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Introduced house sparrows (*Passer domesticus*) have greater variation in DNA methylation than native house sparrows

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Abstract

As a highly successful introduced species, house sparrows (*Passer domesticus*) respond rapidly to their new habitats, generating phenotypic patterns across their introduced range that resemble variation in native regions. Epigenetic mechanisms likely facilitate the success of introduced house sparrows by aiding particular individuals to adjust their phenotypes plastically to novel conditions. Our objective here was to investigate patterns of DNA methylation among populations of house sparrows at a broad geographic scale that included different introduction histories: invading, established, and native. We defined the invading category as the locations with introductions less than 70 years ago and the established category as the locations with greater than 70 years since introduction. We screened DNA methylation among individuals ($n = 45$) by epiRADseq, expecting that variation in DNA methylation among individuals from invading populations would be higher when compared with individuals from established and native populations. Invading house sparrows had the highest variance in DNA methylation of all three groups, but established house sparrows also had higher variance than native ones. The highest number of differently methylated regions were detected between invading and native populations of house sparrow. Additionally, DNA methylation was negatively correlated to time-since introduction, which further suggests that DNA methylation had a role in the successful colonization's of house sparrows.

Key words: epigenetic buffering, epigenetic potential, epiRADseq, phenotypic plasticity

Introduction

The house sparrow (*Passer domesticus*) is a human-commensal songbird that has been introduced or expanded its range across all continents, except Antarctica, within the last 200 years (Anderson 2006; Liebl et al. 2015). Each location of introduction has a unique set of environmental conditions and thus presents novel biotic and abiotic challenges, creating a highly heterogeneous landscape to surmount (Carneiro and Lyko 2020), similar to populations of wild boar (*Sus scrofa*; both introduced and native), which differ in their food consumption among plant, animal, and fungi based on location and season (Ballari and Barrios-García 2014). DNA methylation has been associated to ecologically relevant traits in the highly successful introduced house sparrows and may be involved in the production of rapid phenotypic responses to challenges in the new environment (Schrey et al. 2012; Liebl et al. 2013). House sparrows have phenotypic variation among and within introduced populations (Johnston and Selander 1973; Liebl and Martin 2012; Martin et al. 2017), that can also differ from native populations (Lima et al. 2012). However, the rapidly generated latitudinal clines in phenotypes among house sparrows in the introduced range match those observed among native populations. Introduced populations of house sparrows are genetically differentiated from native populations and the most recently introduced house sparrow population was the most different, having lowest genetic diversity and the most private alleles (Schrey

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et al. 2011). These patterns strongly suggest 1) that plasticity plays an important role in the development of these clines and 2) that such plasticity is at least partially adaptive for introduced house sparrows.

Epigenetic mechanisms have been associated with the global spread of introduced house sparrows, yet their functional role remains speculative and requires mechanistic testing. In introduced house sparrows, observed phenotypic variation among populations has happened so quickly that it is unlikely to have arisen by rapid selection on standing genetic variation alone (Schrey et al. 2011; Liebl et al. 2015). DNA methylation is the most well-studied epigenetic mechanism in vertebrates (Schrey et al. 2013), partly because it has been associated with alterations in gene expression (Nätt et al. 2012) and thus might generate behavioral, physiological, and even organismal variation without changes to DNA sequence (Cubas et al. 1999; Husby 2022). DNA methylation of the genome can happen more rapidly than DNA sequence can evolve, and moreover, it can be reversible and environmentally-induced (Jablonka and Lamb 1989, 1998; Hawes et al. 2018). As such, DNA methylation is likely important to introduced species' responses to their novel environments, as demonstrated in invasive ascidians (*Didemnum vexillum*; Hawes et al. 2019), brown anoles (*Anolis sagrei*; Hu et al. 2019), cane toads (*Rhinella marina*; Sarma et al. 2021), pygmy mussels (*Xenostrobus secures*; Ardura et al. 2017), and Japanese knotweed (*Fallopia japonica*; Richards et al. 2012). Further, epigenetic diversity, measured as haplotype diversity (*h*), increased as genetic diversity, measured as observed heterozygosity (H_o), decreased in recently introduced house sparrows from Kenya (Liebl et al. 2013). Similar trends have been in observed clonally reproducing *Chrosomus eon-neogaeus* from heterogeneous habitats (Massicotte et al. 2011) and in an ancestrally bottlenecked population of freshwater sticklebacks (*Gasterosteus aculeatus*; Ord et al 2023), which suggest that greater epigenetic diversity can provide a source of variation to invading groups to facilitate their response to new environmental conditions. In house sparrows, there is a significant negative correlation between DNA methylation at transcription start sites and gene expression (Lundregan et al. 2022). Also, DNA methylation in the putative promoter of *Toll-like receptor 4* is negatively correlated with gene expression (Kilvitis et al. 2019), suggesting that the expression of this immune response gene is at least partially affected by DNA methylation. These findings support the hypothesis that differences in DNA methylation can underlie changes in transcription, which may contribute to the generation of different phenotypes between introduced and native house sparrows.

