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RESEARCH ARTICLE

High epigenetic potential protects songbirds against pathogenic *Salmonella enterica* infection

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ABSTRACT

Animals encounter many novel and unpredictable challenges when moving into new areas, including pathogen exposure. Because effective immune defenses against such threats can be costly, plastic immune responses could be particularly advantageous, as such defenses can be engaged only when context warrants activation. DNA methylation is a key regulator of plasticity via its effects on gene expression. In vertebrates, DNA methylation occurs exclusively at CpG dinucleotides and, typically, high DNA methylation decreases gene expression, particularly when it occurs in promoters. The CpG content of gene regulatory regions may therefore represent one form of epigenetic potential (EP), a genomic means to enable gene expression and hence adaptive phenotypic plasticity. Non-native populations of house sparrows (*Passer domesticus*) – one of the world's most cosmopolitan species – have high EP in the promoter of a key microbial surveillance gene, *Toll-like receptor 4* (*TLR4*), compared with native populations. We previously hypothesized that high EP may enable sparrows to balance the costs and benefits of inflammatory immune responses well, a trait critical to success in novel environments. In the present study, we found support for this hypothesis: house sparrows with high EP in the *TLR4* promoter were better able to resist a pathogenic *Salmonella enterica* infection than sparrows with low EP. These results support the idea that high EP contributes to invasion and perhaps adaptation in novel environments, but the mechanistic details whereby these organismal effects arise remain obscure.

KEYWORDS: Epigenetics, Phenotypic plasticity, DNA methylation, Invasion, Immunology

INTRODUCTION

As organisms move into new environments, they can be released from native pathogens and/or be exposed to many novel ones (Keane and Crawley, 2002; Lee et al., 2006, 2005; Liu and Stiling, 2006; Martin et al., 2010; Marzal et al., 2011; Torchin et al., 2001). One defense strategy is therefore unlikely to be amenable to all invasions and range expansions. Indeed, the most adaptive immune response could be the most flexible one (Prüter et al., 2020). Phenotypic plasticity – the ability of the same genome to produce a range of phenotypes – is an important mechanism by which populations can respond rapidly to changing conditions (Pigliucci et al., 2006; Snell-Rood et al., 2018). A better understanding of how selection acts on immune plasticity may therefore be important in understanding the molecular underpinnings of successful range expansions and introductions of individuals outside their native ranges.

Phenotypic plasticity can arise through various mechanisms including epigenetic ones such as DNA methylation (Feinberg, 2007). When DNA methylation occurs within regulatory genomic regions (e.g. gene promoters), it can affect phenotypic plasticity via its effects on the transcriptional regulation of gene expression (Bird, 2002; Lemire et al., 2015; Weaver et al., 2004; Zhi et al., 2013). In vertebrates, DNA methylation almost always occurs at the cytosine residue of CpG sites (i.e. adjacent cytosines and guanines linked by phosphates) on the DNA sequence (Feinberg and Irizarry, 2010). DNA methyltransferase enzymes can directly catalyze the addition and removal of methyl groups at a CpG site (Moore et al., 2013; Shi et al., 2021). Importantly, patterns of DNA methylation can be influenced by environmental factors such as pathogen exposure (Law and Holland, 2019; Qin et al., 2021).

The presence of many CpG sites in the promoter of a particular gene may present more opportunities for the *de novo* addition and/or removal of methyl groups. Thus, more CpG sites may represent more chances to adjust gene expression via the regulatory effects of DNA methylation on transcription (Kilvitis et al., 2017; Weber et al., 2007). In other words, more CpG sites may enable an individual to fine-tune or update its phenotype rapidly in response to fluctuating challenges, including changes in pathogen exposure (Levis and Pfennig, 2016; West-Eberhard, 2003). CpG content of promoters therefore represents a form of 'epigenetic potential', or 'EP' for short (Kilvitis et al., 2017).

Previously, we hypothesized that high EP would be favorable in range expansions because it facilitates phenotypic plasticity (Kilvitis et al., 2017). Multiple indirect tests of this hypothesis have supported the role of high EPin the global spread of house sparrows (*Passer domesticus*), one of the world's most successful, introduced vertebrate species (Hanson et al., 2020b,c). First, in the ongoing range expansion across Kenya, EP across a large fraction of the entire genome of house sparrows increased with distance from the site of initial introduction and was selectively favored towards the range edge (Hanson et al., 2022). Second, across the globe, EP was higher in introduced than in native house sparrow populations for two but not a third *Toll-like* receptor (*TLR*) gene (Hanson et al., 2020a). Finally, house sparrows from Tampa, FL, USA, with higher EP in an important microbial surveillance gene (*TLR4*) had greater inducibility and reversibility of *TLR4* expression during an immune challenge in blood, but in spleen and liver in the same birds, *TLR4* expression was higher in birds with low EP (Hanson et al., 2021).

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The expression of *TLR4* is among the most important elements of a protective immune response against bacterial infections (Coburn et al., 2007; Gou et al., 2012). In rodents and chickens, the expression of many factors such as galectin 8 (Hodges and Hecht, 2013), *NOD2*, *NLRP6* and *NLRC4* (Thaiss et al., 2016) can protect against bacterial infections including *Salmonella*. However, the expression of *TLR4* is among the most important elements of a protective immune response against this and other bacterial infections (Coburn et al., 2007). Mouse strains genetically deficient for *TLR4* are highly susceptible to *Salmonella enterica*; likewise, a single nucleotide polymorphism (SNP) in the exon of the ligand-binding domain of *TLR4*, which varies among chicken lines, can affect resistance to and mortality from the bacteria (Leveque et al., 2003). Most *TLR4* expression derives from macrophages and heterophils (Alkie et al., 2019), and these cells as well as lymphocytes are recruited in huge numbers to the gut once *TLR4* is activated and inflammation is initiated locally by gut pathogens. Dendritic cells, too, residing just below the gut epithelium, express abundant *TLR4*, and can further sculpt the local and systemic immune response against *S. enterica* and other infections (Ijaz et al., 2021).

