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RESEARCH ARTICLE

Epigenetic potential affects immune gene expression in house sparrows

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ABSTRACT

Epigenetic mechanisms may play a central role in mediating phenotypic plasticity, especially during range expansions, when populations face a suite of novel environmental conditions. Individuals may differ in their epigenetic potential (EP; their capacity for epigenetic modifications of gene expression), which may affect their ability to colonize new areas. One form of EP, the number of CpG sites, is higher in introduced house sparrows (*Passer domesticus*) than in native birds in the promoter region of a microbial surveillance gene, Toll-like Receptor 4 (*TLR4*), which may allow invading birds to fine-tune their immune responses to unfamiliar parasites. Here, we compared *TLR4* gene expression from whole blood, liver and spleen in house sparrows with different EP, first challenging some birds with lipopolysaccharide (LPS), to increase gene expression by simulating a natural infection. We expected that high EP would predict high inducibility and reversibility of *TLR4* expression in the blood of birds treated with LPS, but we did not make directional predictions regarding organs, as we could not repeatedly sample these tissues. We found that EP was predictive of *TLR4* expression in all tissues. Birds with high EP expressed more *TLR4* in the blood than individuals with low EP, regardless of treatment with LPS. Only females with high EP exhibited reversibility in gene expression. Further, the effect of EP varied between sexes and among tissues. Together, these data support EP as one regulator of *TLR4* expression.

KEY WORDS: Toll-like receptors, CpG sites, Phenotypic plasticity, Immune response, LPS

INTRODUCTION

Phenotypic plasticity, or the ability of a genotype to produce multiple phenotypes across environments, allows organisms to respond to a variety of different biotic and abiotic cues (Pigliucci, 2006). Consequently, plasticity is hypothesized to be advantageous in a variety of contexts, but especially dynamic, spatially heterogeneous or novel environments (Pigliucci, 2006; Lande, 2015, 2009). Phenotypic plasticity can arise through molecular epigenetic mechanisms, such as DNA methylation, which can change gene expression without variation in DNA sequence (Feinberg, 2007). Recently, substantial work has been done on the potential for DNA methylation, induced by environmental factors, to alter evolutionary trajectories. Yet, less attention has been directed to the genetic motifs where methyl marks occur in the genome (Feinberg and Irizarry, 2010). Variations in this substrate could represent vital evolutionary capacitors of phenotypic variation, releasing adaptive plasticity in particular environmental contexts (Ghalambor et al., 2015, 2007). We identified one common genetic motif, the number of CpG sites an individual maintains, as epigenetic potential (EP) (Kilvitis et al., 2017; Feinberg and Irizarry, 2010). We posit EP as a putative form of latent plasticity, which might imbue genotypes with different capacities for epigenetically mediated phenotypic plasticity, making some genotypes advantageous in response to rapidly evolving or varying conditions (Kilvitis et al., 2017).

Within the vertebrate genome, CpG sites are almost exclusively the sites of DNA methylation (Bird, 2002). DNA methylation can induce, enhance, suppress or inhibit gene expression, depending on the location of the CpG site relative to an intron, exon and the transcription start site, and especially its proximity to a transcription factor binding site, promoter or enhancer (Smith and Meissner, 2013). The number of CpG sites in or near regulatory sites are by no means the only factors that affect gene expression, but they may represent important areas where gene expression may be finely titrated, with more CpG sites representing more opportunities for methylation. Moreover, some CpG could serve as a genomic substrate on which the environment could act to alter gene expression within generations and selection later act to affect variation in gene expression across generations (Kilvitis et al., 2017; Branciamore et al., 2010).

EP may be particularly consequential in range expansions (Hanson et al., 2020a,c; Kilvitis et al., 2017; Feinberg and Irizarry, 2010). During these events, organisms must cope with extensive biotic and abiotic challenges while genetic diversity is often decreased (Lee, 2002; Taylor and Hastings, 2005). Individuals with high EP may be able to achieve a wider range of phenotypes than individuals with low EP, as EP may enable them to adjust gene expression more rapidly to prevailing conditions (Kilvitis et al., 2017; Hanson et al., 2020a). Already, EP has been implicated in the spread of house sparrows (*Passer domesticus*), one of the most successful introduced species in the world, into new areas (Hanson et al., 2020b; Anderson, 2006). In an ongoing range expansion in Kenya, EP increased with distance from the site of initial introduction, a trend driven by directional selection acting to preserve the CpG sites at the expanding range edge (Hanson et al., 2020c).

In the above sparrow study, differences in EP among populations were screened from loci scattered across the genome, so it remains obscure whether EP enables plasticity by fine-turning gene expression, as expected. Our interest in the present study was to

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investigate the putative physiological effects of EP, namely that it should provide individuals with nimble control of the expression of particular genes. In the context of invasions, some genes should be more important to fitness than others, particularly those that interface directly with threats inherent to novel areas. One such group of genes is the Toll-like receptors, which recognize evolutionarily conserved pathogen-associated molecular patterns (PAMPs) (Werling et al., 2009). Detection of parasite presence is the integral first step to coping with an infection, which might explain why previous studies observed spatial variation in the expression of one Toll-like Receptor gene, Toll-like Receptor 4 (*TLR4*), among the same Kenyan sparrow populations mentioned earlier (Vaure and Liu, 2014; Martin et al., 2015, 2014, 2017). *TLR4* recognizes lipopolysaccharide (LPS) found on Gram-negative bacteria, but as risk of infections is variable in time and space, greater EP in *TLR4* might give individuals an advantage to adjust the expression (via methylation) of this key gene efficiently, depending on bacterial exposure or other factors implicating infection risk (Brownlie and Allan, 2011; Iwasaki and Medzhitov, 2015). Indeed, functionally relevant variation in *TLR4* expression is partly controlled by DNA methylation. For example, in domesticated chickens, the regulation of *TLR4* expression via DNA methylation was related to susceptibility to *Salmonella* infection (Gou et al., 2012). Additionally, *TLR4* senses damage-associated molecular patterns (DAMPs) resulting from compromised host cells and tissues (Kuzmich et al., 2017; Mckeown-Longo and Higgins, 2017). As such, nimble regulation of *TLR4* might contribute to the control of a range of parasite forms and perhaps even the recruitment of an adaptive immune response (Mukherjee et al., 2016; Olejnik et al., 2018; Zanin-Zhorov and Cohen, 2013).

