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

Lynn B. Martin[1], Kailey McCain[2], Elizabeth Sheldon[3], Cedric Zimmer [4], Melissah Rowe[5], Roi Dor[6], Kevin D. Kohl[7], Jorgen Soraker[8], Henrik Jensen[8], Kimberley Mathot[9], Vu Tien Thinh[10], Phuong Ho[10], Blanca Jimeno[11], Kate Buchanan[12], Massamba Thiam[13], J. Briskie[14], Mark Ravinet[15], and Aaron Schrey[16]

[1]University of South Florida
[2]University of South Florida Health
[3]Sorbonne Université
[4]Université Sorbonne Paris Nord
[5]Netherlands Institute of Ecology
[6]The Open University of Israel
[7]University of Pittsburgh
[8]Norwegian University of Science and Technology
[9]University of Alberta
[10]vietnam national university of forestry
[11]Pyrenean Institute of Ecology
[12]Deakin University Faculty of Science and Technology
[13]Cheikh Anta Diop University of Dakar
[14]University of Canterbury
[15]University of Nottingham
[16]Georgia Southern University - Armstrong Campus

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Abstract

Phenotypic plasticity is a major mechanism whereby organisms adjust their traits to changes in environmental conditions. In the context of range expansions, plasticity is especially important, as plastic changes in traits can lead to rapid adaptation. For these reasons, there has been growing interest in the role of molecular epigenetic processes in range expansions. 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 some invasions. Nevertheless, we know little about how methylation is regulated in wildlife, especially expression of the enzymes responsible for altering methyl marks on the genome. The most important forms of these enzymes in vertebrates are DNA methyltransferase 1, which largely maintains existing methyl marks, DNA methyltransferase 3, which creates most de novo methyl marks, and TET2, which is a major demethylator of CpG motifs, genomic regions where most methyl marks occur. In this study, we compared expression of these genes in three tissues (i.e., gut, liver, and spleen) of house sparrows (Passer domesticus) from 9 locations. Some sparrow populations derived from the native range (i.e., Israel, Netherlands, Norway, Spain, and Vietnam) whereas others were introduced <150 years ago (i.e., Australia, Canada, New Zealand, Senegal). Our hypothesis was that non-native birds and/or birds from sites with comparatively unpredictable climates

would express more of all three genes. We found, however, that DNMT expression differences, while extensive, were reversed of predictions: all three genes were expressed more in sparrows from the native range and from areas with more predictable temperatures. Surprisingly, gene expression was also strongly correlated among populations and within-individuals. Our results reveal no simple role for these enzymes in range expansions, but the appreciable among and within-population variation in these enzymes warrants more detailed investigations.

Introduction:

Most species have narrow geographic ranges, but a few are distributed across much of the globe. Many such species have maintained these 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). Habitats that were historically natural became farms, cities, suburbs, 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 anthropogenic, there is an enduring question: how, mechanistically, do individuals endure such conditions and establish new populations? One favored explanation is phenotypic plasticity (Usui et al. 2023, Chown and McGeoch 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 expansions 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 RNAs activity, and other processes alter the accessibility of the genome to transcription factors (Vogt 2021, Husby 2022b). More importantly, the interplay among these elements and genetic sequence variation partly underlies heterogeneity in phenotype among organisms (Vogt 2017b, Vogt 2021, Bogan and Yi 2024). Many molecular epigenetic processes are also sensitive to current and past environments, such that some organisms will adjust their gene expression contingently, releasing adaptive (or non-adaptive) plasticity when a particular environmental signal induces it (Zhang et al. 2020, Sepers et al. 2019).

For practical reasons, DNA methylation has to date been the molecular epigenetic mechanism that has garnered the most research attention (Husby 2022a, 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 regions of the genome can be methylated, too. Whereas methyl marks also strongly mitigate transposon activity (Marin et al. 2019, 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 and reproductive phenotypes in mammals (Stevenson 2018), and differentiation and activation of various leukocytes in vertebrates (Hong and Medzhitov 2023) among many other traits.

