigenetic processes as the outcome of 'directional induction or bet-hedging stochasticity' (Vogt 2021). We are only just beginning to appreciate, though, that plasticity is probably important to so many biological processes because it underpins organismal agency (Friston 2010, Kirchhoff et al. 2018, Mitchell 2023, Ball 2023). Strong eco-evolutionary roles of plasticity are well known across biological systems (Wade and Sultan 2023), but as we come to understand how methylating enzymes help sculpt the epigenotype in nature, we could be taking a small but important step to revealing how organisms use a variety of entangled, cognitive plasticities (Watson and Szathmáry 2016) to achieve resilience through antifragility (Taleb 2014).

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Author contributions

Lynn B. Martin: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Funding acquisition (lead); Investigation (lead); Methodology (lead); Project administration (lead); Resources (lead); Supervision (lead); Validation (equal); Visualization (lead); Writing – original draft (lead); Writing – review and editing (lead). **Kailey M. McCain**: Investigation (equal); Methodology (equal); Writing – review and editing (equal). **Elizabeth L. Sheldon**: Investigation (equal); Writing – review and editing (equal). **Cedric Zimmer**: Writing – review and editing (equal). **Melissah Rowe**: Writing – review and editing (equal). **Roi Dor**: Writing – review and editing (equal). **Kevin D. Kohl**: Funding acquisition (supporting); Writing – review and editing (equal). **Jorgen S. Soraker**: Resources (supporting); Writing – review and editing (equal). **Henrik Jensen**: Writing – review and editing (equal). **Kimberley J. Mathot**: Formal analysis (equal); Writing – review and editing (equal). **Vu Tien Thinh**: Investigation (supporting); Resources (supporting); **Phuong Ho**: Investigation (supporting); Resources (supporting). **Blanca Jimeno**: Resources (supporting); Writing – review and editing (equal). **Katherine L. Buchanan**: Resources (supporting); Writing – review and editing (equal). **Massamba Thiam**: Investigation (supporting); Resources (supporting). **James V. Briskie**: Resources (supporting); Writing – review and editing (equal). **Mark Ravinet**: Writing – review and editing (equal); **Aaron W. Schrey**: Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Project administration (equal); Resources (equal); Supervision (equal); Writing – review and editing (equal).

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Data availability statement

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.n8pk0p369 (Martin et al. 2025).

Supporting information

The Supporting information associated with this article is available with the online version.

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