During range expansions, flexible adjustments of *TLR4* via EP may be of paramount importance. In the vast majority of cases, parasite diversity and prevalence decrease when hosts leave their native ranges (Torchin et al., 2003; Marzal et al., 2011). Still, no place on Earth is truly without risk of infection, especially because invaders might often need to take more risks than natives to find viable shelter, mates and food. For example, house sparrows at the expanding edge of populations are more exploratory and quicker to consume novel foods than those from longer-established populations (Martin and Fitzgerald, 2005; Liebl and Martin, 2012, 2014). Such behavioral dispositions could enable invading birds to acquire resources quickly, but they also risk exposure to pathogens (Canestrelli et al., 2016). For invading animals then, EP in immune genes including *TLR4* might provide a rapid and labile defense against parasitic threats. In support, EP in the putative promoter of *TLR4* is higher in introduced compared to native house sparrow populations, suggesting it may benefit invaders (Hanson et al., 2020a). Still, it remains to be determined whether and how EP relates to the regulation of *TLR4*.

Here, we took a first step in revealing whether EP affects gene expression in the house sparrow by comparing *TLR4* expression of sparrows with high and low EP in the putative promoter region of *TLR4*, before and after birds were challenged with LPS (a *TLR4* agonist). We then probed whether EP or the composition of particular CpG sites (see below) were stronger predictors of gene expression. We assessed EP effects on *TLR4* expression in blood, liver and spleen as methylation likely differs across cell types and tissues. We expected that EP would be the best predictor of *TLR4* gene expression across all tissues and that EP might imbue individuals with an increased ability to adjust gene expression among tissues. Next, in blood samples, we described dynamic relationships between EP and *TLR4* expression and tested (at least over a short time scale) whether high EP enabled the greatest inducibility and reversibility in gene expression, the crux of our interest in EP. Further, we expected high EP birds administered LPS to show the greatest increase and decrease in expression in the measurement period, whereas we expected modest, if any, temporal changes in TLR4 expression in control birds, particularly in the low EP group. We compared EP effects on both total gene expression (area under the time curve, AUC) as well as the temporal response to LPS itself. In tissues, we could not make directional predictions with respect to EP and/or LPS treatment because we could not sample these tissues repeatedly. However, we did expect tissues (as mentioned above) to manifest different *TLR4* expression with respect to EP, just as we expected low and high EP birds to differ in *TLR4* expression.

MATERIALS AND METHODS

Sample collection

Adult house sparrows [*Passer domesticus* (Linnaeus 1758)] (n=31; 15 males and 16 females) were captured via mist nets in early March 2019 from five different locations across the Tampa Bay area (Florida, USA). An additional six birds were captured, but were not included in the analysis owing to problems during sequencing or during DNA and RNA extraction (see below). A previous study revealed that birds from the Tampa Bay area differed in EP (6–9 CpG sites), but showed no evidence of population genetic structure, so we scattered capture efforts across the city to maximize inter-individual variation in *TLR4* EP and other forms of genetic variation that could affect expression (Hanson et al., 2020a). Our logic was that if EP was important in nature as a driver of *TLR4* expression, we would be able to detect effects of EP on gene expression in spite of other factors that differed among the birds we captured from the wild. As sequencing for EP had to occur after the rest of the study (so as to avoid stress-induced immune suppression associated with captivity, obscuring *TLR4* expression differences), birds were randomly assigned to treatment groups in an attempt to distribute EP comparably across both treatment and control groups ($n_{control}$=14, n_{LPS}=17; Table S1). Captured birds were transferred to a vivarium at the University of South Florida and ∼10 µl blood samples were taken from the brachial vein of each bird. Blood was added to approximately 300 µl of RNAlater (Ambion). Immediately after bleeding, birds were subcutaneously administered either 100 µl of 1 mg ml^{-1} LPS in PBS (*E. coli* 055:B5, Sigma Aldrich L4005) or 100 µl of PBS. LPS solution was stored in a sterile silanized bottle before administration to prevent the binding of LPS to the glass (Martin et al., 2011, 2014). Four hours post-injection, ∼10 µl of blood was taken from the brachial vein and stored in 300 µl of RNAlater. Six hours post-injection, birds were euthanatized via isoflourane overdose and rapid decapitation, and ∼10 µl of blood was again taken and stored in RNAlater. Liver and spleen samples were taken and stored in 500 µl of RNAlater within 10 min of euthanasia. The same region of the liver and the entire spleen were dissected from each bird. All samples were stored at −80°C until further processing. Procedures were approved by the University of South Florida IACUC (number IS00003761).

DNA extraction and genomic sequencing

DNA was extracted using 0.1 g of tissue or 50 µl of whole blood/RNAlater mixture using a DNEasy Blood and Tissue kit (Qiagen). Kilvitis et al. (2019) designed the primers used in this study to encompass the putative promoter region 726 to 1228 nucleotides upstream of the transcription start site, which likely includes regulatory regions and CpG sites that affect expression (Table A1)

(Kilvitis et al., 2019; Meissner et al., 2008; Yang et al., 2014; Landolin et al., 2010). PCR was prepared using 12.5 µl of 2× PCR Master Mix (Promega), 1 µl forward primer (10 µmol l^{-1}), 1 µl reverse primer (10 µmol l^{-1}), 8.5 µl of nuclease-free water and 2 µl of DNA. PCR was conducted on a T100 Thermal Cycler (Bio-Rad). Cycling conditions are described in Kilvitis et al. (2019). PCR products were purified using ExoSAP-IT (Affymetrix). Sequencing was conducted at the Field Museum of Natural History (Chicago, IL, USA) using BigDye Terminator technology with forward primers.

