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House sparrows with high epigenetic potential in the Toll-like receptor 4 promoter are better able to resist a 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. The CpG content of gene regulatory regions may therefore represent one form of epigenetic potential (EP), a genomic means to capacitate gene expression. 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 to native populations. We previously hypothesized that high EP may enable sparrows to balance the costs and benefits of 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 TLR4 were better able to resist a pathogenic Salmonella enterica infection than sparrows with low EP. These results support the premise that high EP contributes to adaptation in novel environments.

INTRODUCTION

As organisms move into new environments, they can be released from native pathogens and/or be exposed to many novel ones (Marzal, et al., 2011; Lee et al., 2005; 2006; Martin et al., 2010). 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 (Zhi et al. 2013; Lemire et al. 2015; Bird 2002; Weaver et al. 2004). In vertebrates, DNA methylation almost always occurs at the cytosine residue of CpG sites (i.e., adjacent cytosines and guanines linked by phosphates) on

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the DNA sequence (Feinberg and Irizarry 2010). DNA methyltransferase enzymes can directly catalyze the addition of a methyl group to a CpG site, which can subsequently be removed by 'erasers' (Moore et al., 2013). Importantly, patterns of DNA methylation can be influenced by environmental cues such as pathogen exposure (Qin et al., 2021; Law and Holland, 2019).

Many CpG sites in the promoter of a particular gene may represent more opportunities for the de-novo addition and/or removal of methyl groups. Thus, more CpG sites may represent more opportunities to adjust gene expression via the regulatory effects of DNA methylation on transcription (Weber et al., 2007; Kilvitis et al., 2017). 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 (West-Eberhard 2003, Levis and Pfennig 2016). CpG content of promoters therefore represents one form of 'epigenetic potential', or 'EP' for short (Kilvitis et al., 2017) (it is recognized that other forms of EP may exist e.g., those concerning histone modification, chromatin structure, or the position, rather than the number of CpG's in the gene promoter).

Previously, we hypothesized that high EP (high promoter CpG content) is favorable in range expansions because it facilitates phenotypic plasticity (Kilvitis et al., .2017). Multiple indirect tests of this hypothesis have been supportive in the global spread of house sparrows, one of the world's most successful, introduced vertebrate species (Hanson et al. 2020b, Hanson et al. 2020c). First, in the ongoing range expansion of house sparrows across Kenya, EP across a large fraction of the entire genome increased with distance from the site of initial introduction and seemed to be selectively favored towards the range edge (Hanson et al., 2022). Second, across the globe, EP was higher in introduced than native house sparrow populations in the promoter of *TLR4* (Hanson et al. 2020a). Third, house sparrows (*Passer domesticus*) with higher CpG content in the regulatory region of an important microbial surveillance gene (*Toll-like receptor 4* (*TLR4*)) had greater inducibility and reversibility of *TLR4* expression during an immune challenge (Hanson et al., 2021)

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). However, *TLR4* expression is also associated with significant host damage via inflammatory over-exuberance (Klein and Diamond, 2008; Kobasa et al., 2007). Selection for plasticity in *TLR4* expression may thus act to balance the costs and benefits of an immune response against pathogens. In the present study, we tested whether high EP in the *TLR4* promoter was associated with an individual's ability to resist (shed less bacteria in their feces due to a more effective gut immune response) and/or tolerate (maintain body mass while infected) a particularly pathogenic serovar of*Salmonella enterica* Typhimurium, a serovar that has caused the mortalities 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 *Salmonella* 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.

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 (Florida, 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 x 38 x 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, Colorado, 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, aluminum foil was placed on the bottom of each cage to collect a fecal sample in order to determine*Salmonella* infection status at the beginning of the experiment. Due to the space constraints of bioBUBBLEs, the experiment was conducted

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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 with the pathogenic serovar among cohorts (linear mixed model, estimate = -0.21 (StDev =0.22), t=-0.98, p=0.34) (Supplementary figure 2).

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 the morning of day 14, preventing the use of tissues from these birds for gene expression analyses. 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 United Kingdom (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).

