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Research article

Simulated bacterial infection induces different changes in DNA methylation between introduced and native house sparrows *Passer domesticus*

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DNA methylation, which can change within-individuals over time and regulate gene expression, is important to many aspects of avian biology. It is particularly important in avian responses to various stressors associated with introductions, such as infection

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and environmental changes. However, it remains unclear whether native and introduced bird populations differ in their epigenetic responses to stressors, and how DNA methylation may contribute to the success of non-native populations because of the limited availability of epigenetic studies. To address this knowledge gap, we used epiRADseq to investigate changes in DNA methylation within-individual house sparrows *Passer domesticus* prior to and eight hours after a simulated bacterial infection. We compare wild-caught house sparrows from introduced populations with those from native populations, assessing the number of genomic locations that exhibit changes in methylation, the magnitude of those changes, and the variance among individuals. Our results show that individuals from introduced populations experience more widespread changes in DNA methylation, with greater magnitude and higher variance, compared to their counterparts from native populations. These findings suggest that DNA methylation plays a significant role in an individual's response to infection. They also indicate that individuals from introduced populations may exhibit distinct epigenetic responses compared to their native counterparts, consistent with the concept of epigenetic buffering.

Keywords: epigenetics, phenotypic plasticity, response to stress

Introduction

There is a growing body of evidence that epigenetics is important to many aspects of avian biology. There are three molecular epigenetic mechanisms: DNA methylation, histone modification, and chromatin structure. All are critical for the function and survival of multicellular species, and therefore, all are important to birds. Of the three, DNA methylation is the most well-studied to date (Schrey et al. 2013). The epigenetic basis of DNA methylation is that methyl groups are added to cytosines generating 5-methylcytosine, commonly in a DNA sequence motif of C followed by G (referred to as a CpG site), which is a locally repressive modification (Kilvitis et al. 2014).

In birds, DNA methylation can regulate gene expression (Kilvitis et al. 2019) and vary both among tissues (Siller and Rubenstein 2019) and developmental stages (Sun et al. 2021). DNA methylation is important for an individual bird's response to stress (Taff et al. 2019, 2024, Siller Wilks et al. 2024) and changes in the environment (Sheldon et al. 2018a, Chen et al. 2024, McNew et al. 2024). For example, DNA methylation differs among post-hatch birds reared at different temperatures (Sheldon et al. 2020), but not in response to all stressors occurring during development (reviewed by Sepers et al. 2023). DNA methylation in birds also varies in response to infection (Lundregan et al. 2022), arsenic contamination (Laine et al. 2021), lead pollution (Mäkinen et al. 2022), and urbanization (Watson et al. 2021). Further, DNA methylation differs among chicks reared in different brood sizes (Sheldon et al. 2018b), among postnatal environments (Sepers et al. 2024), and with early-life conditions (Rubenstein et al. 2016).

A defining characteristic of DNA methylation is that it can change rapidly and dynamically over time and be correlated to changes in RNA expression (Lindner et al. 2021b). This temporal change can be driven by reproductive behavior (Liebl et al. 2021), the initiation of reproduction (Lindner et al. 2021a), seasonal factors (Viitaniemi et al. 2019), and the myriad environmental changes faced by organisms introduced to areas outside their native ranges (Lauer et al. 2024). Introduced species provide a unique opportunity to test if rapid changes in DNA methylation occur in populations with different histories. One suggestion is that birds in introduced populations are successful colonizers because they can use methylation to adjust gene expression rapidly in response to changes in the environment (Kilvitis et al. 2017, Chen et al. 2024). Indeed, phenotypic plasticity is one of the best predictors of the ability of a lineage to thrive outside its native range (Yeh and Price 2004, Price et al. 2008, Lande 2016).

The house sparrow *Passer domesticus* is one of the world's most successful introduced species (Liebl et al. 2015). This success is likely the result of its ability to rapidly respond to new environments (Anderson 2008, Lima et al. 2012, Martin et al. 2014), including through DNA methylation (Schrey et al. 2011, 2012, Liebl et al. 2013, Sheldon et al. 2018a, Kilvitis et al. 2019, Hanson et al. 2022, Lauer et al. 2024). DNA methylation is important to the success of introduced populations. DNA methylation varies among house sparrows from different introduced locations (Schrey et al. 2011, 2012, Sheldon et al. 2018a), and it may compensate for decreases in genetic diversity associated with introduction (Liebl et al. 2013). In house sparrows, DNA methylation varies with time since introduction among putative toll-like receptor (TLR) promoters (Hanson et al. 2022), and DNA methylation of a specific CpG site in the putative TLR-4 promoter is associated with the expression of this gene (Kilvitis et al. 2019). Further, genome-wide DNA methylation is more variable among individuals from introduced locations compared to native locations (Lauer et al. 2024), a pattern that is consistent with epigenetic buffering (O'dea et al. 2016), a mechanism in which individuals responding to a stressor leverage rapid epigenic-based modifications to facilitate resiliency and suppress transposons (Deniz et al. 2019).