High *TLR4* expression thus probably provides protection to infected hosts through various and dynamic means including enhanced phagocytosis and activation of several cell types, which often change over the course of the infection (Coburn et al., 2007; Gou et al., 2012). However, high *TLR4* expression can also cause significant host damage via inflammatory over-exuberance (Klein and Diamond, 2008; Kobasa et al., 2007). Indeed, inflammatory responses mediated by *TLR4* are among the most expensive and self-damaging immune responses available to vertebrates (Lee and Klasing, 2004; Martin et al., 2017). Plasticity in *TLR4* expression should be advantageous because it would balance the costs and benefits of an immune response against pathogens, especially for organisms colonizing areas where threats are evolutionarily novel. In the present study, we tested whether high EP in the *TLR4* promoter of house sparrows was associated with an individual bird's ability to resist (shed fewer bacteria in their feces as a result of a more effective gut immune response) and/or tolerate (maintain body mass while infected) a particularly pathogenic serovar of *Salmonella enterica* Typhimurium. This particular serovar has caused the deaths of thousands of individual passerines worldwide including British house sparrows (Lawson et al., 2014; Mather et al., 2016). Should high EP in *TLR4* in house sparrows be implicated in the control of a *S. enterica* infection, our study would link the patterns of EP in *TLR4* observed in wild native and invasive populations to individual performance and hence support a role for EP in range expansion success.

MATERIALS AND METHODS

House sparrow capture and housing

House sparrows [n=38: females n=11, males n=19, juveniles (sex unknown) n=8] were captured via mist nets from different locations across Tampa Bay (FL, USA) in June 2021. The body mass of each bird was recorded (to 0.1 g) at capture, and thereafter birds were kept in opaque cloth bags until transfer to the Biosafety-level 3 (ABSL-3) facility at the University of South Florida. At the ABSL3, birds were housed in individual cages (33×38×46 cm) surrounded by impervious covers (to reduce seed spillage and fecal transfer among cages) around the lower third of each cage. Cages were then placed next to each other in audial and visual contact inside a secondary containment system (bioBUBBLE, Fort Collins, CO, USA), which further ensured no aerosols or feces could circulate among birds. Food (mixed seeds) and water were provided *ad libitum* throughout the study. Before transferring birds into cages, aluminium foil was placed on the bottom of each cage to collect a fecal sample to determine *Salmonella* infection status at the beginning of the experiment. Because of space constraints of the bioBUBBLE system, the experiment was conducted in 4 cohorts of birds caught from the same population (cohort 1: n=10, cohort 2: n=7, cohort 3: n=13, cohort 4: n=8). There were no statistically significant differences in *Salmonella* burden prior to experimental exposure to the pathogenic serovar among cohorts (linear mixed model, estimate=−0.21, s.d.=0.22, t=−0.98, P=0.34) (for further details, see Figs S1, S2).

For the duration of the experiment, birds were checked twice daily, and any individual showing lethargy or other sickness behaviors was euthanized by isoflurane overdose and rapid decapitation. Four birds were euthanized upon detection of sickness (on days 10, 11, 12 and 13), and two birds were found dead on the morning of day 14, preventing the use of tissues from these birds for gene expression analysis. All remaining birds were euthanized 14 days after pathogenic *S. enterica* exposure. All procedures were approved by the USF Animal Care and Use Committee prior to the start of the study.

Experimental infection and the quantification of *S. enterica* burden over time

For infections, cryopreserved *S. enterica* known to be pathogenic for passerines (Hughes et al., 2008) was defrosted rapidly in a warm water bath and diluted to 10^7 colony-forming units (cfu) in phosphate-buffered saline (PBS). The particular *S. enterica* serovar used in this experiment, Typhimurium isolate 244, was isolated from a greenfinch (*Carduelis chloris*) in northern England in 2006 as part of the Garden Bird Health Initiative investigating 'die-offs' of passerine birds in the UK (Hughes et al., 2008). The isolate has a DT56 phage type and a sequence type (ST) 586 that was associated with invasive salmonellosis in several passerine species (including house sparrows) and shared a common genotype and pulsed field gel electrophoresis pattern indicating a specific epidemic strain associated with passerines (Hughes et al., 2010).

To achieve infections, birds were gavaged with disposable gavage needles with 100 µl of 10^7 cfu *S. enterica* followed by another 100 µl of PBS to flush the needle and ensure each bird received the full dose. This bacterial dosage was used in our experiments as a pilot study showed (i) that *S. enterica* could be detected post-infection in fecal samples, and (ii) that birds did not show overt signs of sickness or die quickly post-exposure. In other words, the choice of concentration for experimental infection was a compromise between the dose being infective but not causing extensive mortality while also being able to detect the bacterial burden in the feces with our qPCR method. It is also consistent with dosages used in comparable experiments (Connolly et al., 2006). The experimental exposure took place on the day of capture, as even short durations in captivity can lead to immune dysregulation in this species (Love et al., 2017; Martin et al., 2011).