CpG site and genetic characterization

Resulting chromatograms from DNA sequences were analyzed manually on Genome Compiler using the Clustal Omega alignment algorithm (Sievers and Higgins, 2014). For five individuals, sequencing was not effective, resulting in regions of the sequence that were not able to be analyzed. As attempts to re-sequence were unsuccessful, these individuals were excluded from the remainder of the study and were not included in any sample size estimates. This criterion was established prior to the start of the study. All single nucleotide polymorphisms (SNPs) and CpG site genomic locations in the *TLR4* promoter were examined across all individuals. CpG sites were counted on each chromosome separately (Hanson et al., 2020a). Individuals were classified into the 'low' or 'high' EP category based on the total number of CpG sites across the entire promoter. Individuals with seven CpG sites were categorized into the 'low' group. As only one individual had nine CpG sites, individuals with eight or nine CpG sites were categorized into the 'high' group. To characterize another potentially important aspect of genetic architecture on gene expression, the specific makeup of CpG sites (what we term CpG composition for the remainder of the paper), individuals were assigned a number to represent the total number of CpG sites and a letter to represent the specific mutation location impacting the CpG site and mutation type. For example, 7a was assigned to individuals that had a C>T mutation impacting the 'C' position of CpG site 5, 7b was assigned to individuals that had an G>A mutation impacting the 'G' position of CpG site 5, and 7c was assigned to individuals that had an G>A mutation impacting the 'G' position of CpG site 2.

While other genetic variation likely plays a role in the regulation of *TLR4* gene expression, we were not able to investigate the effects of all the SNPs observed in this region owing to insufficient power. However, to account for any effects of SNPs that may be linked with mutations in CpG sites (which could confound our EP results), SNPs across the promoter were tested for linkage disequilibrium using GENEPOP version 4.2 with the dememorization number, number of batches and number of iterations per batch all set to 10,000 (Rousset, 2008). To account for multiple comparisons of these SNPs, a Bonferroni correction was conducted using P.adjust in R version 3.6.3 (https://www.r-project.org/). Lastly, as previous work found no population genetic structure in Tampa house sparrows assessed by genetic variation in the *TLR4* promoter and microsatellites, we did not address it here (Hanson et al., 2020a; Schrey et al., 2011).

RNA extraction and real-time quantitative PCR

RNA was extracted from 0.1 g of each tissue or 50 µl of whole blood/RNAlater mixture using TRI-reagent solution (Invitrogen) and was then diluted to 25 ng μl^{-1}. For one individual, RNA extraction was unsuccessful, despite multiple attempts. This individual was excluded from the study and is not reported in any sample size estimates. A reaction using 10 µl of iTaq Universal SYBR Green One-Step Kit (Bio-Rad), 0.3 µl of forward primer, 0.3 µl of reverse primer, 0.25 µl of SCRIPT, 7.15 µl of nuclease-free water and 2 µl of diluted RNA was then used in quantitative reverse transcription PCR (RT-qPCR) (Table A1). We first identified an appropriate housekeeping gene for our samples (see the Appendix) based on consistency of its expression across blood and tissue samples from control and treatment groups across all time points. We considered three potential candidates: *HMBS*, *TFRC* and *RPL13* (Olias et al., 2014), but chose *HMBS*, as its expression was detectable in all samples, was not affected by LPS treatment or EP, and showed the least inter-individual variation across all samples (see the Appendix). Using a Rotor-Gene Q (Qiagen), RT-qPCR was run under the following conditions: 10 min at 50°C for reverse transcription reaction, then 1 min at 95°C for polymerase activation and DNA denaturation, followed by 40 amplification cycles of 15 s at 95°C then 30 s at 60°C. Finally, a melt-curve analysis was performed from 65 to 95°C with 0.5°C increment steps every 3 s. On all plates, nuclease-free water was used as a negative control, a calibrator was included consisting of two pooled samples for each tissue from control birds, and all samples were run in duplicate. *TLR4* expression was assessed at 0, 4 and 6 h post-injection in the blood, and at 6 h post-injection in the liver and spleen.

Data analysis

First, to consider total *TLR4* expression in blood, relative *TLR4* expression values at 0, 4 and 6 h post-LPS were used to calculate the AUC for each individual. Then, to determine whether *TLR4* expression in the blood, liver and spleen was primarily driven by EP or CpG site composition, we first used an information-theoretic approach, assessing the relative support for each model in each tissue separately. Specifically, we fitted two generalized linear models (GLMs) with a gamma error distribution. Models (for each tissue separately) included LPS treatment, sex and either EP or CpG site composition and the two- and three-way interactions among all fixed effects. Best-fit models were identified by comparing corrected Akaike's information criterion (AICc) scores of each model. As the previous analysis showed that EP was the best predictor of *TLR4* expression, we further investigated the effect of EP on *TLR4* expression dynamics in the blood at each time point by fitting generalized linear mixed models (GLMM) with a Gamma error distribution. The models included LPS treatment, sex, EP and time post-injection (0, 4 and 6 h) and the two- and three-way interaction among all fixed effects. Individual identity of each bird was specified as a random effect. GLMs were run using the GENMOD procedure and GLMMs with the GLIMMIX procedure in SAS University Edition (SAS Institute Inc., Cary, NC, USA). *Post hoc* comparisons were performed using Tukey–Kramer multiple comparison adjustments to obtain corrected *P*-values. All figures were created in GraphPad Prism Version 8.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Best-fit models for TLR4 expression

We first asked whether EP (number of CpG sites) was related to CpG site composition (specific makeup of CpG sites), as such a relationship could affect gene expression and hence our functional interpretation of EP. Total CpG sites ranged from seven to nine. Across all individuals, five possible CpG site compositions occurred (7a, 7b, 7c, 8 and 9; Table S1), and 11 SNPs were identified across all individuals. Two pairs of SNPs were found to be significantly linked (SNPs 2 and 4: P=0.015, SNPs 1 and 5: 0.002) and one pair of SNPs was marginally non-significantly linked