Our objectives were to investigate the change in DNA methylation within individuals in response to a simulated bacterial infection. We compared patterns of DNA methylation among wild-caught individuals from both the introduced and native range of house sparrows before and after exposure to a highly immunostimulatory element of *E. coli* (i.e. lipopolysaccharide, LPS). We characterized the number of CpG sites with significant change in DNA methylation before and after LPS exposure, the direction of the change, and the magnitude and variance of the change. We hypothesized that individuals from introduced populations would

change DNA methylation at more CpG sites, with greater magnitude, and greater variance, indicative of an 'introduced-bird' phenotype of higher reliance on epigenetic mechanisms and supporting epigenetic buffering.

Material and methods

Sample collection and simulated bacterial infection

House sparrows were collected from four locations in their native range: Israel (n = 6), Norway (n = 6), Spain (n = 6), and Vietnam (n = 6), and three locations in their introduced range: Australia (n = 3), Canada (n = 6), Senegal (n = 6) between October 2021 and February 2023 (Table 1). Individuals were identified as male or female, and we classified each introduced site by the date of first introduction of sparrows to the country: Australia 1860s (Sheldon et al. 2018a), Canada early 1900s (Grinnell 1919, Anderson 2008), and Senegal 1970s (Hanson et al. 2020a; Table 1). We captured adult house sparrows via mist netting from sunrise to 11:00 during the non-breeding seasons of 2020–2023. Upon capture, we took a 50 µl blood sample from the brachial vein of each bird and stored it in a cryovial with 300 µl of DNA/RNA Shield (Zymo). Immediately after this, we injected each bird with 100 µl of 1 mg ml^{-1} LPS (from *E. coli* 055:B5; Fisher L4005) in sterile saline subcutaneously over the breast muscle (following McCain et al. 2025). Post-injection, we housed birds individually in wire songbird cages (35.6 × 40.6 × 44.5 cm) with food and water ad libitum. Although individually housed, the birds could hear and see one another. Eight hours post-injection, we took an additional 10 µl of blood from the brachial vein. The LPS dosage for simulated infection and the eight-hour-post-injection window were confirmed to affect immune surveillance gene expression in the house sparrow (McCain et al. 2025) and have been used in many previous studies of the inflammatory response of this species (Coon et al. 2011, Martin et al. 2011, 2017). All animal research procedures adhered to local animal research guidelines and were approved in advance by both the USF-IACUC (IS00011653) and the relevant authorities in the country of origin. We extracted DNA samples using the DNeasy Kit (Qiagen). Thus, we had paired 0- and 8-h samples for each individual to screen changes in DNA methylation.

Data collection

We used epiRADseq (Schield et al. 2016) to screen variation in DNA methylation among house sparrows on the Ion Torrent PGM platform (Thermo Fisher Scientific). epiRADseq is a ddRADseq protocol, developed for species without well-annotated genomes. This method 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. The enzyme thus 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. epiRADseq generates data in which zero read count result for an individual is meaningful, and therefore, we did not use cutoffs for differences in methylation.

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) 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 manufacturer protocols of the Ion PGM-Hi-Q-View OT2-200 kit on the Ion Express OneTouch2 platform. We sequenced resultant fragments following manufacturer protocols of the Ion PGM-Hi-Q-View Sequencing 200 Kit using an Ion 316v2 BC Chips.

The epiRADseq technique is a vast improvement on MS-AFLP (Schrey et al. 2013), yet it maintains many of the same limitations (i.e. anonymous CCGG sites, analysis focused on variable sites among individuals) and benefits (i.e. not requiring a reference genome, using standard RNA-seq analysis methods, and being economical) of MS-AFLP. We believe that epiRADseq is best used to ask questions about variation in DNA methylation among experimental units, rather than to address specific questions about the functional role of DNA methylation at the molecular level. Importantly, epiRADseq is not comparable to bisulfite- or enzymatic-methyl sequencing-like approaches. As such, we have intentionally maintained a separation of our analysis to

Table 1. Summary of house sparrow samples screened for change in DNA methylation eight hours after lipopolysaccharide injection. The country of origin, date of introduction, date of collection, number of 0- and 8-h pairs screened, with the mean change in DNA methylation and the variance in change of DNA methylation.