On days 3, 6, 9, 12 and 14 after exposure to *S. enterica*, foil was again placed on the bottom of each cage to collect fecal samples over time. This method allowed us to quantify bacterial burden (i.e. the amount of *S. enterica* DNA detected in the feces) in each bird over the course of infection. After collection, each fecal sample was diluted (1:5 mass to volume) in PBS and kept at −80°C until the extraction of *S. enterica* DNA. To extract *S. enterica* DNA from fecal samples, a DNA/RNA-free bead was first added to each microtube with diluted feces, then each sample was agitated for 2 min at 2000 rpm on a Bead Mill 24 homogenizer (Fisherbrand). Then, 50 µl of each homogenate was processed for genomic DNA

extraction using a QIAmp Powerfecal pro DNA kit (Qiagen) following the manufacturer's protocol. *Salmonella enterica* burden in each fecal sample was then determined via quantitative real-time PCR (qPCR).

For qPCR, DNA from the same strain used to infect birds (isolate 244) was extracted from 100 µl of cultured bacteria (10^7) using a DNEasy Blood and Tissue kit (Qiagen) and quantified using a Qubit Fluorometer and Quant-iT dsDNA HS assay kit (Invitrogen). Following Park et al. (2008), bacterial DNA was diluted to 4×10^5, 4×10^4, 4×10^3, 4×10^2, 4×10^1 and 0 genome equivalents per 5 µl. Genome equivalents were calculated using the following equation: DNA genome equivalent=$(A\times6.022\times10^{23})(660\times B)^{-1}$, where A is the DNA concentration and B is the length of genomic DNA (Park et al., 2008). Primers and a FAM-probe (TaqMan) validated by Park et al. (2008) for the detection and quantification of *S. enterica* were then used on these standards to create a standard curve. All qPCR amplifications were performed in a total volume of 25 µl in duplicate on a Rotor-Gene Q system (Qiagen). Each reaction contained 12.5 µl of TaqMan master mix (TaqMan Universal PCR Master Mix, Applied Biosystems), 1 µl of each primer (10 µmol l^{-1}), 0.5 µl of probe, 5 µl of DNA and 5 µl of nuclease-free water. Thermal cycling conditions were a first-step for 2 min at 50°C followed by 10 min at 95°C, then a second step of 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence signals were measured at the end of the extension step for each cycle, and the *Salmonella* burden in each sample determined by relating Ct values from samples against the standard curve.

Controlling for pre-existing *Salmonella* burden

Because our study dealt with wild-caught birds, we expected that some birds could already be infected with *Salmonella* sp. or have been previously infected and recovered. While it is not possible to determine unequivocally whether birds have been previously infected and have cleared any *Salmonella* variant, we were able to determine whether birds were currently infected by at least some type of *Salmonella* sp. at the beginning of the experiment and take this pre-existing infection condition into account in our analyses. To do this, we collected a fecal sample at capture to quantify pre-experimental *Salmonella* sp. burden using qPCR. We included this pre-existing burden measure in all relevant analyses and treated it as a continuous rather than a binary 'infected versus uninfected' variable. Importantly, we used a specific serovar of *S. enterica* in our experiments. This serovar was isolated from a greenfinch in the UK in 2006. Thus, Tampa Bay house sparrows in our study were unlikely to have been pre-infected with this serovar because of geographic distance.

Quantification of body mass and *S. enterica* tolerance

The body mass of each bird (to 0.5 g) was also recorded at capture (before experimental infection), 24 h after infection, and 3, 6, 9, 12 and 14 days after infection. This approach allowed us to determine how body mass changed over the course of the infection. Comparing each individual's rate of change in body mass relative to its rate of change in *S. enterica* burden allowed us to estimate a form of each birds 'tolerance' to the infection (i.e. an individual's capacity to maintain body mass while infected) (Burgan et al., 2018). We also recorded the occurrence of sickness and mortalities during the experiment.

Quantification of EP

After euthanasia, the whole gut and a piece of the liver were collected and stored in RNA later at −80°C for less than 1 month. Samples were thawed, and DNA was extracted from ∼0.1 g of house sparrow liver tissue using a DNAEasy Blood and Tissue kit (Qiagen). Kilvitis et al. (2019) designed the primers used in this study to encompass the putative promoter region 726–1228 nucleotides upstream of the *TLR4* transcription start site, which includes regulatory regions and CpG sites that affect expression (Table S3). Each PCR reaction contained 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 run on a T100 Thermal Cycler (Bio-Rad). Cycling conditions included an initial denaturation at 95°C for 2 min followed by 35 cycles at 94°C for 40 s, annealing at 62°C for 40 s and extension at 72°C for 150 s, and a final extension at 72°C for 5 min. PCR products were purified using ExoSAP-IT (Affymetrix), and Sanger sequencing using BigDye Terminator technology with forward primers was conducted at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign (Urbana, IL, USA), on an Applied Biosystems 3730xl DNA Analyzer. We did not map the sequenced region to chromosomes or align sequences; neither action was necessary as a BLAST search of our regions indicated no homology with other loci. The chromatograms from DNA sequences were then analyzed manually on Unipro UGENE (Okonechnikov et al., 2012). When sequencing was ineffective (i.e. regions of the target sequence could not be analyzed), samples were re-sequenced. This re-sequencing was rare, and ultimately high-quality sequences were obtained for all birds.