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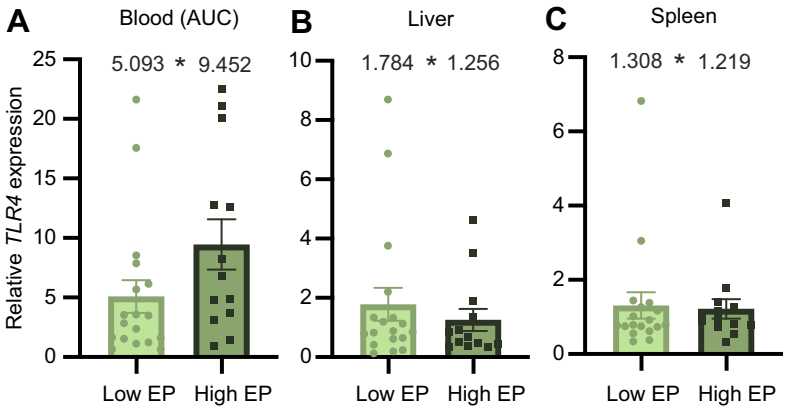

Fig. 1. Epigenetic potential (EP) predicts tissue-specific *TLR4* expression in house sparrows. (A) High EP individuals had significantly higher *TLR4* AUC (area under the time-series curve) expression in the blood (β=1.32 [0.05–2.58], *P*=0.0075) compared with low EP individuals. Conversely, high EP individuals expressed less *TLR4* in the (B) liver (β=1.27 [0.22–2.31], *P*=0.0177) and (C) spleen (β=−1.12[−1.97–−0.28], *P*=0.0092) compared with low EP individuals. Means are displayed beneath panel labels. Asterisks denote a significant difference between EP group, error bars are ±s.e.m. and samples sizes are reported in Table S1.

(SNPs 1 and 4: *P*=0.055). Although we were unable to investigate the relationship between expression and all observed genetic variation, we found that none of the linked SNPs overlapped CpG sites (e.g. mutations were not at the C or G position of the CpG sites). EP consistently appeared as the best predictor for *TLR4* expression [blood (AUC): AIC_c=194.99, w_i=1.0; liver: AIC_c=70.56, w_i=1.0; spleen: AIC_c=97.99, w_i=1.0; Table S2].

EP effects on TLR4 expression in blood and organs

In the blood, individuals with high EP had larger AUC for *TLR4* expression than low EP individuals (β=1.32 [0.05–2.58], *P*=0.0075; Figs 1A, 3A, Table S3). AUC for relative *TLR4* expression in blood was not influenced by sex, nor other interactions with fixed effects (Table S3). Treatment with LPS had a marginally non-significant effect on *TLR4* AUC expression, tending to increase expression (β= 0.22 [−1.05–1.48], *P*=0.0588; Table S3).

TLR4 expression in the liver was predicted by EP (β=1.27 [0.22–2.31], *P*=0.0177; Fig. 1B), but this relationship differed between the sexes (sex×EP, β=−1.62 [−3.00–0.23], *P*=0.0221; Fig. 4, Table S3). Although the interaction was significant, subsequent tests for multiple comparisons revealed no specific significant differences between the sexes of different EP level. Treatment with LPS significantly increased *TLR4* relative expression in the liver (β=−0.80 [−1.32–−0.29], *P*=0.0005). However, *TLR4* expression in the liver was not predicted by the interaction between treatment and EP, nor any other term in the model (Table S3).

TLR4 expression in the spleen was influenced by EP (β=−1.12 [−1.97–−0.28], *P*=0.0092; Fig. 1C), but this relationship differed between LPS and control groups (treatment×EP, β=2.20 [0.99–3.41], *P*=0.0004; Fig. 2, Table S3). High EP controls expressed more *TLR4* than low EP controls (*z*=2.66, *P*=0.0391) and low EP individuals administered LPS expressed more *TLR4* than low EP controls (*z*=−3.43, *P*=0.0033; Fig. 2, Table S4). Sex was also a significant predictor of *TLR4* expression in the spleen, with males expressing more *TLR4* than females (β=−0.88 [−1.48–−0.28], *P*=0.0039; Table S3).

Dynamic effects of EP on TLR4 expression in the blood

To evaluate whether EP affected reversibility in *TLR4* expression, we also investigated dynamic changes in expression in the blood after LPS or vehicle administration. *TLR4* expression in the blood changed over time, but this effect was different by sex and EP (time×sex×EP, $F_{3,65.37}$=5.59, *P*=0.0018; Fig. 3B, Table S5). *Post hoc* comparisons revealed that there were no significant differences between males and females of any EP level at 0 or 4 h, but at 6 h, females with high EP had lower expression than males with high EP (*t*=−4.56, d.f.=80.33, *P*=0.0013) and females with low EP had marginally lower expression than males with high EP (*t*=−3.34, d.f.=80.33, *P*=0.0568; Fig. 3B, Table S6). Across time points but within the same sex and EP categories, females with high EP significantly increased *TLR4* expression by 4 h post-injection (*t*=−6.02, d.f.=62, *P*=<0.0001) and significantly decreased expression to baseline levels at 6 h compared with samples at 4 h (*t*=4.80, d.f.=62, *P*=0.0006) as there was no difference in gene expression at 0 and 6 h (*t*=−1.22, d.f.=62, *P*=0.9854; Fig. 3B, Table S6). In females with low EP, there was a marginally non-significant difference in expression between 0 and 4 h post-injection (*t*=−3.32, d.f.=62 *P*=0.0594), no difference between 4 and 6 h (*t*=0.53, d.f.=62, *P*=1.0) and no difference between 0 and 6 h (*t*=−2.79, d.f.=62 *P*=0.2075) Fig. 3B, Table S6. In males with high EP, there was no difference between expression between 0 and 4 h (*t*=−2.64, d.f.=62, *P*=0.2805) or between 4 and 6 h (*t*=−0.61, d.f.=62, *P*=1.0), but there was a marginally non-significant increase in expression between 0 and 6 h (*t*=−3.25, d.f.=62, *P*=0.0727, Fig. 3B, Table S6). In males with low EP, there was a significant increase in expression by 4 h (*t*=−4.50, d.f.=62, *P*=0.0016), with no change between 4 and 6 h (*t*=0.68, d.f.=62, *P*=0.9999, Fig. 3B, Table S6). Low EP males never returned to baseline levels of expression, as they still had significantly more expression at 6 h than prior to treatment (*t*=−3.83, d.f.=62, *P*=0.0142, Fig. 3B, Table S6). Here, treatment with LPS (but no subsequent interactions) increased *TLR4* expression overall ($F_{1,31}$=6.37, *P*=0.0170; Table S5).