Country	Date of introduction	Date collected	No. of pairs	Mean change	Variance change
Introduced					
Australia	1860s	02/2023	3	0.059	0.002
Canada	1900s	12/2023	6	−1.511	13.179
Senegal	1970s	10/2021	6	12.994	388.840
Native					
Israel		08/2022	6	0.055	0.013
Norway		04/2022	6	−0.455	1.783
Spain		10/2022	6	0.006	0.023
Vietnam		05/2022	6	−0.012	0.017

that typically expected of these techniques, in order to avoid confusion or overinterpretation of our results.

Data analysis

We demultiplexed runs and conducted quality control with Torrent Suite ver. 4.4.3. We retained bases above the AQ20 confidence threshold. We trimmed sequences to 100 bp targeting the higher quality sequence at the 5′ end. We performed a de novo assembly and constructed a pseudo-reference using Geneious Prime ver. 2022.1.1 (Dotmatics). We mapped individual sequences with BWA Galaxy ver. 0.7.17.4 (Li and Durbin 2009, 2010). We used featureCounts Galaxy ver. 1.6.4+galaxy1 (Liao et al. 2014) to determine read counts of fragments within 100 bp bins spanning the pseudo-reference. The 100 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 5000 sequencing reads or higher. Second, we standardized all statistics by sequence read count at the individual sample level.

We used edgeR, Galaxy ver. 3.24.1+galaxy1 (Robinson et al. 2010), to detect differently methylated regions (DMR), between the 0- and 8-h samples, with a false discovery rate (FDR) of 0.05. We first compared all samples between 0- and 8-h; we then repeated the comparison separately for individuals from native and introduced populations to determine if there were genomic regions that differ only within these categories. We determined the number of DMRs in each comparison and identified DMR that were shared or unique to a particular comparison.

We assessed if the change in DNA methylation was best characterized by sex, native or introduced status, or country of capture. For every house sparrow, we calculated the change in DNA methylation between the 0- and 8-h sample for all bins with significant differences as identified by the edgeR analyses. We standardized each count for each bin by sequencing depth as (observed count for bin x / total read count) × 1000. We constructed a generalized linear mixed model with the *glmer* function in the package 'lme4' in RStudio (Bates et al. 2015, www.r-project.org) using a Gamma error distribution with a logit link function. We set the absolute value of change in DNA methylation as the dependent variable, used fixed effects of sex, and status (native or introduced), and used country of capture as a random effect. We also performed an ANOVA for the change in DNA methylation in individuals among countries of collection using the *aov* function in R and ran a Tukey HSD as post hoc analysis with the function *TukeyHSD* in the 'car' package (Fox et al. 2012). Statistical tests 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 generated a pseudo-reference of 17 532 684 bases. At the individual-level, between 5095 and 89 777 CCGG sites were resolved. We constructed a dataset of all individuals at 0- and 8-h, and a dataset of the magnitude of change between temporally paired individual samples at 0- and 8-h.

House sparrows from introduced populations had more significant differences in DNA methylation between 0- and 8-h samples, with a stronger magnitude of change, compared to house sparrows from native populations (Table 1, Fig. 1). We detected nine differently methylated CpG sites among all samples with a magnitude of change ranging between −2.01 and 4.37 (Fig. 2). In this comparison, the individuals from introduced populations had a magnitude of change ranging between −5.24 and 11.41, and the individuals from native populations had a magnitude of change ranging between −0.63 and 0.08 (Fig. 2).

We detected a qualitatively similar, but quantitively stronger, pattern when we analyzed individuals from introduced and native populations separately. We detected 35 differently methylated CpG sites among only individuals from introduced populations with a magnitude of change ranging between −9.31 and 11.41 (Fig. 1). We detected only one differently methylated CpG site among only individuals from native populations with a magnitude of change of −0.38 (Fig. 1). We were not able to directly compare the numerical results between native and introduced, due to the separate analyses. However, we detected more significant CpG sites in the individuals from introduced populations and found higher magnitude and variance in change among the individuals from introduced populations.