All CpG sites in the putative *TLR4* promoter were quantified across all individuals, counting CpG sites on each homologous region (Hanson et al., 2021). In our study, across all birds, EP in *TLR4* ranged from 6 to 10 CpG sites (EP=6–10; Fig. S3): 76.3% of birds either had EP=7 or EP=8, and the remaining 23.7% of birds had EP=6, EP=9 or EP=10 (Fig. S3). In previous work that also quantified EP in *TLR4* in house sparrows from Tampa, we also found that 96.7% (30 out of 31 birds) of birds had either EP=7 or EP=8, and only one bird had EP=9 (Hanson et al., 2021). In this prior study, the binary form of EP was the best predictor of *TLR4* expression (Hanson et al., 2021). In addition to treating EP as a continuous variable, we also assessed whether 'CpG identity' was related to *TLR4* expression, asking whether the specific location of the CpG polymorphism(s) was associated with expression. However, EP as a binary variable was consistently the best predictor for *TLR4* expression (Hanson et al., 2021).

Quantification of *TLR4* expression

Whole gut tissue from each bird was left to thaw on a dissection board placed in a tray filled with ice. When thawed, each gut was opened along its length and the contents washed out with distilled water. The small intestine was then separated into three sections: proximal, medial and distal. From the middle portion of each section, we collected a transverse fragment (about 1 mm wide) and immediately placed it into a microtube on dry ice. We also collected a section from a cecal segment of the gut and processed it in the same way. All gut samples were then stored at −80°C until RNA extraction. RNA was extracted from each gut sample separately using a TRI-reagent extraction method; each extract was then diluted to 25 ng μl^{-1} (Hanson et al., 2021). From each extracted RNA sample, we measured *TLR4* mRNA abundance using one step RT-qPCR. All RT-qPCR reactions (20 µl) were run in duplicate alongside non-template controls (NTC) and no reverse transcriptase controls (NRT) on a Rotor-Gene Q system (Qiagen). Each reaction contained 10 µl of iTaq Universal SYBR Green One-Step Kit (Bio-Rad), 0.3 µl of forward primer (10 µmol l^{-1}), 0.3 µl of reverse primer (10 µmol l^{-1}), 0.25 µl of reverse transcriptase (or 0.25 µl

nuclease-free water for NRTs), 7.15 µl of nuclease-free water and 2 µl of diluted RNA (or 2 µl of nuclease-free water for NTCs).

Thermal cycling conditions were: 10 min at 50°C for the 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. Melt-curve analyses were performed from 65 to 95°C with 0.5°C increments every 3 s. A calibrator (i.e. a mix of RNA from a homogenate of the four different gut samples from four individuals) and an internal reference gene (*hydroxymethylbilane synthase*, *HMBS*) (Zimmer et al., 2021) were run on all plates to calculate mRNA abundance using the comparative Ct method ($2^{\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). *TLR4* expression could not be estimated in four birds because of RT-qPCR failures.

Statistical analyses

All statistical analyses were conducted in R 4.1.2 (http://www.R-project.org/). Using the R packages 'olsrr' and 'lmtest', we visually inspected plots of residual versus fitted values and conducted Shapiro–Wilk and non-studentized Breusch–Pagan analyses. These tests indicated that the residuals of our three regression analyses (Tables 1, 2 and 3) were normally distributed and homoscedastic (Razali and Wah, 2011) (Table S1). Because we observed tissue-specific effects in our previous study (Hanson et al., 2021), we examined whether *TLR4* expression was affected by gut region, EP in *TLR4*, or their interaction using a linear mixed model (LMM) (Table 3). In this model (Table 3), *TLR4* expression was included as the dependent variable and gut region (i.e. proximal, medial, distal and cecal), EP (i.e. high or low) and their interaction were included as fixed effects.

Association between *S. enterica* resistance, EP and *TLR4* expression

Our first goal was to investigate whether EP in *TLR4* and/or *TLR4* expression was related to resistance of *S. enterica*. Here, we quantified resistance as the ability of individuals to limit the absolute amount of and the increase in *S. enterica* shed in their feces over time. We used the lme4 package in R to conduct our first LMM with *S. enterica* burden as the dependent variable (Table 1). We included EP in *TLR4* (treated as a binary term, i.e. high or low EP), *TLR4* expression (averaged across four gut tissues, as EP in *TLR4* effects on *TLR4* expression were not dependent on gut region; see Results), and their interaction with day of sampling as fixed effects. This approach allowed us to simultaneously detect the effects of EP in *TLR4* and *TLR4* expression on absolute *S. enterica* burden (at each day of sampling) and on the change in *S. enterica* burden over time. In all our models, we also included body mass at capture and pre-existing *Salmonella* burden as fixed effects. In all models, we also included the status of the bird (male, female or juvenile) as a fixed effect, and bird ID as a random effect to account for within-individual differences.

Association between *S. enterica* tolerance, EP and *TLR4* expression

Our second goal was to assess whether EP in *TLR4* and/or *TLR4* expression was associated with tolerance of *S. enterica*. Here, we quantified tolerance as the relationship between the body mass of a bird and its *S. enterica* burden over the course of the infection (an approach similar to that which we used for West Nile virus responses in this species; Burgan et al., 2018; Kernbach et al., 2019). We characterized individuals better able to maintain body mass while infected as more tolerant of the *S. enterica* infection. We fitted this second LMM with body mass as the dependent variable (Table 2). We included EP in *TLR4*, average *TLR4* expression, and their interaction with *S. enterica* burden^2 as fixed effects. We fitted a quadratic effect of burden (i.e. *S. enterica* burden^2) as a fixed effect and an interaction term (instead of an untransformed value of *S. enterica* burden) because *S. enterica* effects on body mass appeared to be non-linear (results of the untransformed value of *S. enterica* burden are presented in Table S2). This approach allowed us to simultaneously detect potential effects of EP in *TLR4* and *TLR4* expression on body mass (at each day of sampling) and the rate of change in body mass with burden over time.