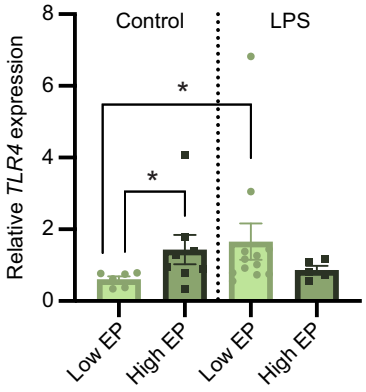

Fig. 2. EP and treatment predict *TLR4* expression in the spleen of house sparrows. EP effects on splenic *TLR4* expression differed between the LPS-treated (right of the dotted line) and control groups (left of the dotted line; EP×treatment, β=2.20 [0.99–3.41], *P*=0.0004). Asterisks denote differences detected by pair-wise contrasts, error bars are ±s.e.m. and samples sizes are reported in Table S1.

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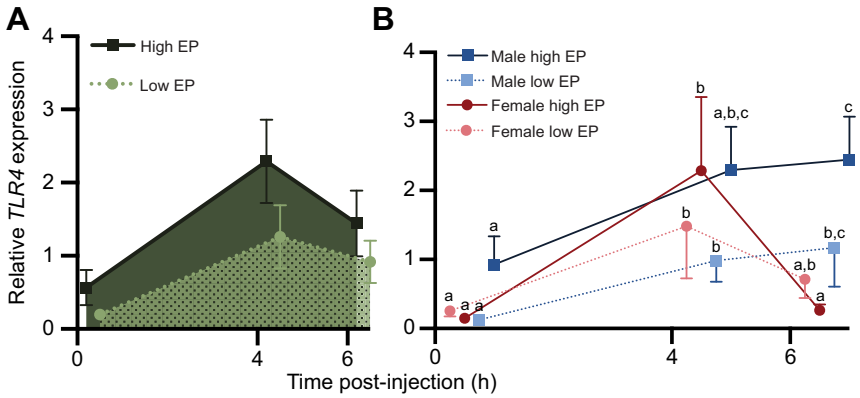

Fig. 3. EP influences the inducibility and reversibility of *TLR4* expression in the blood of house sparrows. (A) Across all individuals regardless of sex or treatment, high EP individuals had higher levels of induced *TLR4* expression (AUC) compared with low EP individuals (β=1.32 [0.05–2.58], *P*=0.0075). (B) *TLR4* expression differed over time between males and females with different EP levels ($F_{3,65.37}$=5.59, *P*=0.0018). Within time points, all individuals had similar levels of expression at the time of injection and after 4 h. Across time points, high EP females showed more inducibility and reversibility of expression. Conversely, males of EP show a delayed response and no reversibility, only moderately increasing their expression by 6 h post injection. Low EP females show less inducibility and reversibility in expression than high EP females. Low EP males show inducibility, but not reversibility. Overall, only high EP females show evidence of inducibility and reversibility of *TLR4* expression. Note that for B, we only display significant or marginally non-significant differences (indicated by different letters) within the same time points and across time points for the same sex and EP level groups (Table S6). Significant differences exist across time points between sexes of different EP level, but they are not meaningful for this investigation. Error bars are ±s.e.m. and samples sizes are reported in Table S1.

DISCUSSION

Our goal was to investigate whether and how one form of EP, the number of CpG sites in the putative promoter region of *TLR4*, affects gene expression in the blood, liver and spleen of house sparrows. Here, we used LPS to simulate an immune challenge, which transiently upregulates *TLR4*, in order to ascertain whether birds with high EP regulated *TLR4* expression differently. As the regulation of gene expression is quite complex, we first asked which gene regulatory factor was the most important for TLR4 regulation among EP and CpG site composition. As anticipated, EP was the best predictor of *TLR4* expression (in all tissues we considered) compared with CpG site composition (Table S2). Across all tissues, we saw an effect of EP, but the directionality of this effect was tissue-specific (Fig. 1). Surprisingly, treatment with LPS had a weaker effect on *TLR4* expression than we anticipated and have observed previously. Only in the spleen did we find that treatment modulated the effect of EP, a finding we expected to see across tissues (Fig. 2). When we investigated the influence of EP on gene expression in the blood, we found that individuals with high EP had expressed more *TLR4*, regardless of treatment with LPS (Figs 1A, 3A). Further, sex had a significant effect on *TLR4* expression across all tissues, and in the blood and liver, the influence of EP on expression also differed by sex (Fig. 3B, Fig. 4, Tables S3 and S5). Lastly, we found that only female house sparrows with high EP exhibited both inducibility and reversibility of *TLR4* expression in the blood, although these effects were independent of LPS treatment (Fig. 3B). In males, overall *TLR4* expression tended to be higher, probably because of the lack of reversibility over the period considered (Fig. 3B).