Further, house sparrows from introduced populations had more uniquely differently methylated CpG sites. Among all significant tests, there were four differently methylated CpG sites shared between the 'all individuals' and the 'introduced' analysis; and there were five unique differently methylated CpG sites for 'all individuals' analysis, 31 unique differently methylated CpG sites for the 'introduced' analysis, and only one unique differently methylated CpG site for the 'native' analysis.

In a GLMM, we detected that the change in DNA methylation was best characterized by native or introduced status, and that one country of capture contributed most strongly to differences among locations, Senegal. Treating country of capture as a random effect did not contribute to explaining variance in the change in DNA methylation among individuals leaving a residual variance of 2.093. Native house sparrows had less change of DNA methylation compared to introduced individuals (−2.84, SE = 0.468, t-value = −6.08, $p < 0.0001$; Table 2). We failed to detect significant relationships between sex and change in DNA methylation. Change in DNA methylation also differed among countries ($p = 0.0291$; Table 3). Tukey HSD results

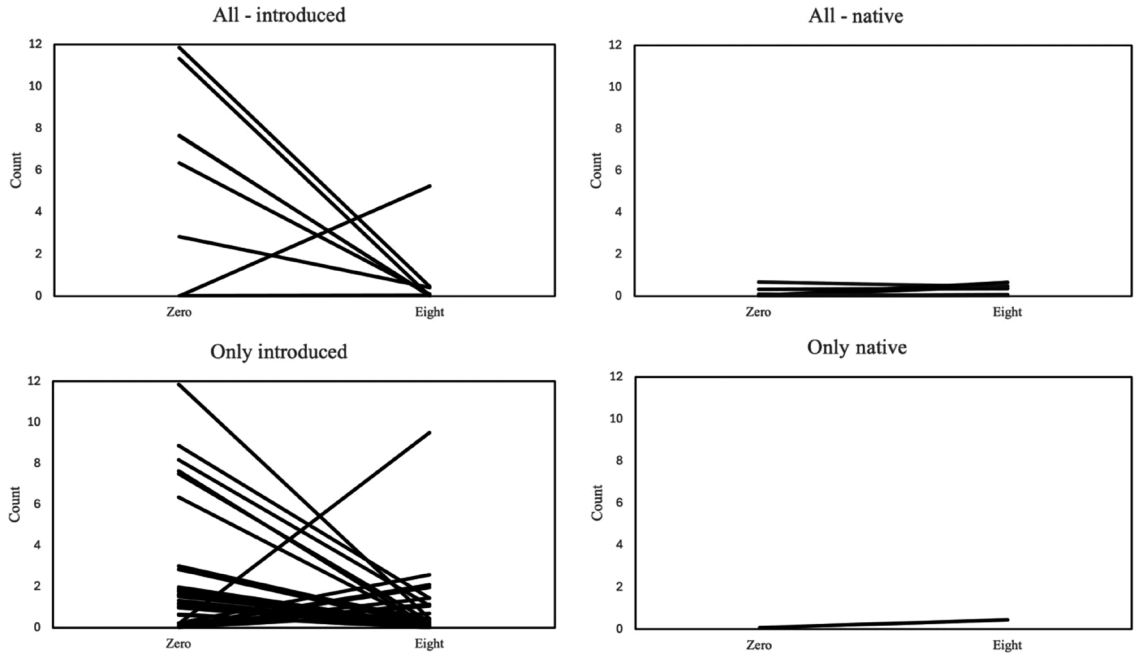

Figure 1. Introduced house sparrows had more sites with significant change in DNA methylation and a larger magnitude of change in DNA methylation between 0- and 8-h after lipopolysaccharide injection. Change in DNA methylation estimated via standardized change in count data of epiRADseq data for house sparrows. Results from three separate analysis between 0- and 8-h samples are presented: 1) all individuals presented for introduced and native samples, 2) analysis for only introduced individuals, and 3) analysis for only native individuals.

detected that significant differences were driven by individuals from Senegal, which had higher mean change in DNA methylation compared to individuals from Canada (difference = 7.193, p = 0.018). No other pairwise geographic comparisons were significant.