In the context of range expansions, DNA methylation and the genetic substrates on which it acts (i.e., CpG motifs) seem to determine which and by what means certain individuals come to comprise new populations (Chen et al. 2024, Kilvitis et al. 2017). Our focal species in the present study, 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 (Hanson et al. 2022, Hanson et al. 2020a, Sheldon et al. 2018). In Kenya, for instance, where the species arrived probably via human shipping activity, we found a strong inverse correlation between genetic and epigenetic variation among populations (Liebl et al. 2013). In this study, it was speculated that the pattern arose because DNA methylation rescued some populations from extinction by enabling some individuals to mitigate the effects of lethal alleles or inbreeding depression, which are common in small populations. A subsequent study revealed more direct support for DNA methylation in this range expansion: CpG count in the genomes of individual

birds (what was 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 in ˜1950) (Hanson et al. 2022). The rationale was that fewer CpG sites would mean fewer opportunities for 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 specifically was to test the hypothesis that introduced populations would express more of the enzymes important to the maintenance, addition and subtraction of methyl marks on the genome than native populations (Hanson and Liebl 2022, Vogt 2017a). 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, with marks later being maintained by DNMT1 (Wu and Zhang 2010). DNMT3 can also methylate the genome later in life, but contingent on environmental exposures to a variety of factors (Hanson and Liebl 2022). Finally, TET2 is responsible for inactivating methyl marks, functionally eliminating methylation from a previously methylated region (Wang et al. 2018). Historically, methylation was thought to be a quite stable epigenetic mark, but extensive recent work has revealed methylation can be quite labile (see also Schrey et al., this issue). In some tissues and contexts, it is even reversible (Wu and Zhang 2010, Stevenson 2018), especially during early phases of development (Vogt 2021, Vogt 2017a).

We tested our hypothesis by comparing DNMT/TET gene expression among 9 different populations of house sparrows (Table 1), choosing specific populations depending on whether they: i) had independent introduction histories, ii) are definitively native or not, and iii) are sufficiently large to enable sample collection (Hanson et al. 2020b). We also selected these populations to test 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. High latitude or altitude environments, for instance, are much more dynamic and unpredictable than near-equatorial and low elevation ones (Hau 2001). Extremes of climatic unpredictability exist generally across the globe, and these conditions are well described by Colwell's indexes (Colwell 1974). Colwell's indexes represent day-to-day predictability of temperature and precipitation relative to the local annual cycles; high values represent comparatively predictable conditions and low values represent unpredictable ones. Recently, Colwell's indices have been calculated at a 0.5°spatial resolution across the world using climate data from 1901-2012 (Jiang et al. 2017, Harris et al. 2014). We predicted both DNMTs and TET gene expression would be higher in more dynamic environments. We also expected that human-habitat modification could influence expression of these genes. Urbanization is evolutionarily novel, so birds dwelling in or near cities should express more DNMTs or TET2 to help birds adjust their phenotypes adequately to unnatural conditions. Finally, individual-level factors such as sex 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 2021, Vogt 2017a). In the case of genetic ancestry, similarity in gene expression could come from shared 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, climatic predictability, and tissue identity would be the strongest predictors of variation in enzyme expression (Mishra et al. 2020, Coyle et al. 2020). Tissues are comprised of many cell types, each of which derives from different types and degrees of epigenetic activity that various cells experience during differentiation. We expected that the effects of other factors such as individual sex, genetic ancestry, urbanization, latitude, and altitude

would be detectable but comparatively weaker than the above forces.

Methods:

Bird capture, husbandry and tissue collection:

We captured adult house sparrows using mist nets from sunrise until 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 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. Birds were housed individually in wire songbird cages (approx. 35.6 x 40.6 x 44.5 cm) with food and water provided *ad libitum* , while maintaining visual and vocal contact. Forty-eight hours post-injection, between 0700 and 1000, 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 animal procedures complied with local ethical guidelines, 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 permits.