Geographic patterns of variation in DNA methylation also indicate that it is likely contributing to introduction success of house sparrows. House sparrows in more recently invaded locations have higher diversity in DNA methylation across their genomes compared to individuals in more established locations (Liebl et al. 2013). Further, geographically and temporally separated introductions of house sparrows have different patterns of DNA methylation (Schrey et al. 2012; Sheldon et al. 2018a), and DNA methylation varies within introduced populations (Liebl et al. 2013; Sheldon et al. 2018a). The presence of variation in DNA methylation within and among introduced house sparrow populations suggests that it contributes to their post-introduction response. At the same time, introduced populations are less genetically differentiated than native ones (Schrey et al. 2011; Lima et al. 2012; Sheldon et al. 2018a). The weight of evidence, so far, suggests that plasticity via DNA methylation is associated with phenotypic diversification in house sparrow colonization of most new areas. The current study examines if this idea holds at a broader geographic scale than previously tested.

The latent potential of an individual's genome to have different epigenetic states, defined as epigenetic potential (Kilvitis et al. 2017), could also contribute to the success of introduced house sparrows. Epigenetic potential is variable among species, individuals, and genes (Sheldon et al. 2023), and individuals with more epigenetic potential are expected to have greater capacity to use DNA methylation to fine-tune their gene expression. In house sparrows, epigenetic potential varies among individuals and among genes within individuals and is related to gene expression (Hanson et al. 2020a, 2021). Importantly, epigenetic potential is highest in house sparrows from more recently colonized locations (Hanson et al. 2022), which suggests that in the most challenging locations, house sparrows benefit from high epigenetic potential. House sparrows with high epigenetic potential in the *TLR4* promoter are also more resistant to infection by a novel pathogen (*Salmonella enterica*) than house sparrows with low epigenetic potential (Sheldon et al. 2023), which supports the idea that epigenetic potential is associated with at least one evolutionarily salient form of novelty in the house sparrow. Thus, theoretically, epigenetic potential establishes the conditions for plasticity, changes in DNA methylation states actualize the plasticity, and plasticity facilitates the success of introduced house sparrows.

Our objective was to assess how DNA methylation varied among populations of house sparrows spanning four continents, including six introduced and two native locations. We grouped populations of house sparrow into three introduction categories: invading, established, and native, to account for the known differences in genetic diversity and genetic differentiation among native and introduced populations (Schrey et al. 2011). We separated the introduced house sparrows into invading and established groups to account for the genetic differences detected among individuals in recently introduced areas (lowest genetic diversity, most different genetic differentiation) and those from populations that have existed in the introduced range for an extended period of time (genetic diversity lower than native, but not as low as invading; genetic differentiation from invading and native; Schrey et al. 2011, 2012). We defined the invading category as the locations with introductions less than 70 years ago, to account for the largest genetic differences detected in the most recently introduced populations of house sparrows (Schrey et al. 2011). We defined the established category as the locations with greater than 70 years since introduction, and native as birds from the native range. Previous studies of DNA methylation in house sparrows focused on single locations (Liebl et al. 2013; Kilvitis et al. 2019; Hanson et al. 2022; Siller 2022), comparisons between only two introductions (Schrey et al. 2012), or comparisons among introductions on the same continent (Sheldon et al. 2018a). We expect that introduced populations of house sparrows will have greater variance in DNA methylation because they face the most challenging environments.