Discussion

House sparrows from the introduced range had a stronger and more variable epigenetic response to simulated bacterial infection within 8-h compared to individuals from the

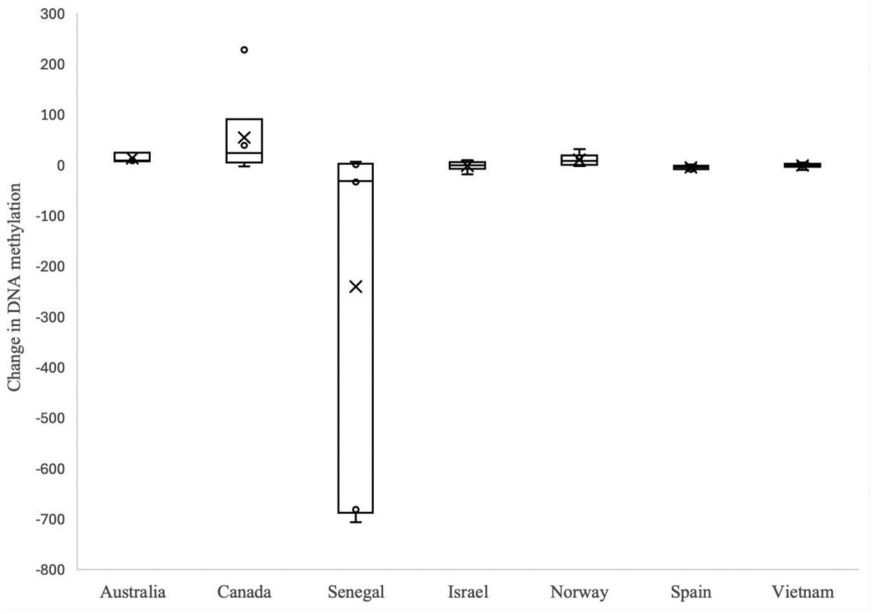

Figure 2. Differences in change in DNA methylation between 0- and 8-h post-simulated bacterial infection among house sparrows from introduced locations (Australia, Canada, and Senegal) and native locations (Israel, Norway, Spain, and Vietnam) displayed as a box and whisker plot.

Table 2. Summary of results testing house sparrows for factors that contributed to changes in DNA methylation between 0- and 8-h post-simulated bacterial infection using GLMM. The R^2 for marginal values was 0.642. For each factor, the estimated value (Estimate), SE, value of t-test (t), and the p-value are provided. The native group was set as the reference for the native or introduced fixed effect.

Fixed effects	Estimate	SE	t	p-value
Intercept	0.7764	0.5785	1.342	0.180
Sex (male)	0.3316	0.5040	0.658	0.511
Native or introduced	−2.8410	0.4676	−6.076	< 0.00001

native range. Between 0- and 8-h post-simulated bacterial infection, DNA methylation differed at more genomic locations, with a greater magnitude of change, in introduced than native house sparrows. These changes in DNA methylation occurred in both directions, with some sites gaining methylation and others losing methylation. Introduced individuals had more unique genomic locations (n = 35) that were differently methylated, while only a single unique genomic location was differently methylated in native individuals. Further, the native or introduced status of individuals was the strongest predictor of change in DNA methylation, and individuals from Senegal, the most-recently introduced, non-native population, made the strongest contribution to differences among geographic locations. It is possible that the change in DNA methylation we observed over the 8-h time series might not reflect an immune response per se, but could also reflect response to the stress of being brought into captivity, or a combination of the two. As the present study did not include a group that was brought into captivity and not exposed to the simulated bacterial infection, we cannot directly estimate response to handling stress or captivity alone. Yet, as all individuals were handled in a similar manner, and we detect clear epigenetic differences between native and introduced house sparrows, these results support the hypothesis of an epigenetically mediated invader phenotype present among introduced house sparrows, which provided a mechanism for plasticity in response to novel stressors (Sheldon et al. 2023). Further, it supports the hypothesis that epigenetic potential and epigenetic buffering likely play a role in the manner of this response (O'dea et al. 2016, Lauer et al. 2024).