At 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 *Salmonella* 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 minutes 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 protocol. *S. enterica* burden in each fecal sample was then determined via quantitative Real-Time qPCR (qRT-PCR).

For qRT-PCR, 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 detection and quantification of *S. enterica* were then used on these standards to create a standard curve. All qRT-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 mastermix (TaqMan Universal PCR Master Mix, Applied Biosystems), 1 µl of each primer (10 µmol l), 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

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min at 50@C followed by 10 min at 95@C, then a second step of 40 cycles of 95@C for 15 sec and 60@C for 1 min. Fluorescence signals were measured at the end of the extension step for each cycle, and burden in each sample determined by relating Cts 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 whether birds have been previously infected and 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 qRT-PCR. We included this pre-existing burden measure in all relevant analyses, and treated it as a continuous rather than a binary 'infected vs 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 be pre-infected with this particular serovar due to geographic distance

Quantification of body mass and *S. enterica* tolerance

The body mass of each bird was also recorded at capture (before experimental infection), 24 hours 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 *Salmonella* 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 Epigenetic Potential (EP)

After euthanasia, the whole gut was collected and stored in RNA later at -80°C for less than one 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 to 1228 nucleotides upstream of the *TLR4* transcription start site, which includes regulatory regions and CpG sites that affect expression (Supplementary Table 3). Each PCR reaction contained 12.5 µl of 2× PCR Master Mix (Promega), 1 µl forward primer (10 µmol l), 1 µl reverse primer (10 µmol l), 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 94degC for 40 s, annealing at 62degC for 40 s and extension at 72degC for 150 s, and a final extension at 72degC 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 resequencing 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 chromosome separately (Hanson et al. 2021). In our study, across all birds, EP in *TLR4* ranged from six to ten CpG sites (Supplementary Figure 3): 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 (Supplementary Figure 3). In previous work that also quantified EP in *TLR4* in house sparrows from Tampa, FL, USA, and there 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, Hanson et al. (2021) also assessed whether 'CpG identity' was related to *TLR4* expression; asking if the specific location of the CpG polymorphism(s) was associated

with expression. However, EP as a binary variable consistently was the best predictor for *TLR4* expression (Hanson et al., 2021).

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Quantification of *TLR4* expression

Whole gut 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 -80degC 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 qRT-PCR. All qRT-PCR 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), 0.3 µl of reverse primer (10 µmol l), 0.25 µl of reverse transcriptase, 7.15 µl of nuclease free water, and 2 µl of diluted RNA or 2 µl of nuclease free water for NTCs. For NRTs, reverse transcriptase was replaced by nuclease free water.

Thermal cycling conditions were: 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. Melt-curve analyses were performed from 65 to 95 *C with 0.5 *C increment step every 3 s. A calibrator (i.e., a mix of RNA from an 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 ($2^{\Delta\Delta^{\hat{}}\tau}$) (Livak and Schmittgen 2001). *TLR4* expression could not be estimated in four birds due to qRT-PCR failures.

Statistical analyses

All statistical analyses were conducted in R 4.1.2 (R Development Core Team, 2021). Using the R packages 'olsrr' and 'lmtest', we visually inspected residual vs fitted values plots 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) (Supplementary Table 1). Because tissue specific effects have been observed in a 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, Figure 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 were 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 below), 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.

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

Our second goal was to assess whether EP in *TLR4* and/or *TLR4* expression were 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 have 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 linear mixed model 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 fit 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 Supplementary Table 2). 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 rerun 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.

RESULTS

The effects of EP in*TLR4* 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 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, Supplementary Figure 1). However, pre-existing*Salmonella* infection were not related to EP in *TLR4* (Low EP: estimate: -0.29451 (SD = 0.258), t = -1.141, p = 0.256), *TLR4*expression (*TLR4* expression: estimate: 0.139 (SD = 0.218), t = 0.43400, p= 0.529), resistance or burden in our study (Table 1, 2).

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, Figure 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.

Table 1. Effects of epigenetic potential and *TLR4*expression on resistance to experimental *S. enterica* exposure in house sparrows. Significant effects are indicated by an asterisk (*). Bird ID was modeled as a random term. SE = standard error, SD = standard deviation. N = 34 birds, Av = average.