Methods

House sparrows screened

We screened DNA methylation in house sparrows ($n = 45$; Table 1, Fig. 1) that were collected from two locations in their native range, France ($n = 4$) and Turkey ($n = 8$), and six locations in their introduced range: British Columbia ($n = 4$), Brazil ($n = 3$), Florida, USA ($n = 4$), Kenya ($n = 14$), Panama ($n = 4$), and South Africa ($n = 4$). We used the documented year of introduction to categorize introduced house sparrows as invading (less than 70 years since introduction) or established (more than 70 years since introduction). The invading locations were Panama 1980 (Hanson et al. 2020b) and Kenya 1950 to 2005. In Kenya, a range of dates is provided for time of arrival, as house sparrows originally were introduced to the port city of Mombasa, then largely spread north-westward towards the border with Uganda: Mombasa 1950, Voi 1960, Nairobi 1990, Nakuru 2000, Garissa 2000, and Kakamega 2005 (Coon and Martin 2014). The established locations were Florida, USA, 1886 (Peña-Peniche et al. 2021), South Africa 1900 (Liebl et al. 2015), Brazil 1905 (Lima et al. 2012), and British Columbia 1915 (Hanson et al. 2020b). We extracted DNA from blood samples stored in 100% ethanol or dried on Whatman paper using the DNeasy Kit (Qiagen, Valencia CA USA).

Table 1. House sparrow sample locations, grouped by introduction category based on year of initial introduction (year) and those from the native range, with sample sizes (*n*).

Location	Year	*n*	Mean methylation (variance)
Invading			0.864 (0.006)
Panama	1980	4	0.862 (0.0003)
Kenya	1950 to 2005	14	0.864 (0.0080)
Established			0.913 (0.002)
British Columbia	1915	4	0.864 (0.0004)
Brazil	1905	3	0.870 (0.000005)
South Africa	1900	4	0.949 (0.000006)
Tampa, FL, USA	1886	4	0.958 (0.00011)
Native			0.963 (0.0004)
France	…	4	0.960 (0.0003)
Turkey	…	8	0.964 (0.0004)

Mean methylation was estimated for each location. Variance in methylation is included for both introduction category and sample locations.

Next-generation sequencing

We used epiRADseq (Schield et al. 2016) to screen variation in DNA methylation among individuals on the Ion Torrent PGM platform (Thermo Fisher Scientific, Waltham, MA). epiRADseq is a ddRADseq protocol, developed for species without well-annotated genomes, that uses a DNA methylation sensitive restriction enzyme, *HpaII*, which fails to cut when its CCGG restriction site is modified by DNA methylation at the internal CG. This generates a variable fragment library among individuals based on the DNA methylation state of the *HpaII* restriction site. If the site is methylated, no fragments are generated to be sequenced. Thus, variation in DNA methylation is assayed as read count variation among individuals, which estimates the differences in DNA methylation of the screened CCGG sites. Only variable sites are meaningful to this analysis, as 100% methylated sites among all individuals will not appear in the library and 0% methylated sites are expected to sequence in ratios predicted by the amount of sequence generated for each individual, thus not have different frequencies. epiRADseq generates data in which a zero read count result for an individual is very meaningful. As such, we did not use cutoffs for differences in DNA methylation.