Association between house sparrow mortality, *S. enterica* infection, EP and *TLR4* expression

In an additional analysis, we asked how EP was related to mortality by *S. enterica*. We used a multivariate Cox proportional hazard regression to assess whether the probability of death was associated with EP, average *TLR4* expression across all tissue types in the gut, average *S. enterica* burden across the entire infection (this model was also re-run with maximum *S. enterica* burden with analogous results), the status of the bird (male, female or juvenile) and absolute body mass lost over the experiment (Table 4). The interaction between EP and *S. enterica* burden was also included, as this allowed us to detect potential effects of EP on mortality in relation to *S. enterica* burden. Additionally, the interaction between absolute body mass loss and *S. enterica* burden was included so that we could test whether mortality was associated with burden-related body mass changes.

Table 1. Effects of epigenetic potential and *TLR4* expression on resistance to experimental *Salmonella enterica* exposure in house sparrows

Dependent variable: *S. enterica* burden (log_{10} genomic equivalents)					
	Estimate (s.e.)	*t*-value	*P*-value	Variance	s.d.
Fixed effects					
EP (low)	−0.793 (0.572)	−1.384	0.169		
Day of sampling	0.187 (0.041)	4.483	<0.001*		
Average *TLR4* expression	0.089 (0.133)	0.669	0.505		
Pre-existing *Salmonella* burden	0.058 (0.091)	0.636	0.531		
Body mass at capture	−0.083 (0.087)	−0.951	0.351		
Juvenile status	−0.202 (0.410)	−0.495	0.625		
Male status	0.108 (0.319)	0.340	0.737		
EP (low)×Day	0.150 (0.058)	2.599	0.011*		
Average *TLR4* expression×Day	−0.021 (0.012)	−1.686	0.095		
Random effects					
Bird ID				1.021	1.011
Marginal R^2=37.02, Conditional R^2=47.52					

Significant effects are indicated by an asterisk. Bird ID was modeled as a random term. *N*=34 birds. EP, epigenetic potential.

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Table 2. Effect of EP in *TLR4* and *TLR4* expression on *S. enterica* tolerance in house sparrows

	Dependent variable: mass (g)				
	Estimate (s.e.)	*t*-value	*P*-value	Variance	s.d.
Fixed effects					
EP (low)	−0.907 (0.615)	−1.475	0.148		
S. enterica burden^2	−0.164 (0.069)	−2.363	0.021*		
Average *TLR4* expression	0.201 (0.143)	1.401	0.169		
Pre-existing *Salmonella* burden	−0.505 (0.297)	−1.700	0.103		
Mass at capture	0.489 (0.168)	2.897	0.007*		
Juvenile	−0.365 (0.786)	−0.465	0.645		
Male	0.600 (0.625)	0.959	0.345		
EP×*S. enterica* burden^2	0.067 (0.040)	1.643	0.106		
TLR4×*S. enterica* burden^2	−0.029 (0.036)	−0.809	0.422		
Random effects					
Bird ID				1.6727	1.2933
Marginal R^2=0.2769245, Conditional R^2=0.78997					

Tolerance was defined as maintenance of body mass within individuals across varying levels of *S. enterica* infection. Significant effects are indicated by an asterisk. Bird ID was modeled as a random term. *N*=34 birds. ^2 indicates quadratic effects.

RESULTS

The effects of EP in the *TLR4* promoter on *TLR4* expression were not dependent on gut region (Table 3). Therefore, to simplify our models, we included average *TLR4* expression across all gut regions for each individual bird in our LMMs. Just over half (*n*=21) of the birds were infected with a detectable form of *Salmonella* sp. at the time of capture (i.e. prior to experimental infection; Fig. 1). However, pre-existing *Salmonella* infection was not related to EP in *TLR4* (low EP: estimate: −0.29451, s.d.=0.258, *t*=−1.141, *P*=0.256), *TLR4* expression (*TLR4* expression: estimate: 0.139, s.d.=0.218, *t*=0.43400, *P*=0.529), resistance or burden in our study (Tables 1 and 2). Pre-existing infection was also not associated with bird body mass at capture (estimate=−0.169, *t*=0.331, *P*=0.492).

Association between *S. enterica* resistance, EP and *TLR4* expression

Low EP in *TLR4* was positively associated with a faster rate of *S. enterica* burden increase over time, indicated by the positive effect of the interaction between low EP and day of sampling on *S. enterica* burden (Table 1, Fig. 1). High EP birds were therefore more resistant than low EP birds. The status of the bird (male, female or juvenile), body mass at capture and pre-existing *Salmonella* burden were unrelated to *S. enterica* infection dynamics (Table 1). Bird ID explained 10.5% of variance in *S. enterica* burden whereas the majority (37.0%) of variance was explained by the fixed effects (Table 1). These patterns were analogous when treating EP as a continuous variable.

Association between *S. enterica* tolerance, EP and *TLR4* expression

House sparrow body mass was affected by exceptionally high *S. enterica* burden, indicated by the negative quadratic effect of *S. enterica* bacterial burden on absolute mass loss (Table 2, Fig. 2). However, this effect was not dependent on EP in *TLR4* (Table 2). Similarly, body mass also tended to decrease with increasing *S. enterica* burden when it was not treated as a quadratic effect (i.e. when non-quadratic values of *S. enterica* burden were used), but this effect was non-significant (Table S2). Body mass at capture had an effect on body mass change over the course of the experiment, with smaller birds tending to lose more mass than large birds (Table 2). Bird ID explained 27.69% of variance in body mass in the model; fixed effects explained 51.30% (Table 2).