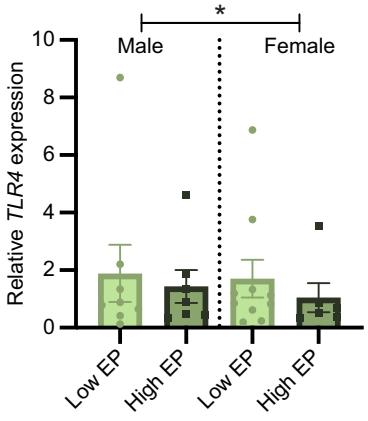

Fig. 4. EP and sex predict *TLR4* expression in the liver of house sparrows. *TLR4* expression in the liver (*n*=31) was influenced by EP, but the effect differed between males (left of the dotted line) and females (right of the dotted line; β=−1.62 [−3.00–0.23], *P*=0.0221). *Post hoc* comparisons revealed no significant pairwise differences between groups. Asterisk denotes a significant difference between the sexes, error bars are ±s.e.m. and samples sizes are reported in Table S1.

Epigenetic potential is the best predictor of gene expression

Of course, many genetic and environmental factors will affect the expression of *TLR4* as well as probably most genes in free-living animals. We argue that EP persists in populations partly because it imbues organisms with a latent capacity for phenotypic plasticity. We do not expect it is the only factor that affects TLR4 expression in this species, but as discussed in the Introduction, we do expect that it is particularly important for vertebrates expanding their ranges or living in otherwise spatiotemporally variable environments (Kilvitis et al., 2017). As each CpG site represents a place for DNA methylation to occur, more CpG sites should allow for greater control of gene regulation, facilitating the malleability of gene expression in response to salient stimuli. However, variation in EP in the present study revealed a potential confounding factor: the number of CpG sites (EP) was different among individuals, but the specific CpG sites making up the total number, or CpG site composition, was also different among individuals. Many studies have found that gene expression is driven by methylation at specific CpG sites; methylation at some sites is fixed whereas dynamism in methylation at other loci is unrelated to gene expression (Lanata et al., 2018). For example, in Kenyan house sparrows, DNA methylation at one CpG site in the putative *TLR4* promoter

(examined here) was inversely correlated with hepatic gene expression (Kilvitis et al., 2019). If a mutation occurred within that one functional CpG site, gene expression might be affected. Generally, though, the loss of a CpG site corresponds to the loss of ability for methylation to occur (Zhou et al., 2015; Zhi et al., 2013), so we first asked whether EP or CpG site composition was a better predictor of *TLR4* expression in any tissue. We found that EP was indeed a better predictor across all tissue types than CpG site composition, giving credibility to the idea that it is in fact EP, rather than specific sites within the promoter, that is influencing the expression of this gene in these sparrows (Table S2). Owing to insufficient power, we could not investigate other genetic differences between individuals, which may also influence gene expression; however, we did investigate whether SNPs were linked to CpG sites. We did not find evidence of this, again indicating that EP is the important factor affecting *TLR4* expression.

Epigenetic potential predicts tissue-specific expression

One might expect that given such strong and diverse protective effects, *TLR4* should be expressed at all times at high levels on many cell types. However, *TLR4* instigates such broad immune responses that the imposition of costly investments of energy and resources might favor rapid and reversible as opposed to constitutive expression (Sadd and Schmid-Hempel, 2009; King and Swanson, 2013; Zuk and Stoehr, 2002; Martin et al., 2017). Too little *TLR4* may render an organism susceptible to infection, but too much might be physiologically wasteful and or even risky in terms of inflammatory self-damage (Olejnik et al., 2018; Perrin-Cocon et al., 2017). Indeed, *TLR4* overactivation can lead to sepsis and death, which altogether suggests that it should be extremely important to regulate *TLR4* expression so as to balance protection against collateral damage and wastefulness (Kuzmich et al., 2017). Responses may thus need to be sculpted to cell types and tissues, as each differ in likelihood of exposure to Gram-negative bacteria (Adelman and Hawley, 2017).

The elegance of EP is that it is encoded in the genome, putatively allowing differential regulation of gene expression across scales from single cells to whole tissues. As EP was revealed to be the best predictor of *TLR4* expression that we considered, we explored the effects EP on each tissue, too. We found an effect of EP on *TLR4* expression across the blood, spleen and liver (Fig. 1). In the blood, high EP individuals had higher relative *TLR4* AUC expression than low EP individuals (Figs 1A, 3A). Conversely, in the liver and the spleen, high EP individuals had lower relative *TLR4* expression (Fig. 1B,C). Other studies have found similar tissue specificity in *TLR4* expression (Nishimura and Naito, 2005; Zarember and Godowski, 2002). This outcome is not surprising, as blood, liver and spleen are made up of very different cell types and proportions, lending to their differences in gene expression, function and subsequent immune responses (Robinson et al., 2016; Lewis et al., 2019). Spatiotemporal heterogeneity also exists within and among tissues (Gough et al., 2017). For example, subpopulations of cell types within tissues can differ in respect to *TLR4* expression, often driven by different exposure to microbiome constituents and other factors (Wang et al., 2010; Price et al., 2018; Chaussé et al., 2011; Pioli et al., 2004). Importantly, differences in expression can be dynamically mediated by rapid changes in DNA methylation, and patterns of DNA methylation are often tissue- and cell-specific (Bruniquel and Schwartz, 2003; Husby, 2020). While we do not yet know whether the directionality or rapidity of these changes is adaptive or protective, or even whether they are driven via DNA methylation, EP may be one mechanism by which house sparrows and perhaps other vertebrates manifest immune responses differently across cellular, tissue and organismal levels.