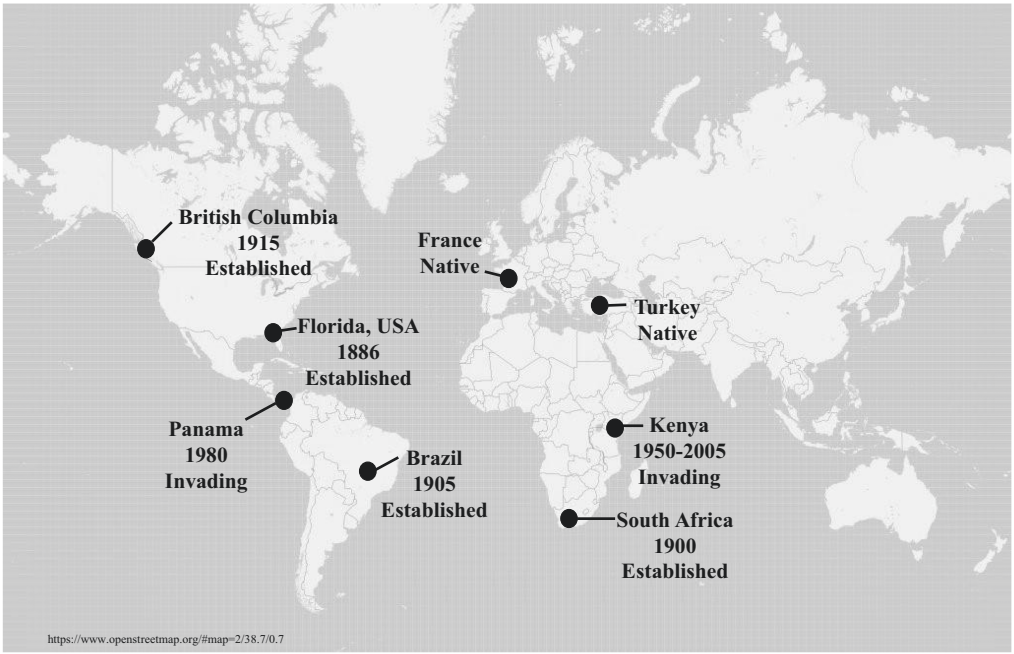

Fig. 1. House sparrow sampling locations, year of initial introduction, and defined introduction category (invading, established, native). Map data from OpenStreetMap (© OpenStreetMap).

The epiRADseq technique is a vast improvement on MS-AFLP (Schrey et al. 2013), yet it maintains many of the benefits and limitations of MS-AFLP. The benefits are: it does not require a reference genome, can be used among vastly different organisms, follows a simplified RNA-seq style analysis approach, and is economical. The limitations are: it screens anonymous CCGG sites, it focuses on sites that are variable among the screened individuals, and it is not comparable to bisulfite sequencing-like approaches. epiRADseq generates data in which zero read count result for an individual is very meaningful, and therefore, we did not use cutoffs for differences in methylation. Additionally, epiRADseq targets CCGG sites with variable DNA methylation among individuals. As such, it should be used to ask questions about variation in DNA methylation among experimental units, rather than specific questions about the functional role of DNA methylation at the molecular level (e.g. studies interested in the effect of DNA methylation on gene expression, or characterizing DNA methylation patterns across genomic elements, etc.).

We followed a genotype-by-sequencing (GBS) protocol developed for the Ion Torrent platform (Mascher et al. 2013), substituting the DNA methylation sensitive restriction enzyme *HpaII* for *MspI* (New England Biolabs, Ipswich, MA) to construct the epiRADseq library. After restriction digestion, we ligated Ion Torrent IonXpress barcoded adaptors and y-adapters. We ran emulsion polymerase chain reactions (PCR) following manufacturers protocols of the Ion PGM-Hi-Q-View OT2-200 kit on the Ion Express OneTouch2 platform. We sequenced resultant fragments following manufacturers protocols of the Ion PGM-Hi-Q-View Sequencing 200 Kit using an Ion 316v2 BC Chips.

Data analysis

We demultiplexed runs and conducted quality control with Torrent Suite version 4.4.3. We trimmed sequences to 150 bp. We performed a de novo assembly and constructed a pseudo-reference using Geneious Prime v. 2022.1.1 (Dotmatics). We mapped individual sequences with BWA Galaxy Version 0.7.17.4 (Li and Durbin 2009, 2010). We used featureCounts Galaxy Version 1.6.4 + galaxy1 (Liao et al. 2014) to determine read counts of fragments within 150 bp bins spanning the pseudo-reference. The 150 bp bins were used to count fragments among individuals ultimately to represent variation in DNA methylation among the CCGG sites screened. For a fragment to be sequenced, it had to have a non-methylated CCGG site. Counting matches to the bins across the pseudo-reference equates to variation in DNA methylation among the CCGG sites. As epiRADseq generates data with the zero read count result indicating DNA methylation, we used two approaches to control for sequencing coverage differences. First, we only analyzed individuals with 5,838 sequencing reads or higher, and second, we only analyzed results from the first 20,000 bins, and removed the other bins, as they had progressively lower coverage.