RNA extraction and gene expression analyses:

We extracted RNA from liver, gut, and spleen tissue samples of each sparrow using a standard phenol:chloroform protocol (Sambrook and Russell, 2012). 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, 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; see Supplemental Table 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 (version 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 predictability indices). Yet other factors (e.g., genetic group membership) were determined from ongoing projects (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 (e.g., obvious human-built structures, which could be confirmed by higher-resolution images assessable in Goole Earth).

Table 1 also lists dates of birds capture from each location. Whereas time of year (i.e., phase of the breeding season) could have affected gene expression, we could not design our study to avoid any such effect 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 of many sparrow populations is unknown (e.g., Senegal, Vietnam). We did our best to sample birds outside what we expected to be the breeding season for each population, 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 shaping gene expression across the populations in our study, 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 samples from the same individuals and individuals sampled in the same countries, respectively. We also calculated the adjusted repeatability for random effects (individual and country) following published methods (Nakagawa and Schielzeth 2010). 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% CI for repeatability estimates. These analyses revealed significant gene:tissue interactions, therefore, we analyzed gene expression separately in subsequent analyses to simplify interpretation.

Next, we determined the set of variables that best-explained among country variation in gene expression for each gene by model selection using the "dredge" function from the MuMIn package in R (Barton and Barton 2015). First, we constructed 3 separate global models (i.e., one for each gene) with gene expression as the response variable. All global models included the same explanatory variables: tissue, 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 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 study population (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% confidence intervals.

Results:

First, we evaluated whether there were 2-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 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 consistent predictors of expression for all three genes: tissue, population type, temperature predictability, genetic group, and latitude; urbanization was a significant predictor for TET2 only. 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 the same set of factors explained variation in gene expression for 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 (see Figure 3). Controlling for tissue type, in countries where house sparrows exhibited high average expression of DNMT1, they also had 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 = 0.00, -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/TET expression could affect plasticity in free-living vertebrates (Sharma et al. 2018, Cardoso-Júnior 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 these enzymes played some role in the geographic distribution of this species, but both introduction history and especially temperature predictability had strong directional effects that ran counter to our predictions. Moreover, many other conspicuous factors were minimally predictive (e.g., sex, precipitation predictability), but for yet other factors, strong variation was also observed, namely heterogeneity in expression among tissues.

Variation in enzyme gene expression is not surprising among tissues. In the vertebrate nervous system, DNMT1 facilitates neuronal stability (Feng et al. 2010), underpins survival of new neurons (Noguchi et al. 2015), and regulates the differentiation of stem and progenitor cells (Fan et al. 2005). In the immune system, though, DNMT1 helps maintain multipotency of hematopoietic stem cells (HSCs) and lymphoid cell differentiation, and DNMT3 is critical for HSCs to maintain a self-renewal capacity (Suarez-Alvarez et al. 2012). DNMT1 also plays a strong role in thymopoiesis (i.e., circulating CD4+ and CD8+ T cell numbers) and T-cell derived immune pathology (Lee et al. 2001). TET2 prevents widespread gene enhancer hypermethylation, which can lead to leukemia (Rasmussen et al. 2015).

Differences in expression among countries where birds were captured, however, are harder to explain, as they largely run counter to our initial hypotheses. On the other hand, they are consistent with another study we conducted of house sparrows invading Senegal. There, we detected a similar pattern of DNMT differences among populations (Kilvitis et al. 2018). In that study, we asked whether hippocampal DNMT1 and 3 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 that specific brain region was expected to be an important mechanism whereby neurogenesis was regulated in coordination with glucocorticoid hormones (Liebl and Martin 2014, Liebl and Martin 2012, Martin et al. 2017). Whereas we detected a main effect of 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.

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

In the present study, we detected a significant effect of native versus non-native population status on the expression of all three genes. As with the trend in Senegal, older (native) populations expressed *more* DNMT1 and DNMT3 as well as TET2 than non-native birds. What are reasonable explanations for this pattern, so strong but reverse of expectations? There might be some insight to gain by comparing the most import-

ant *other* drivers of gene expression among the factors we considered (Fig. 1). After tissue, temperature predictability and genetic group were the strongest predictors of expression with native/non-native status significant but a fraction as informative. We cannot explain the effect of genetic group 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.