Our results expand previous research on the importance of DNA methylation in the response to infection in supporting the immune response of house sparrows in multiple contexts. In response to a parasite infection, DNA methylation differed between infected and non-infected house sparrows, and among individuals sampled temporally after infection, at the nestling stage (10–14 days old) and at the fledged juvenile stage (26–37 days; Lundregan et al. 2022). Lundregan et al. (2022) found that DNA methylation of the Nuclear Receptor Subfamily 1 Group D Member 1 (NR1D1: a nuclear receptor active in circadian rhythms, metabolism, and inflammation) differed between infected and uninfected individuals and was correlated to recruitment. Our findings are congruent in detecting DNA methylation changes post-infection, yet over a much shorter timeframe. Further, introduced house sparrows had higher expression of pathogen surveillance genes and cytokine responses genes, to the simulated bacterial infection investigated in the present study (McCain et al. 2025). In introduced house sparrows, as TLR-4 expression increased, IL-1β and IL-10 responses decreased, which was not detected in native sparrows. Our results suggest that the observed differences in immune response in introduced house sparrows are related at the group level to changes in DNA methylation. In addition, introduced house sparrows with higher epigenetic potential (estimated by the number of CpG sites in the promoter of TLR-4) had higher resistance to infection by *Salmonella enterica* compared to individuals with lower epigenetic potential (Sheldon et al. 2023). As epigenetic potential measures the genetic capacity for an individual to adopt different DNA methylation states, our results suggest that both an individual's inherent capability to be methylated differently, and it actually being methylated differently, are important factors in the response to infection. Integrating these findings demonstrates that both epigenetic potential and the actual changing of DNA methylation state is important in the response to infection. Also, these studies indicate that it is highly likely that the difference in change of DNA methylation we detected between introduced and native individuals would ultimately support introduction success.

Table 3. Results from ANOVA testing change in DNA methylation in house sparrows between 0- and 8-h post-simulated bacterial infection among countries of capture. The ANOVA table reports the sum of squares, mean squared, degrees of freedom (df), *F*-value (*F*) and p-value.

	Sum of squares	Mean squared	df	*F*	p-value
Country	200.8	33.47	6	2.74	0.029
Residuals	391.0	12.22	32		

Our results also provide new context for previous findings of the role of DNA methylation in the success of introduced house sparrows, by finding supporting results in how individuals change over time. Differences in DNA methylation occur within and among introductions of house sparrow (Liebl et al. 2013, Sheldon et al. 2018a), and these differences manifest across the edge-core axis of introduction (Hanson et al. 2020b). Further, DNA methylation differs between introduced and native individuals, with those from more recent introductions having greater variance in DNA methylation (Lauer et al. 2024). The present temporal study found congruent results within individuals over time: detecting differences in DNA methylation among introductions and between introduced and native individuals. The individual-level response to simulated bacterial infection suggests that the larger patterns detected may, in part, be shaped by individual responses.

Our results also provide new information in the study of how DNA methylation changes over time in birds. We document substantial, rapid changes in DNA methylation state in response to simulated bacterial infection (within 8 h), which, to our knowledge, is the shortest time frame studied. In aviary-controlled conditions, temporal changes in DNA methylation

were detected in great tits *Parus major* among three time points, 21 days apart, across a breeding season. Time points targeted the initiation of gonadal development, nest building, and initiation of egg laying (Linder et al. 2021a, 2021b). Changes in DNA methylation in liver and blood were correlated, and DNA methylation near transcription start sites was correlated to decrease in gene expression. In captive great tits from aviary conditions, changes in DNA methylation in blood were detected both between temperature treatments and temporally across four selected time points, which roughly spanned reproductive behaviors of initiation of reproduction, through 50% of individuals laying eggs (Viitaniemi et al. 2019). A relatively large number of small magnitude changes in DNA methylation were detected and there was a large variation in the change over time given a relative low level of methylation, with a large amount of among-individual variation. In wild collected chestnut-crowned babblers *Pomatostomus ruficeps*, DNA methylation in blood differed among individuals sampled at hatching, fledgling, and 1-year (Liebl et al. 2021). In this cooperative breeder, first year dispersers had a greater number of loci that changed DNA methylation state between hatchling and fledgling, and had lower DNA methylation, compared to non-dispersers before fledgling but not as hatchlings or adults. Together, these studies show within-individual change in DNA methylation is critically important to the response to environment, and coordination of temporally variable behaviors.

While we demonstrate a clear individual response in DNA methylation to simulated bacterial infection, it is important to note that DNA methylation is active in multiple different contexts within individuals, and even within cells (Sheldon et al. 2022, Chen et al. 2024). Thus, not all individuals in all introduced areas are expected to show identical change in DNA methylation, or even that the response in DNA methylation would be expected to be directional in general. Rather, it is likely that maintaining, or increasing, the ability to change, is of primary importance to introduced species. The potential for change and the response to immediate local stressors might best be detected in variance of DNA methylation among introduced individuals, or in targeted analysis of the regulation of specific genes. Also, it is highly likely that histone modification is another critically important epigenetic mechanism to this process (Ray et al. 2024). We encourage investigations in all three areas, to provide additional insights into the response of individuals to stress and to the success of the house sparrows as introduced species.

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Author contributions

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Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.qjq2bvqs2 (Schrey et al. 2025).

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