We used edgeR, Galaxy Version 3.24.1 + galaxy1 (Robinson et al. 2010), to detect differently methylated regions (DMR), which indicate specific regions of the genome that had significantly different levels of DNA methylation among individuals, with a False Discovery Rate (FDR) of 0.05. First, we tested for the presence of DMRs among individuals between all introduced versus native birds. We then tested for DMR with edgeR in an analysis based on introduction categories (invading, established, or native) to further understand how populations of house sparrows respond to introduction with DNA methylation. To characterize the pattern of change in DNA methylation among introduction categories, we defined DMR that were shared in comparisons among multiple categories, and DMR that were unique to a particular comparison.

For every house sparrow, we also calculated an estimate of total methylation, standardized by sequencing depth. We divided the total number of binned reads by the total number of sequences observed for each individual, then subtracted this ratio from one. We compared total methylation estimates among introduced and native birds, and by the different introduction categories (i.e. invading, established, or native). We then compared mean total methylation and variance in total methylation among populations and population categories using *t*-tests and *f*-tests, respectively. For introduced house sparrows, we also tested for a relationship between total methylation scores and the year of introduction among individuals using Pearson's correlations. Statistical tests of total methylation used alpha = 0.05 and were corrected by the sequential Bonferroni method when appropriate (Rice 1989).

Results

Screening DNA methylation using the epiRADseq method on the Ion Torrent PGM in house sparrows ($n = 45$) generated a pseudo-reference of 11,371,350 bases. The observed sequences represented 76 to 71,214 CCGG sites per individual.

Introduction-based DMR

Thousands of differences in DNA methylation were present among all introduction categories, and the greatest DMRs were found between the introduction categories that were the most different from each other (i.e. invading house sparrows and native house sparrows). There were 8,734 DMR between introduced and native house sparrow groups. Further, there were 19,874 DMR among all birds after separating house sparrows into invading, established, and native categories (Fig. 2). The highest number of DMR, 11,538, occurred between invading and native group; 4,226 DMR occurred between invading and established house sparrows; and 4,110 DMR occurred between established and native house sparrows (Fig. 2). There were shared and unique DMR across all combinations of comparisons among introduction categories (Fig. 2). Invading house sparrows had the most DMR and the most unique DMR (Fig. 2). Yet, DMR were shared among all comparisons.

Introduction-based total methylation

DNA methylation was related to introduction category (Fig. 3); introduced populations of house sparrows had a lower mean total methylation than those from the native range (*t*-test $P = 0.00028$; Table 1). In other words, a given CCGG site in introduced birds was less likely to be methylated than a given CCGG site in native birds. Mean total methylation was also lower in invading (mean total methylation = 0.864; Table 1) than established house sparrows (mean total methylation = 0.913; *t*-test invading vs. established $P = 0.020$), lower in invading than native house sparrows (mean total methylation = 0.963; *t*-test invading vs. native $P = 0.0001$),

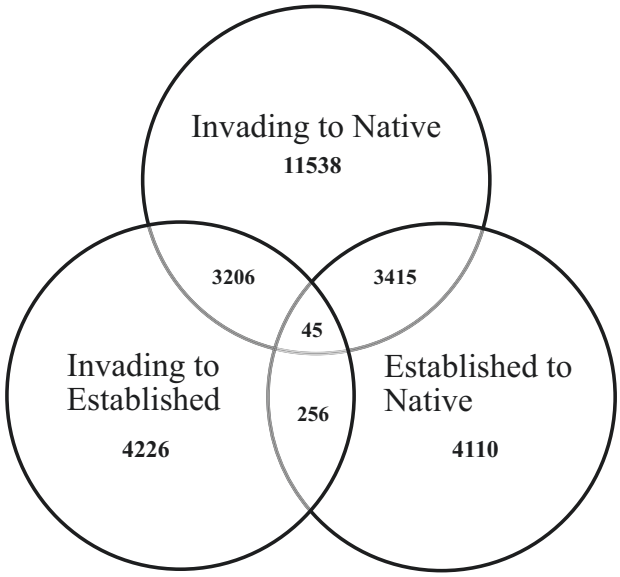

Fig. 2. Venn diagram of the number of differently methylated regions, indicating different CCGG restriction sites, and the pattern of sharing among house sparrows grouped by comparisons among introduction categories. Statistical significance for all differently methylated regions was determined by an FDR of 0.05.