Dependent variable: *S.enterica* burden ($\log_{10}$ genomic equivalents)	Dependent variable: *S.enterica* burden ($\log_{10}$ genomic
Fixed effects	estimate (SE)
Epigenetic potential (low)	-0.793 (0.572)

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Day of sampling	0.187 (0.041)
Av. *TLR4* expression	0.089 (0.133)
Pre-existing *Salmonella* burden	0.058 (0.091)
Body mass at capture	-0.083 (0.087)
Juvenile status	-0.202 (0.410)
Male status	0.108 (0.319)
Epigenetic potential (low) * Day	0.150 (0.058)
Av. *TLR4* expression*Day	-0.021 (0.012)
Random effects	variance
Band ID	1.021
	marginal R^2
	37.02

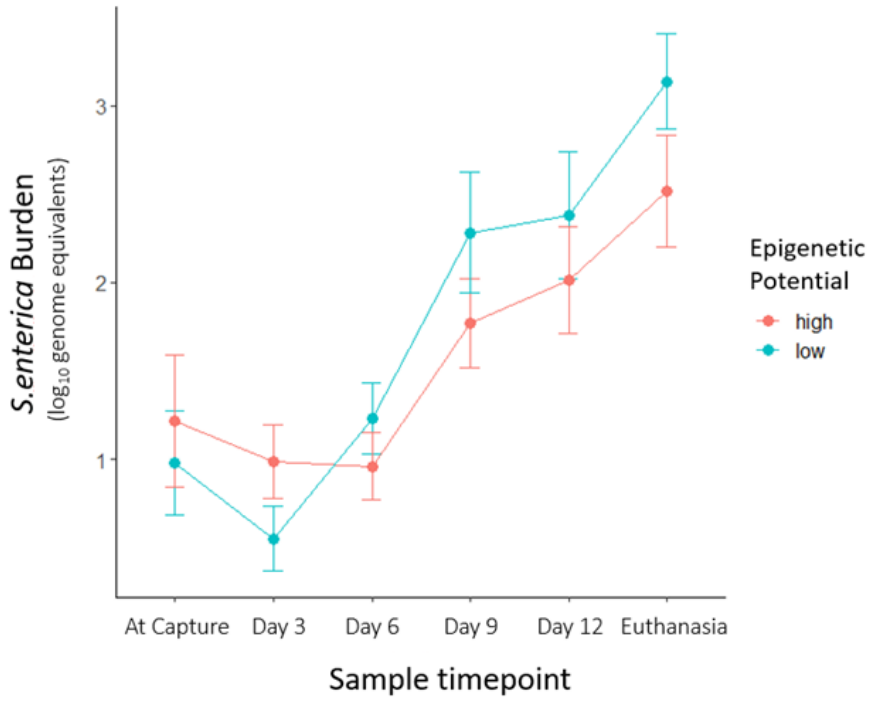

Figure 1. Changes in *S. enterica* burden over time in experimentally infected house sparrows. High EP in *TLR4* was associated with a slower rate of increase in shedding of *S. enterica* in feces across the experiment (i.e., higher resistance to*S. enterica* infection). Error bars depict means +/- 1 SE for*S. enterica* burden (i.e., $\log_{10}$ genome equivalents) at each day of feces collection. 'Euthanasia' occurred on day 14 of the experiment.

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; Figure 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 (Supplementary Table 2). Body mass at capture had an effect on body mass change over the course of the experiment with smaller birds tending to

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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).