Surprisingly, treatment with LPS did not seem to release latent plasticity in *TLR4* expression as we expected. We predicted treatment with LPS to push the system such that both inducibility and reversibility of gene expression could be examined, at least in the blood, where we could measure expression across multiple time points. Across the tissues, we expected to see that EP modulated expression in response to LPS, but we only saw this effect in the spleen (Fig. 2). There, low EP individuals treated with LPS expressed more *TLR4* than low EP controls (Fig. 2). Additionally, high EP controls expressed more *TLR4* than low EP controls (Fig. 2). Perhaps we saw an interaction between EP and LPS in the spleen, as this tissue filters blood-borne pathogens and exhibits high constitutive expression of *TLR4* (Lewis et al., 2016; Vaure and Liu, 2014). In the blood (AUC) and liver, individuals treated with LPS expressed more *TLR4*, but this effect was not statistically significant in the blood (AUC) (Table S3). Previous studies have shown that *TLR4* expression increases in mice rapidly in response to restraint stress and in house sparrows kept in captivity for several weeks, as well as to other stressors (Zhang et al., 2008; Gárate et al., 2014; Martin et al., 2011). We propose that the unavoidable stress of capture, restraint and repeated sampling in the present study may have obscured some of the effects of LPS across tissues. Overall, while we expected to find EP-dependent LPS effects on *TLR4* expression, we instead found that EP seems to capacitate gene expression differently across each tissue.

Effects of epigenetic potential on the inducibility and reversibility of TLR4 expression

Much of our interest in EP lies in its potential to modulate the inducibility and reversibility of gene expression. Related to this, we were also interested in the range of expression achieved by high and low EP individuals. As we were constrained to measuring expression in the liver and the spleen only once at the end of the experiment, largely when we expected the effects of LPS to be diminished, we focused on inducibility and reversibility in the blood. We first used AUC to investigate the range of expression over the entire time course of the experiment. Individuals with high EP had almost double the mean amount of AUC expression in the blood of low EP birds (Figs 1A, 3A). LPS effects on *TLR4* expression did not interact with EP; high EP birds expressed more *TLR4* independently of LPS treatment (Table S3). Nevertheless, EP did influence the range of expression released over time. Similar outcomes have been seen in other systems, with CpG site content in promoter regions and proximal to the TSS found to correlate positively with gene expression and promoter activity (Yang et al., 2014; Landolin et al., 2010).

This investigation into AUC expression could not reveal whether EP effects are typically induced or reversible. Presently, EP seems to underlie constitutive differences in expression among individuals; however, there were no significant differences in *TLR4* expression between individuals at 0 h (Fig. 3B, Table S6). Thus, high EP individuals did not constitutively express more *TLR4* than low EP individuals, suggesting that the response was induced and related to EP, but not to treatment with LPS. Further, we found some evidence for reversibility effects by EP, but in a sex-specific way (Fig. 3B). Females with high EP increased *TLR4* expression by 4 h, but decreased expression between 4 and 6 h, leading to similar levels of *TLR4* expression as seen prior to LPS (Fig. 3B). In other words, high EP females showed both inducibility and reversibility (independent of treatment with LPS). In comparison, high EP males only

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increased expression after 6 h post-injection. In this case, males only showed inducibility (again independent of LPS treatment), but not reversibility (Fig. 3B). Both low EP females and males exhibited similar sex-specific trends as their high EP counterparts, but their responses were lower in magnitude (Fig. 3B). It is possible that if we had extended the duration of the experiment, males would have eventually returned to baseline expression levels. Altogether though, EP seemed to capacitate a wider range of induced *TLR4* expression in all birds, but only reversibility in female house sparrows. As described earlier, for invading animals, EP in *TLR4* might provide a rapid and labile defense against unfamiliar parasites, especially as *TLR4* is one of the first points of contact between host immune system and pathogens (Hanson et al., 2020a). A wider range of induced *TLR4* expression may be advantageous when coping with unfamiliar environments or novel parasites, as the optimal level of expression necessary to combat infection may be variable.

Sex-specific effects of TLR4

One unexpected finding in this study was the effect of sex on *TLR4* expression across tissues. Life history trade-offs have long been studied as a driver of variation in immunity, and *TLR4* expression has been shown to differ between the sexes in other systems, but the directionality is inconsistent (e.g. baseline versus LPS-induced, tissue/cell types, etc.) (Klein and Flanagan, 2016; Roberts et al., 2012, 2013; Temple et al., 2008; Han et al., 2020; Marriott et al., 2006). Sex hormones affect *TLR4* expression, but results are similarly inconsistent (Calippe et al., 2010). Similar to the effects of EP in the blood mentioned above, we found that *TLR4* expression in the liver varied by sex and EP (although this effect was weak given that *post hoc* analysis revealed no pair-wise differences) (Fig. 4). In the spleen, males expressed more *TLR4* than females, regardless of EP (Table S3). This study was conducted early in the breeding season for Tampa house sparrows, when sex hormones could be elevated (Hegner and Wingfield, 1986). Perhaps the relationships we identified between sex, EP and *TLR4* expression will differ across breeding and non-breeding seasons. Further, sex-specific differences in DNA methylation have been found in other studies, including other passerines (Verhulst et al., 2016). As DNA methylation can contribute to sex differences in expression, EP may provide additional substrate for the flexible modulation of gene expression between the sexes (Inoshita et al., 2015).

Conclusions

EP was related to *TLR4* gene expression in the blood, liver and spleen of house sparrows (Fig. 1). We expect that these relationships will have implications for phenotypic plasticity in perhaps this and other species, as EP was related to the range of gene expression achievable over time in the blood. EP also behaved differently among tissues and between sexes, providing an additional (and unexpected) context-dependency in expression that could also play a role in range expansions. Surprisingly, the effects of EP acted largely independently of LPS treatment, except in the spleen (Fig. 2). Additionally, we only found evidence of reversibility in gene expression in females with high EP; in both sexes, though, high EP was associated with greater *TLR4* inducibility (Fig. 1A and Fig. 3). Overall, our results are consistent with our initial hypothesis that EP may provide a latent, genomic capacity for flexible modulation of gene expression, but further work is needed to understand whether and how gene expression in sparrows is altered by DNA methylation, how much of the influence of EP on expression is regulated versus stochastic, what other genetic factors affect the expression of *TLR*s and other genes integral to the control of bacterial infections, and what functional role EP in *TLR4* in particular plays in house sparrows in mitigating infection risk.