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 and in Senegal: birds from sites where plasticity should be more favorable (i.e., the youngest populations) expressed *less* of the enzymes that regulate methylation. Our expectation 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 less predictable (McCaw et al. 2020). Even the genetic group effect could be an echo of temperature predictability: despite our ambitious sampling effort, we only collected tissues from 9 countries. More importantly, sparrows from Israel, Senegal and Vietnam are also members of genetic group 2, the most predictable sites we studied (Table 1). Our current country set prevents us from disentangling genetic history and temperature predictability. Nonetheless, we suspect that temperature predictability is the driving force here. Genetic group 1 includes birds from Spain, one of the more predictable climates, but also birds from the Netherlands and Norway, the least predictable climates, and native/non-native status either relates to introduction history alone or is also partly entangled with temperature predictability.

Another potentially useful way of updating our hypotheses given our results involve *kinds* of plasticity that might be fostered by methylation. Our initial hypothesis was based on environmentally-sensitive, reversible plasticity. Our results may reflect, though, the dispositions of populations to realize canalized, developmental plasticities to predictable cues over development, not plasticity activated and suppressed to match current conditions (Vogt 2017a, Vogt 2017b). In support, there was a consistent effect of latitude on expression of all three genes. 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 indexes, which capture climatic *unpredictability* . Perhaps DNMT/TET expression is more important for plastic responses to predictable than unpredictable environmental change (McCaw et al. 2020, Lynch et al. 2016). There could also be an upper limit on the rate at which environmental variation can be transduced into DNMT expression, then methylation, and ultimately reversible 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 play some role in reversible plasticity, too, but the consistent effects of latitude on expression could suggest that DNMT 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 find this possibility (i.e., the patterns we observed largely being due to the age of the birds we studied) worthy of future study but unsatisfying. First, latitude effects on expression were very small compared to other forces. If seasonality is so important, its effects would probably have been of comparable strength to the other factors. Second, DNMT and TET2 expression (Lynch et al. 2016) and resultant methylation (Sheldon et al. 2020) can in fact be quite dynamic in many vertebrates (see also Schrey et al., this issue). 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. Finally, DNMTs in other species can in fact play roles over the timescales and contexts that underpin our hypotheses (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. One thing to mention as well is that we gave all birds in our study a small dose of lipopolysaccharide (LPS) to induce an immune response that is the subject of other projects. We could not study untreated birds for ethical and practical reasons, but it is possible that birds not treated with LPS could have shown different patterns.

A final surprising result that warrants attention and might even be related to the unexpected outcomes we observed among populations are the strong within-individual and among-population correlations in gene expression (Fig. 2). 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 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 among individuals and populations, which could constrain expression variation consistently, despite the utility of plasticity for unpredictability in some sites.

Conclusion

Clearly, our study raises more questions than it answers. Several drivers of expression were identified, but in a direction opposite of predictions (e.g., tissue, temperature predictability, native/non status, urbanization). Likewise, factors expected to have some explanatory power on expression (e.g., sex of bird, precipitation predictability) had little to none. 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 involving transcriptional repression of DNMT expression or exclusion of DNMTs from nuclei so methyl marks naturally degrade (Law and Jacobsen 2010) 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 ecological contexts (Luo et al. 2012). Genetically-modified animals cannot ethically be released from captivity, but creative approaches to studying sparrow domestication or the colonization of captive spaces could be insightful. Relatedly, DNMT/TET expression could be studied in embryonic and nestling birds (Wilks et al. 2023, Siller Wilks et al. 2024). Our bias to studying adults here probably missed important among and within population differences, 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 of great tits (Sepers et al. 2023) revealed nine SNPs in DNMT3a, two of which were associated with methylation of two distant CpGs. One of these CpGs occurred in an exon of the gene, SELPLG, and another intronically in CTNNA3. Whether these SNPs affect expression of these genes, the expression of DNMT3 itself, or the physiological functions any 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).