Association between EP and *TLR4* expression across the gut

TLR4 expression differed among gut regions; expression was higher in the cecum and distal regions than in the proximal and medial regions (Table 3). EP in the *TLR4* promoter also affected *TLR4*

Table 3. Effect of EP in *TLR4*, gut region and their interaction on *TLR4* expression in house sparrows 14 days after experimental infection with *S. enterica*

	Dependent variable: *TLR4* expression (relative quantity)				
	Estimate (s.e.)	*t*-value	*P*-value	Variance	s.d.
Fixed effects					
TLR4-EP (low)	1.782 (0.724)	2.458	0.017*		
Medial tissue	0.270 (0.398)	0.679	0.499		
Distal tissue	0.908 (0.401)	2.261	0.026*		
Cecum tissue	1.257 (0.405)	3.099	0.003*		
Juvenile	−0.186 (0.932)	−0.200	0.843		
Male	−0.405 (0.735)	−0.551	0.586		
Final *Salmonella* burden	0.139 (0.218)	0.636	0.529		
EP (low)×Medial tissue	−0.518 (0.617)	−0.840	0.403		
EP (low)×Distal tissue	−0.054 (0.619)	−0.088	0.930		
EP (low)×Cecum tissue	−0.493 (0.619)	−0.797	0.428		
Random effects					
Bird ID				2.590	1.610
Marginal R^2=0.175, Conditional R^2=0.711					

Significant effects are indicated by an asterisk. Bird ID was modeled as a random term. *N*=34 birds.

Journal of Experimental Biology (2023) 226, jeb245475. doi:10.1242/jeb.245475

Table 4. Results of a multivariate Cox proportional hazards model describing the effects of EP in *TLR4*, average *S. enterica* burden, average body mass loss, their interactions and bird status on mortality of house sparrows experimentally infected with *S. enterica*

	Dependent variable: mortality		
	Estimate (s.e.)	*z*-value	*P*-value
Fixed effects			
EP (low)	0.014 (1.079)	0.012	0.990
Average *S. enterica* burden	0.135 (0.541)	0.250	0.803
Average *TLR4* expression	−0.020 (0.101)	−0.199	0.842
Juvenile	0.428 (1.535)	0.765	0.445
Male	0.131 (0.425)	0.306	0.759
Absolute mass loss	0.155 (0.322)	0.480	0.631
EP×Average *S. enterica*	−0.116 (0.603)	−0.193	0.847
Average *S. enterica*× Absolute mass loss	0.072 (0.137)	0.527	0.598

Significant effects are indicated by an asterisk. Bird ID was modeled as a random term. *N*=34 birds.

expression; sparrows with high EP expressed lower levels of *TLR4* (mean=1.613, range=0.01–9.221, s.d.=1.445) than birds with low EP (mean=2.992, range=0.08–9.57, s.d.=2.546) (Fig. 3, Table 3), but this EP effect did not differ among gut regions (Table 3). Within-individual differences (i.e. bird ID) explained 36.23% of the variance in *TLR4* expression whereas fixed effects explained 30.66% of the variance (Table 3).

Association between house sparrow mortality, *S. enterica* infection, EP and *TLR4* expression

Of the 38 birds in our experiment, only six died. A multivariate Cox proportional hazard regression revealed that mortality was not related to EP in *TLR4*, average (or maximum) *S. enterica* burden or their interaction. Mortality was also not associated with absolute body mass or its interaction with *S. enterica*, *TLR4* expression or the status of the bird (male, female or juvenile) (Table 4).

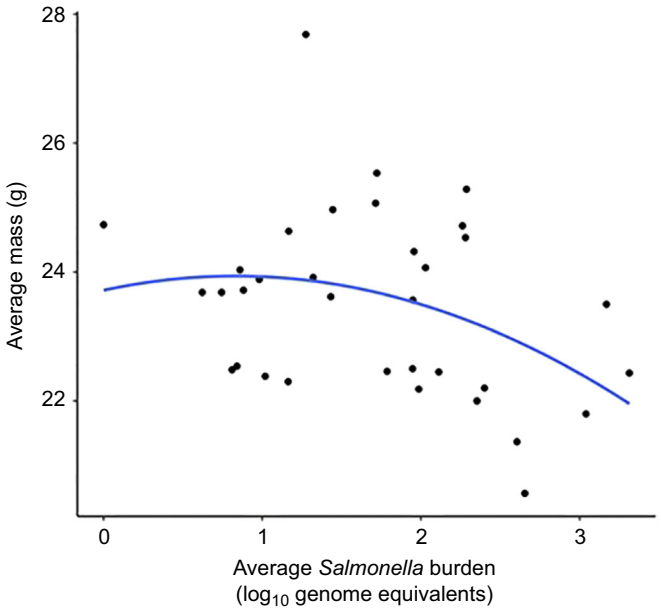

Fig. 2. House sparrow body mass in relation to *Salmonella enterica* burden. Average house sparrow body mass over the course of the infection was affected by exceptionally high *S. enterica* burden (log_{10} genome equivalents^2, where ^2 indicates quadratic effects) (LMM, *n*=34 birds).