Table 2. Effect of epigenetic potential in *TLR4* and *TLR4* expression on *S. enterica* tolerance (i.e., maintenance of body mass within individuals across varying levels of *S. enterica* infection) in house sparrows. Significant effects are indicated by an asterisk (*). Bird ID was modeled as a random term. SE = standard error, SD= standard deviation. N =34 birds, Av =average,

Dependent variable: Mass (grams)	Dependent variable: Mass (grams)	Dependent variable: Mass (grams)	Dependent v
Fixed effects	estimate (SE)	t-value	*p*-value
Epigenetic Potential (low)	-0.907 (0.615)	-1.475	0.148
S. enterica burdenˆ2	-0.164 (0.069)	-2.363	0.021*
Av. *TLR4* expression	0.201 (0.143)	1.401	0.169
Pre-existing *S.enterica* 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* burdenˆ2	0.067 (0.040)	1.643	0.106
*TLR4***S. enterica* burdenˆ2	-0.029 (0.036)	-0.809	0.422
Random effects	variance	SD	
Band ID	1.6727	1.2933	
	marginal R2	conditional R2	
	0.2769245	0.78997	

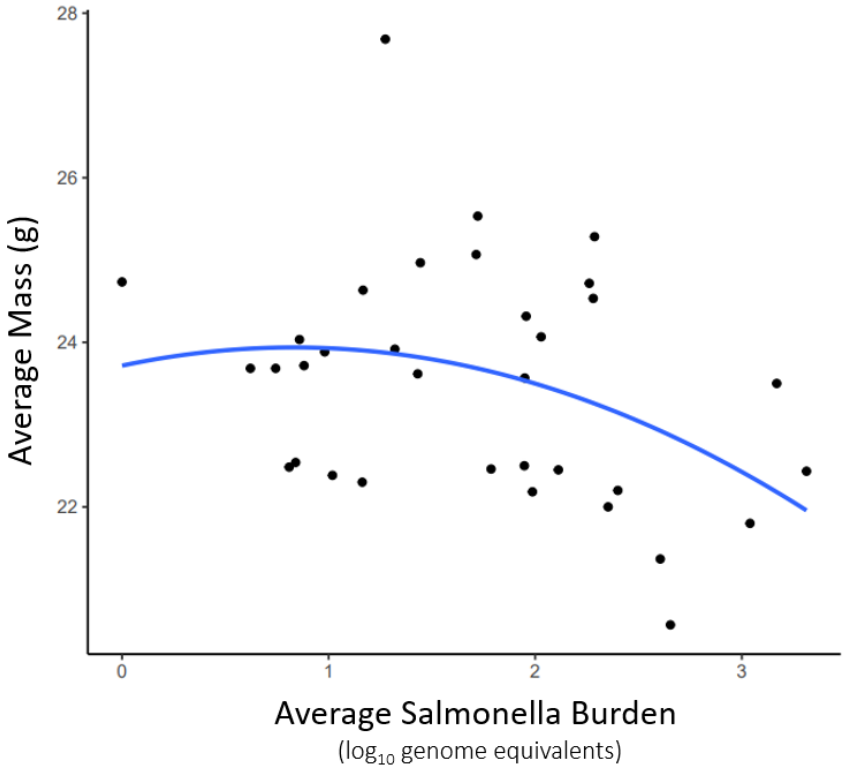

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Figure 2. Average house sparrow body mass (grams) over the course of the infection was affected by exceptionally high*S.enterica* burden ($\log_{10}$ genome equivalentsˆ2) (n =34 birds).

Associations between EP and TLR4 expression across the gut

TLR4 expression differed among gut regions; expression was higher in the cecum and distal regions compared to the proximal and medial regions (Table 4). EP in the *TLR4* promoter affected *TLR4* expression; sparrows with high EP expressed less *TLR4* (mean =1.613, range = 0.01-9.221, SD=1.445) than birds with low EP (average =2.992, range=0.08-9.57, SD=2.546) (Figure 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).

Table 3. The effects of EP in *TLR4* , gut region, and their interaction on *TLR4* expression in house sparrows 14 days after experimental infection with *S. enterica* . Significant effects are indicated by an asterisk (*). Bird ID was modeled as a random term. SE = standard error, SD = standard deviation. N = 34 birds.