Appendix

Housekeeping gene validation

We considered three potential housekeeping genes based on genes that have been previously validated in passerine species (Table A1) (Olias et al., 2014). In order to determine the best housekeeping gene between *HMBS*, *TFRC* and *RPL13* for our experiment, we ran RT-qPCR from samples across different tissues (blood, liver, spleen and gut), treatment groups (LPS and control) and EP levels (high and low). Then, we determined whether expression of each of these genes was affected by treatment and EP using generalized linear mixed models (GLMMs). For each gene, the cycle threshold (C_t) value was the dependent variable and tissue, treatment, EP and their two- and three-way interactions were added as fixed factors. Individual identity was specified as random factor to take into the non-independence of tissue from a same individual. We also used RefFinder algorithm to produce comprehensive ranking of the considered genes. This algorithm uses four programs (Delta Ct method, Normfinder, Best Keeper and geNorm) to assign a weight value to each gene and calculates the geometric mean of the weights to create the overall final ranking (Xie et al., 2012).

HMBS was detectable in all 35 samples while *RPLC13* was detectable in only 28 samples and *TFRC* in 29 samples. *HMBS* showed the lowest inter-individual variation across all samples (mean±s.d. C_t=24.9±3.1) compared with *RPL13* (28.8±4.0) and *TFRC* (25.4±5.2). Additionally, *HMBS* expression was not affected by treatment or EP, but differed between tissues (Table A2). Among tissues, expression in the gut was higher than liver and spleen ($t \geq 4.15$, $P \leq 0.0006$), and expression in blood was higher than in the spleen (t=2.86, P=0.343). On the contrary, *RPL13* expression was influenced by the interaction between treatment, tissues and EP

Table A1. Primers for sequencing and RT-qPCR with their original references listed

Primer	Function	Primer sequence (forward/reverse)	Reference
TLR4 promoter	PCR, sequencing	GGGATTTTGTAGAACTTGCCAAATTT AAAGCTCCCTGCCTTCATTTAGTCTG	Kilvitis et al. (2019)
TLR4	RT-qPCR	GCTCCTGTGTGTACCTGGAC ACAACACAACCACTGGGGAG	
HMBS	RT-qPCR (housekeeping gene)	CTGAAGAGAATGGGCTGGGA TCTTGGTCTTTGGCACGAAC	Olias et al. (2014)
TFRC	RT-qPCR (housekeeping gene)	GGAACTTGCCCGTGTGATC GTAGCACCCACAGCTCCGT	
RPL13	RT-qPCR (housekeeping gene)	CCACAAGGACTGGCAGCG ACGATGGGCCGGATGG	

(Table A3) and *TFRC* expression by treatment, tissue, and the interactions between treatment and tissue, treatment and EP, and tissue and EP (Table A4).

RefFinder confirmed that *HMBS* is the best housekeeping gene for our samples as it is ranked first in the comprehensive ranking based on the geometric variance calculated by RefFinder (Table A5). *HMBS* also ranks first in three (Delta Ct method, Best Keeper and geNorm) of the four programs used by RefFinder, while *RPL13* ranks first in Normfinder (Table A5).

Table A2. Result of the GLMM for *HMBS* expression

Effect	d.f.	*F*	*P*
Treatment	1,35	0.21	0.6483
Tissue	3,35	10.17	<0.0001
EP	1,35	0.44	0.5104
Treatment×Tissue	3,35	2.00	0.1322
Treatment×EP	1,35	0.76	0.3880
Tissue×EP	3,35	0.15	0.9307
Treatment×Tissue×EP	2,35	1.23	0.3054

Table A3. Result of the GLMM for *RPL13* expression

Effect	d.f.	*F*	*P*
Treatment	1,28	0.22	0.6401
Tissue	3,28	1.35	0.2795
EP	1,28	0.00	0.9480
Treatment×Tissue	3,28	2.26	0.1028
Treatment×EP	1,28	0.49	0.4880
Tissue×EP	2,28	0.45	0.6417
Treatment×Tissue×EP	1,28	6.09	0.0200

Table A4. Result of the GLMM for *TFRC* expression

Effect	d.f.	*F*	*P*
Treatment	1,29	5.50	0.0260
Tissue	3,29	3.54	0.0268
EP	1,29	0.04	0.8475
Treatment×Tissue	3,29	3.36	0.0322
Treatment×EP	1,29	2.97	0.0956
Tissue×EP	3,29	3.53	0.0270
Treatment×Tissue×EP	1,29	0.60	0.4437

Table A5. Results from RefFinder algorithm for *HMBS*, *RPL13* and *TFRC* expression

Gene	RefFinder geometric mean	Delta Ct mean stddev	Best Keeper stddev	Normfinder Stability value	geNorm Stability value
HMBS	1.32 (1)	7.67 (1)	2.58 (1)	5.228 (2)	0.148 (1)
RPL13	6 (2)	7.98 (2)	2.93 (2)	4.633 (1)	2.265 (2)
TFRC	7.74 (3)	13.52 (3)	11.44 (3)	11.342 (3)	9 (3)

For each gene, RefFinder calculates the geometric mean and provides the mean standard deviation from the Delta Ct method, the standard deviation from Best Keeper and the stability value from Normfinder and geNorm. Values are presented with ranking in parentheses for each method.

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Competing interests
The authors declare no competing or financial interests.

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Conceptualization: H.E.H., C.Z., A.W.S., L.B.M.; Formal analysis: H.E.H., C.Z.; Investigation: H.E.H., C.Z., B.K., A.W.S., J.D.M.; Writing - original draft: H.E.H.; Writing- reviewing and editing: H.E.H., C.Z., B.K., A.W.S, J.D.M, L.B.M.; Project administration: L.B.M.

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Data availability
DNA sequences can be accessed via GenBank accession numbers MW557619 MW557649. Supporting data can be accessed at https://doi.org/10.6084/m9.figshare.13670119.v1.

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