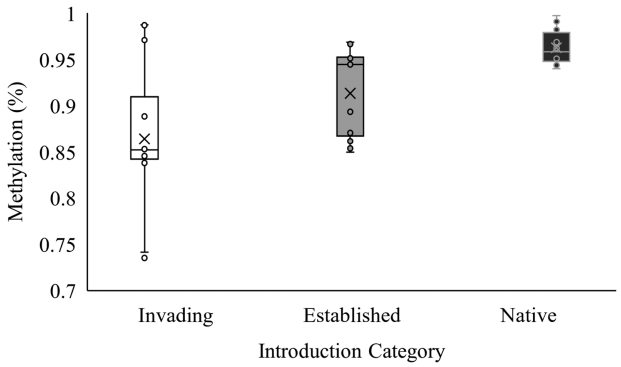

Fig. 3. Variance in DNA methylation is higher in the introduced house sparrows (invading and established) compared to the native house sparrows. Variance among all categories was significant at alpha = 0.05.

and lower in established than native house sparrows (*t*-test $P = 0.001$). Finally, individual-level total methylation estimates were correlated to year of introduction (including natives: $r = -0.505$, $P = 0.0002$, $n = 45$; excluding natives: $r = -0.614$, $P = 0.0001$, $n = 33$).

Variance in total methylation decreased with increasing time-since introduction in the introduced house sparrows, with native house sparrows showing the lowest variance in total methylation (Table 2). Variance in total methylation ranged from 0.0039 to 62.96, with house sparrows from introduced populations having higher variance in total methylation than native house sparrows (*f*-test $P = 0.00007$; Table 1). Invading house sparrows had the highest variance in total methylation (0.006) and native house sparrows had the lowest variance in total methylation (0.0004; Table 1). Invading (*f*-test $P = 0.00003$) and established (*f*-test $P = 0.005$) house sparrows had higher variance in total methylation than native house sparrows.

Discussion

Introduction history explained the patterns of DNA methylation among populations of house sparrows across multiple continents. Invading populations had the lowest mean total methylation, and highest variance in total methylation. Variance in DNA methylation also decreased with time-since introduction in non-native populations. These findings significantly extend those of Schrey et al. (2012), which detected differences in DNA methylation between population of house sparrow from Tampa and Kenya, and those of Sheldon et al. (2018a), which found epigenetic differentiation among populations of house sparrow from separate introductions into Australia. Also, the higher variance in DNA methylation among individuals in more recently introduced locations corresponds with increased epigenetic potential found in more recently introduced populations. Specifically, Liebl et al. (2013) found that epigenetic diversity compensated for decreased genetic diversity among populations of introduced house sparrow in Kenya. Integrating our findings of greater variance in DNA methylation among populations of house sparrows from more recently introduced locations with the findings of higher epigenetic potential in those from more recently introduced locations (Hanson et al. 2021, 2022) suggests that house sparrows with more epigenetic potential manifest greater diversity in epigenetic states, which might reflect the utility of plasticity for adjusting to novel challenges in new areas.

The presence of both shared and unique changes in DNA methylation among introduction categories indicates that DNA methylation in house sparrows responds to introduction in at least three fundamental ways: one being a consistent response to introduction, the second being location specific, and the third being hypervariable. The consistent response across categories suggests that house sparrows have a set of loci that are consistently methylated differently in response to introduction. For example, introduction likely presents a consistent set of challenges that must be overcome in all cases. A loose corollary situation may be seen in the rapid response to domestication in chickens (*Gallus gallus*), which is facilitated by DNA methylation (Nätt et al. 2012). The location specific response (i.e. unique DNA methylations across populations) suggests that house sparrows methylate different loci in response to the challenges associated with introduction into different areas. For example, house sparrows in Kenya manifest different patterns of DNA methylation compared to those from the United States or Australia (Schrey et al. 2012; Liebl et al. 2013; Sheldon et al. 2018a). The hypervariable response (i.e. the middle of the Venn diagram) indicates that some loci vary in DNA methylation state among individuals across all categories. Also, we note that it is likely that DNA methylation responds to habitat variation at a finer scale than was captured by our sampling design. For example, these loci could respond to specific stimuli such as differences in temperature (Sheldon et al. 2020), nest habitat conditions (vonHoldt et al. 2023), brood size (Baker 1995; Sheldon et al. 2018b), and reproductive behavior (Liebl et al. 2021).