Dependent variable: *TLR4* expression (RQ)	Dependent variable: *TLR4* expression (RQ)	Dependent variable: *TLR4* ex
Fixed effects	estimate (SE)	t-value
TLR4-EP (low)	1.782 (0.724)	2.458
Medial tissue	0.270 (0.398)	0.679
Distal tissue	0.908 (0.401)	2.261
Cecum tissue	1.257 (0.405)	3.099
Juvenile	-0.186 (0.932)	-0.200
Male	-0.405 (0.735)	-0.551
Final Salmonella burden	0.139 (0.218)	0.636
EP (low)*Medial tissue	-0.518 (0.617)	-0.840
EP (low)*Distal tissue	-0.054 (0.619)	-0.088
EP (low)*Cecum tissue	-0.493 (0.619)	-0.797
Random effects	variance	SD
Band ID	2.590	1.610
	marginal R^2	conditional R^2
	0.175	0.711

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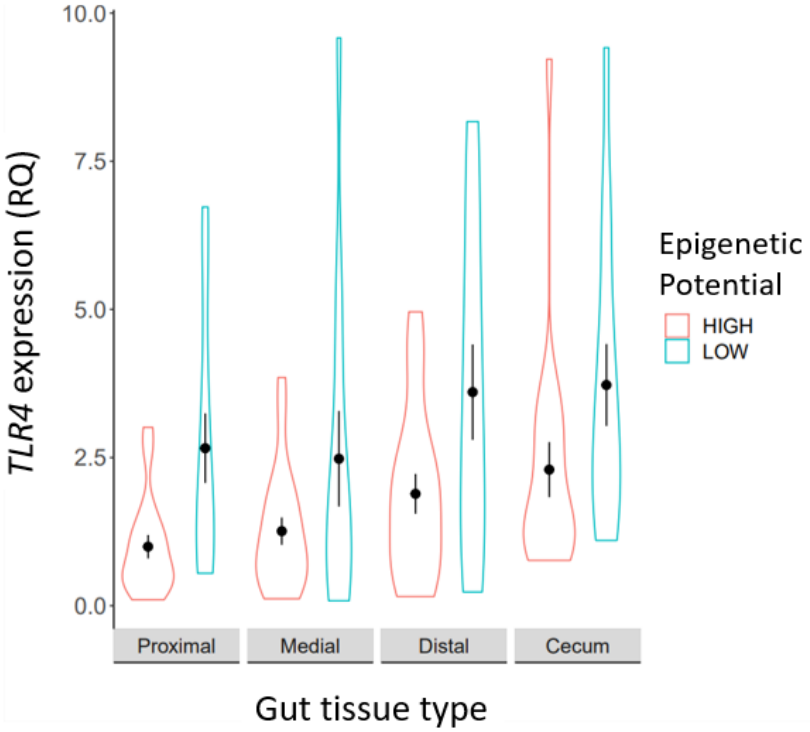

Figure 3. *TLR4* expression was higher in low EP house sparrows, but EP effects were not dependent on gut region. However, *TLR4* was expressed more in the distal and cecal portions of the gut than the other two regions. Error bars depict means +/- 1SE for the relative quantification (RQ) of *TLR4* expression.

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

Of the 38 birds included in our experiment, only six died during the experiment. A multivariate Cox proportional hazard regression revealed that mortality was not related to EP in *TLR4* , average (or maximum) *S. enterica* burden nor their interaction. Mortality was also not associated with absolute body mass or its interaction with*S. enterica* , *TLR4* expression nor the status of the bird (male, female or juvenile) (Table 4).

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* . Significant effects are indicated by an asterisk (*). Bird ID was modeled as a random term. SE = standard error, SD = standard deviation. $N = 34$ birds.

Dependent variable: Mortality	Dependent variable: Mortality	Dependent variable: Mortality	Dependent var
Fixed effects	estimate (SE)	z-value	p-value
Epigenetic Potential (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

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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, and survive a pathogenic *Salmonella* infection. We found that birds with high EP in *TLR4* shed less 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 of 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, and body mass was generally unaffected by *S. enterica* until the shed bacteria reached very high levels (>3 logs). Our study provides empirical and conceptual support for the idea that one form of epigenetic potential contributes to the efficacy of control of an important pathogen. 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 defense to *S. enterica*

S. enterica serotype Typhimurium is a Gram-negative bacterial taxon that imposes major losses to 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 other routes (i.e., persistence in the soil or on surfaces for weeks to months) (Tizard 2004, Hilbert et al. 2012). 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, Hughes et al. 2010).