DISCUSSION

The central aim of our experiment was to investigate whether one form of EP, the number of CpG sites in the promoter region of *TLR4*, affected the capacity of house sparrows to resist, tolerate or survive a

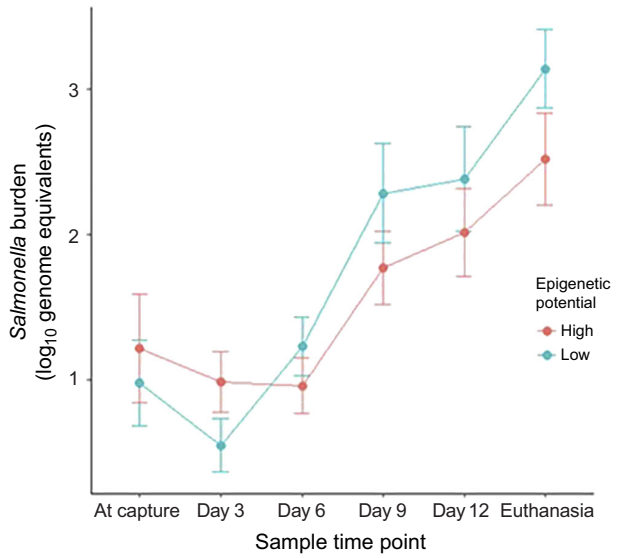

Fig. 1. Changes in *Salmonella* burden over time in experimentally infected house sparrows. High epigenetic potential (EP) in *TLR4* was associated with a slower rate of increase in shedding of *Salmonella* in feces across the experiment (i.e. higher resistance to *Salmonella* infection). Data are means±1 s.e.m. from LMM for *Salmonella* burden (i.e. log_{10} genome equivalents) on each day of feces collection (*n*=34 birds). Euthanasia occurred on day 14 of the experiment.

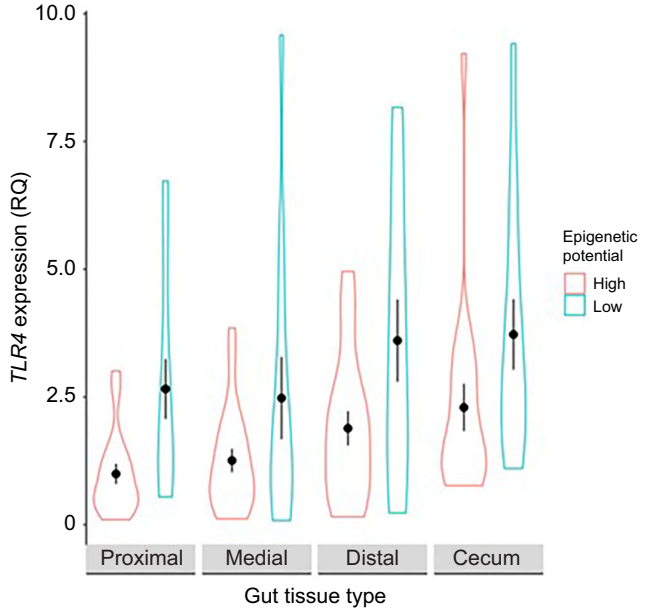

Fig. 3. Association between EP and *TLR4* expression across the gut. *TLR4* expression was higher in low EP house sparrows, but EP effects were not dependent on gut region. However, *TLR4* was expressed more in the distal and cecal portions of the gut than in the other two regions. Data are means±1 s.e.m. from LMM for the relative quantification (RQ) of *TLR4* expression (*n*=34 birds).

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pathogenic *Salmonella* infection. We found that birds with high EP in *TLR4* shed fewer bacteria than low EP birds. As bacteria shed in feces likely relates to the burden in the gut at or near the time of fecal sampling, high EP in *TLR4* was thus related to higher host resistance to this pathogen. In showing that high EP is associated with host resistance, our results support the hypothesis that EP in *TLR4* could be an important target for pathogen-driven selection. That said, tolerance and mortality were not associated with EP in *TLR4* in our study; indeed, body mass was generally unaffected by *S. enterica* until the shed bacteria reached very high levels (>3 log) and mortality overall was very low for what was a pathogen to English house sparrows. As we did not measure methylation because our study design was not conducive to capturing the expected dynamic interplay among EP, methylation, gene expression and *S. enterica* resistance, we cannot be sure that EP operates in the manner our hypothesis portrays it to do. Nevertheless, our study provides empirical and conceptual support for the idea that one form of EP contributes to the efficacy of control of an important pathogen, a finding that supports our hypothesis about why EP is important in range expansions of this species (Hanson et al., 2021, 2022; Kilvitis et al., 2017). Below, we discuss the potential ecological and immunological ramifications of these results for this and other range-expanding animals.

TLR4 expression is a key element of the vertebrate immune response to *S. enterica*

Salmonella enterica serotype Typhimurium is a Gram-negative bacterial taxon that has a major impact on human, wildlife and livestock health (Mahmoud, 2012; Malik et al., 2021). It can infect and be transmitted by many host species, typically after exposure in food or water, and it is most often transmitted directly via excretion in feces but also via other routes (i.e. persistence in the soil or on surfaces for weeks to months) (Hilbert et al., 2012; Tizard, 2004). Significant to this study, the *S. enterica* serovar studied here has been responsible for large die-offs of wild birds in the past (Hughes et al., 2008, 2010). In rodents and chickens, many factors (Thaiss et al., 2016) can protect against bacterial infections including *Salmonella*, but the expression of *TLR4* is among the most important (Coburn et al., 2007). Exactly which of the above mechanisms described in the Introduction (i.e. the types of leukocytes conferring protection against *Salmonella*, methylation of CpGs among and within those cell types, or both) was potentiated by high EP in *TLR4* is unknown. Nevertheless, EP played an important role in the control of this pathogen. More work will be necessary to elucidate the complex molecular and cellular mechanisms whereby EP provides protection.

Could high EP defend against *Salmonella* via the dynamic regulation of *TLR4*?