The pattern of more recently introduced populations of house sparrows having the highest variance in DNA methylation may be explained by epigenetic buffering. Epigenetic buffering is a phenomenon whereby a group of individuals endures challenging conditions and avoids extirpation because individual organisms somehow manifest high epigenetic

Table 2. Comparisons of mean and variance in total methylation among house sparrows.

	France	Turkey	Kenya	South Africa	British Columbia	Florida, USA	Panama	Brazil
France	…	0.8552	0.0211	0.0257	0.8418	0.4293	0.9934	0.0304
Turkey	0.3664	…	**0.0007**	0.0145	0.9570	0.2978	0.8627	0.0219
Kenya	0.0.0271	**0.0030**	…	**0.0002**	0.0303	**0.0048**	0.0214	**0.0011**
South Africa	0.1369	0.0947	0.04140	…	0.0179	0.1037	0.0253	0.5758
British Columbia	**0.0002**	**<0.0001**	0.4993	**<0.0001**	…	0.3285	0.8482	0.0237
Florida, USA	0.4577	0.3200	0.0281	0.0675	**<0.0001**	…	0.4248	0.0815
Panama	**0.0001**	0.0175	0.4798	**<0.0001**	0.4300	**<0.0001**	…	0.0301
Brazil	**0.0002**	**<0.0001**	0.4619	**<0.0001**	0.3426	**<0.0001**	0.2526	…

The *P*-values for the *t*-tests are presented below the diagonal and those for the *f*-tests are presented above the diagonal. Values in bold indicate statistical significance after sequential Bonferroni correction.

variation (O'Dea et al. 2016; Hawes et al. 2018). For example, great tits (*Parus major*) in urban environments had more variation in DNA methylation compared to those in rural environments (Watson et al. 2021). Also, invasive mussels (*Xenostrobus securis*) altered DNA methylation in response to higher stress environments (Ardura et al. 2018). If epigenetic buffering is important, variance in DNA methylation should be higher in more resource heterogeneous, or stressful, locations (O'Dea et al. 2016). The reduction in variance of total DNA methylation for the established house sparrows may indicate directional selection occurs on this state (Gould 1988), which would suggest that the established populations have existed in their introduced locations long enough for selection to specialize individuals to their new location and stabilize DNA methylation on the states conferring higher fitness. Interestingly, higher epigenetic potential among individuals from recently introduced areas (Hanson et al. 2020a, 2022) would facilitate epigenetic buffering at the population level. Here, epigenetic potential provides the necessary conditions for epigenetic regulation of phenotypic plasticity and phenotypic accommodation (Baldwin 1896; Ghalambor et al. 2007; Foster et al. 2015). DNA methylation would actualize epigenetic potential into a particular and presumably adaptive plastic response. Thus, having more CpG sites increases the potential for the gene of an individual to be regulated variably by DNA methylation; when a given gene is methylated epigenetic potential becomes actualized into a particular phenotypic state. Ultimately, at the population level, higher epigenetic potential in individual organisms would generate more phenotypic variation among organisms, buffering the population against highly heterogeneous environments expected of new areas.

In conclusion, we found more variance in DNA methylation in the most recently introduced populations of house sparrows, possibly as evidence of epigenetic buffering via selection on epigenetic potential. These patterns may suggest that introduced species are using DNA methylation to fine-tune gene expression in their new environments. Here, we used anonymous markers to characterize the pattern of DNA methylation in house sparrows across the broadest scale studied to date. We note that future studies would benefit from identifying the DNA methylation patterns and epigenetic potential in specific genes. We propose that epigenetic buffering extends beyond DNA methylation to include the other epigenetic mechanisms, in particular histone modifications. We also suggest that DNA methylation-based epigenetic buffering is active in other introduced species and in other cases of responses of species to heterogeneous environments.

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Data Availability

We have deposited the raw data underlying these analyses to the SRA database (BioProject accession PRJNA1020895).

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