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 (Mitchell 2023, Kirchhoff et al. 2018, Friston 2010, 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) and thus endure natural and ongoing anthropogenic change.

Figure legends:

Figure 1: Factors that predict DNMT1, DNMT3 and TET2 gene expression in house sparrows from across the globe. Visual summary of fixed-effects estimates from model selection results presented in Table 2.

Figure 2: Correlations in gene expression levels ($\log_{10}$ transformed) for DNMT1, DNMT3 and TET2: A) across 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.

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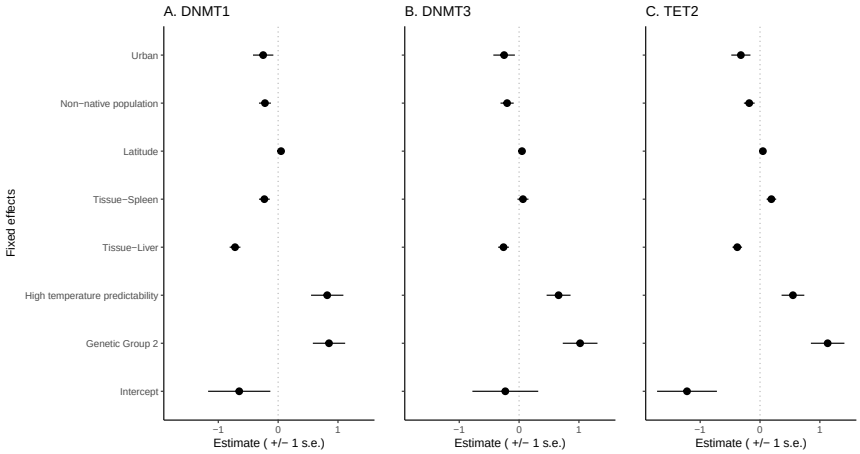

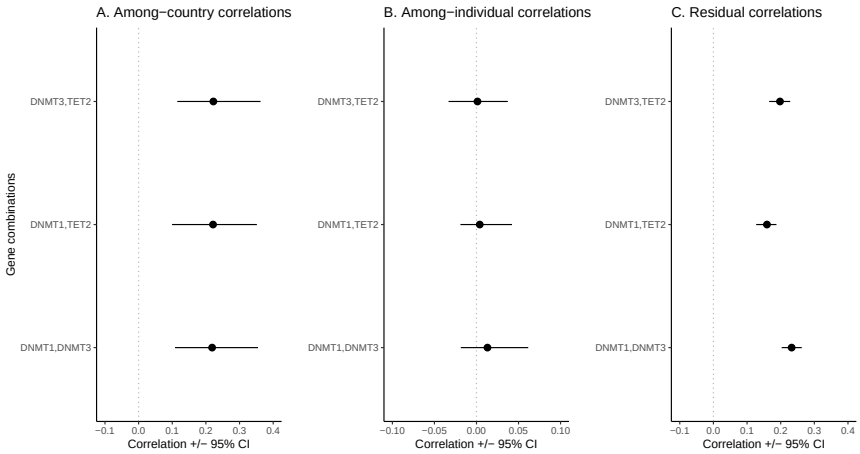

Table 1. Key characteristics of study sites

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

^population is native or non-native (i.e., recently introduced by humans)

**genetic groups based on genome sequence data from Jack's paper

[ǂ]indices taken from Jiang et al., 2017; denote climatic predictability (temperature and precipitation) at a capture site Italicized text denotes 'low predictability' sites

[$]urbanization is fraction of a 10km radius circle centered on capture site estimated using Google Earth. Italicized text denotes low urbanization sites

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Table2. Summary statistics for best-fit models of gene expression predictors.docx available at https://authorea.com/users/563499/articles/1269156-temperature-predictability-and-introduction-history-affect-the-expression-of-genes-regulating-dna-methylation-in-a-globally-distributed-songbird