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 (Coburn et al. 2007). Mice strains genetically deficient for *TLR4* are highly susceptible to *S. enterica* ; likewise, a SNP in exon of the ligand-binding domain of *TLR4* , which varies among chicken lines, can affect resistance of and mortality to the bacteria (Leveque et al. 2003). Most *TLR4* expression derives from macrophages and heterophils (Alkie et al. 2019), and these cells and lymphocytes are recruited in huge numbers to the gut once *TLR4* is activated and inflammation initiated locally. Dendritic cells, too, residing just below the gut epithelium, express abundant *TLR4* , and can further sculpt the local and systemic immune response against *Salmonella* infections (Ijaz et al. 2021). High *TLR4* expression thus provides protection through various and dynamic means including enhanced phagocytosis and activation of several cell types, which often change over the course of the infection. Exactly which mechanism(s) was potentiated by high EP in *TLR4* is unknown, but clearly EP played an important role in the control of this pathogen.

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 at the end of our experiment. This result is similar to the patterns observed in spleen and liver in another study on house sparrows such that EP effects vary among tissues and time (Hanson et al., 2021). The current results are also intriguing because *high TLR4* expression is more commonly associated with bacterial resistance (Gou et al., 2012). However, our hypothesis did not predict that high EP in *TLR4* may be protective because it imbues high, constitutive expression of *TLR.* Rather, we expected that it would facilitate *TLR4* expression *plasticity* and perhaps reversibility by increasing the potential for DNA methylation modifications. In other words, we predicted that high EP in *TLR4* is associated with *Salmonella* resistance through a greater propensity to tune *TLR4* expression (turn up and down) over the course of an infection, rather than enduringly elevating it.

We suggest that the above scenario is the likely adaptive/functional one for two reasons. First, house sparrows with high EP in *TLR4* have already been shown to have greater inducibility and reversibility of *TLR4* expression across repeated blood samples (Hanson et al., 2021). Second, the ability to flexibly regulate *TLR4* expression should better balance the costs and benefits of infection. Indeed, inflammatory

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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), but of course under-expression of *TLR4* could leave the organism vulnerable to infection and low fitness including death. Here, we were only able to measure gut *TLR4* expression once in the gut, 14 days after the onset of an infection (due to the destructive nature of sampling the gut tissue). Further experimentation, for example, sampling the gut from different birds at different stages of an infection, is therefore required to add insight into the pathway by which EP may have affected*S. enterica* resistance via the dynamic regulation of *TLR4*expression.

Ecological implications of EP in *TLR4*

Interestingly, our results indicate that EP in *TLR4* may help protect organisms from*S. enterica* by enhancing host resistance, but not to the point of sterilizing the gut of this pathogen. In addition to providing a level of protection against pathogens, high levels of EP among range-expanding/non-native house sparrows (Hanson et al., 2021) may also afford these populations an indirect means to outcompete resident host species, by exposing their competitors to pathogens (Martin et al. 2010a, Coon and Martin 2014, Martin et al. 2014, Martin et al. 2015, Martin et al. 2017). Indeed, house sparrows have already been implicated as major reservoirs and potential progenitors of *Salmonella*epidemics that have drastically reduced the population sizes of birds (e.g., greenfinches) (Tizard 2004; Hernandez et al. 2016). Somewhat surprisingly, most Tampa house birds were able to tolerate and survive infection with a pathogen that is lethal to other songbirds including European house sparrows. Perhaps this outcome is a vestige of past selection for North American birds, which occurred at the time of introduction from ancestral Europe.

Conclusions and further work

Besides more work on EP in *TLR4* , in other genes and other invading or range-expanding species, we should next ask 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 outputs by affecting CpG methylation in *TLR4* (Takahashi et al. 2011). High EP in *TLR4* could thus potentially facilitate microbiota-induced epigenetic changes by providing more genomic substrate for modifications 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 showed 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., 2021; Hanson et al., 2022).

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