Whereas our study showed that high EP in *TLR4* was linked to high *S. enterica* resistance, it also showed that high EP in *TLR4* was linked to low *TLR4* expression in the gut tissue measured at the end of our experiment. This result conflicts with observations we have made in blood, but it partly resembles the patterns observed in spleen and liver (Hanson et al., 2021). The current results are also intriguing because high *TLR4* expression is more commonly associated with bacterial resistance in chickens (Gou et al., 2012). However, our hypothesis did not predict that high EP in *TLR4* would be protective because it imbues high, constitutive expression of *TLR*. Rather, we expected that it would facilitate *TLR4* expression plasticity (perhaps including reversibility) by increasing the potential for DNA methylation modification. In other words, we predicted that high EP in *TLR4* is associated with *Salmonella* resistance through a greater propensity to tune *TLR4* expression (increase and decrease) over the course of an infection, rather than enduringly elevating it. Indeed, in the previous study on blood (Hanson et al., 2021), this higher reversibility is exactly what we observed and what we would expect here too had our study design allowed us to describe *TLR4* expression dynamics (and associated methylation) in the gut and other relevant lymphoid tissues or cells. However, the heterogeneity of effects of EP across cell types suggests a new but not altogether incompatible mechanistic possibility for EP: EP might enable cell types to have different and perhaps more suitable levels of gene expression for pathogen control.

Presently, we favor the possibility that *TLR4* expression reversibility is the adaptive/functional mechanism because the ability to flexibly regulate *TLR4* expression should better balance the costs and benefits of an immune response against a pathogen. However, we were only able to measure *TLR4* expression in the gut once, 14 days after the onset of an infection (because of the destructive nature of sampling the gut tissue), so we cannot really test this idea in this study. Indeed, further experimentation – for example, sampling the gut from different birds at different stages of an infection – will be critical to revealing the specific pathway by which EP may have affected *S. enterica* resistance. Such a study could also help elucidate the specific manner by which methylation mediates the effect of EP on *Salmonella* resistance. As many types of leukocytes will be integral to successful control of the bacteria, it will be important to consider both the timing post-infection and cell/tissue type in any measurements of methylation. It will be useful to test directly whether EP enables a given cell to express an optimal amount of *TLR4* as it encounters or receives signals about *Salmonella* from other host cell types and/or if cell types vary in their propensity to upregulate and downregulate gene expression via DNA methylation.

Ecological implications of EP in *TLR4*

Our results indicate that EP in *TLR4* protects Tampa house sparrows from *S. enterica* by enhancing resistance, but not to the point of sterilizing the gut of this pathogen. High levels of EP among range-expanding/non-native house sparrows (Hanson et al., 2021) may also afford certain sparrow populations with an indirect means to outcompete resident host species, exposing their competitors to microbes that cause no pathogenic effects to themselves but potentially harm these other host species (Coon and Martin, 2014; Martin et al., 2010, 2014, 2017, 2015). Indeed, house sparrows have already been implicated as potential progenitors of *Salmonella* epidemics that have drastically reduced the population sizes of other birds (e.g. greenfinches) (Hernandez et al., 2016; Tizard, 2004). Somewhat surprisingly, too, most sparrows in this study were able to tolerate and survive infection with the same microbe that was lethal to other songbirds including European house sparrows. Perhaps this outcome is a vestige of past selection for *TLR4* EP in North American birds, which occurred at the time of their introduction from ancestral Europe.

Conclusions and further work

Besides investigating EP in other genes and other invading or range-expanding species, it would be beneficial to resolve how *TLR4* expression and symbiosis in the gut are interrelated. For example, gut microbiota-derived metabolites (e.g. short chain fatty acids) can modulate gut transcriptional output by affecting CpG methylation in *TLR4* (Takahashi et al., 2011). High EP in *TLR4* could thus

potentially facilitate microbiota-induced epigenetic changes in sparrows by providing more genomic substrate for modification of methylation profiles. Host cells largely regulate their sensitivity to commensal microbes via DNA methylation and its subsequent effects on defensive gene expression (Thaiss et al., 2016). As host intestinal epithelial cells (IECs) form a physical barrier with, sense signals from, and secrete peptides directed at microbes (Alenghat and Artis, 2014), the potential epigenetic mechanisms whereby IECs and other host cells cope with resident Gram-negative bacteria might have been exploited by some individual birds to affect how they combat gut pathogens (Takahashi et al., 2011). Although the mechanisms whereby EP fostered resistance and the selective value of EP remain unresolved, here we have shown that EP in *TLR4* was positively associated with *Salmonella* resistance in the house sparrow. Our findings support the hypothesis that variation in EP could be adaptive for hosts encountering novel and dynamic pathogen risk scenarios (Hanson et al., 2022, 2021), but much more mechanistic work, especially focused on methylation among various lymphoid tissues, is crucial.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.Z., H.H., B.K., L.B.M.; Methodology: C.Z., H.H., B.K., L.B.M.; Validation: C.Z., H.H., B.K., L.B.M.; Formal analysis: E.S.; Investigation: C.Z., H.H., B.K., A.S., D.R., P.W., A.L.W., L.B.M.; Resources: P.W., L.B.M.; Data curation: E.S., C.Z.; Writing - original draft: E.S., C.Z.; Writing - review & editing: E.S., C.Z., H.H., A.S., D.R., P.W., A.L.W., L.B.M.; Visualization: E.S.; Supervision: C.Z., L.B.M.; Project administration: C.Z., L.B.M.; Funding acquisition: L.B.M.

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Data availability
Data are available from figshare at https://doi.org/10.6084/m9.figshare.23596692.

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