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Immune gene expression and epigenetic potential affect the consumption of risky food by female house sparrows

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ABSTRACT

When organisms move into new areas, they are likely to encounter novel food resources. Even if they are nutritious, these foods can also be risky, as they might be contaminated by parasites. The behavioural immune system of animals could help them avoid the negative effects of contaminated resources, but our understanding of behavioural immunity is limited, particularly whether and how behavioural immunity interacts with physiological immunity. Here, we asked about the potential for interplay between these two traits, specifically how the propensity of an individual house sparrow (*Passer domesticus*) to take foraging risks was related to its ability to regulate a key facet of its immune response to bacterial pathogens. Previously, we found that sparrows at expanding geographic range edges were more exploratory and less risk-averse to novel foods; in those same populations, birds tended to over-express *Toll-like receptor 4* (*TLR4*), a pattern-recognition receptor that distinguishes cell-wall components of Gram-negative bacteria, making it the major sensor of potentially lethal gut microbial infections including salmonellosis. When we investigated how birds would respond to a typical diet (i.e., mixed seeds) spiked with domesticated chicken faeces, birds that expressed more *TLR4* or had higher epigenetic potential for *TLR4* (more CpG dinucleotides in the putative gene promoter) ate more food, spiked or not. Females expressing abundant *TLR4* were also willing to take more foraging risks and ate more spiked food. In males, *TLR4* expression was not associated with risk-taking. Altogether, our results indicate that behaviour and immunity covary among individual house sparrows, particularly in females where those birds that maintain more immune surveillance also are more disposed to take foraging risks.

1. Introduction

Food consumption is inherently risky because of the potential for food contamination by parasites. Animals therefore face a trade-off between food acquisition and infection avoidance (Sarabian et al., 2018). To counter such risk, animals have evolved a behavioural immune system (Schaller and Park, 2011), a first line of defence against infection, which operates by facilitating behavioural avoidance or mitigation of risk (Poirotte et al., 2019; Sarabian et al., 2018). To date, our understanding of behavioural immunity is limited, particularly whether and how behavioural immunity interconnects with physiological immunity (Sarabian et al., 2018). In some contexts, both behavioural and physiological immunity will be critical for individual survival, such as in the context of invasions and range expansions. Animals moving into new areas must find and consume novel but otherwise nutritious food, so their need to eat unfamiliar foods should often expose them to risk they would probably be able to avoid in familiar areas (Canestrelli et al., 2016; Liebl and Martin, 2012; Martin and Fitzgerald, 2005). This dilemma raises the question: do animals willing to take feeding risks have immune systems better able to mitigate that risk?

The vertebrate immune system is comprised of various barriers to living and non-living threats. Regarding bacteria, one of the most

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common groups of infectious organisms found in food, a particularly important barrier to infection is the expression of the Toll-like receptors (TLRs) (Werling et al., 2009). TLRs are found on the membranes and in the cytosol of many leukocytes, in all cases serving as pathogen-recognition receptors (PRRs) that bind pathogen-associated molecular patterns (PAMPs). PAMPs including lipopolysaccharide, peptidoglycan, and others reliably indicate the presence of microbes (Brownlie and Allan, 2011; Iwasaki and Medzhitov, 2015). Whereas pathogenic, commensal and even mutualistic microbes all express PAMPs, PAMPs remain among the most important instigators of vertebrate innate immune responses (Iwasaki and Medzhitov, 2015). Indeed, a useful way to conceive TLRs is as major surveillance mechanism that activates appropriate immune responses given the identity of the PRR-PAMP association (Janeway and Medzhitov, 2002).

TLRs, as with most aspects of the immune system, are inducible such that various stimuli elicit dynamic changes in their expression. The time scale of such plasticity spans both long- and short-time periods; early-life infection can enduringly alter *TLR* expression, but exposure to live bacteria or PAMPs just minutes to hours earlier can change *TLR* expression, too. In several vertebrate species, some of this plasticity, particularly long-term changes, is mediated by DNA methylation (Foster and Medzhitov, 2009). Methylation of promoters or other regions of TLRs is a form of epigenetic variation that can adjust gene expression by changing the accessibility of transcription factors to the genome (Feinberg, 2007). In domesticated chickens, for instance, DNA methylation-associated plasticity in *TLR* expression was associated with the ability of individuals to cope with experimental *Salmonella* infections; birds with high methylation expressed less *TLR4* and succumbed faster to bacterial infection than those with low DNA methylation (Gou et al., 2012).

In the present study, we asked how foraging disposition was related to the genomically-encoded ability of individual house sparrows (*Passer domesticus*) to modulate gene expression via DNA methylation, one form of what we term epigenetic potential (EP) (Hanson et al., 2020; Hanson et al., 2022; Hanson et al., 2021; Kilvitis et al., 2017). 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). EP recognizes that organisms will often vary in the number and location of CpG sites, where methyl marks bind, in their genomes. The number of CpG sites in or near regulatory sites is not the only factors influencing gene expression, but CpG sites may constitute important areas where gene expression may be finely regulated with more CpG sites allowing increased DNA methylation possibilities. This variation in EP can be evolutionarily relevant as it is genomically-encoded and hence provide organisms with a heritable propensity to alter gene expression (Branciamore et al., 2010; Hanson and Liebl, 2022; Hanson et al., 2022; Kilvitis et al., 2017). It is also probably a major capacitor of phenotypic plasticity, as it should influence the ability of an individual to adjust gene expression contingent on context (Ghalambor et al., 2015; Hanson and Liebl, 2022; Hanson et al., 2021). In one recent study, we found that house sparrows with higher EP in the putative *TLR4* promoter (i.e., relatively more CpG sites) expressed more *TLR4* than those that did not (Hanson et al., 2021). Just one additional CpG site enabled some birds to express more *TLR4* than birds with fewer sites; critically, it was not the identity of a CpG site that influenced expression, but the total number of CpG sites in the putative promoter (Hanson et al., 2021).

Here, we investigated whether variation in EP in *TLR4* and/or variation in *TLR4* expression were associated with the propensity to take foraging risks among individual house sparrows. This work was also partly motivated by our prior discoveries in two independent invasions (i.e., Panama and Kenya); sparrows in those new populations took more feeding risks than birds from older populations (Liebl and Martin, 2014; Martin and Fitzgerald, 2005). Doing so, generally, should dispose invading birds to greater infection risk. Introduced sparrows especially at the range edge also show higher EP (Hanson et al., 2020; Hanson et al., 2022). Furthermore, in Tampa sparrows, we showed that individuals with high EP in the putative *TLR4* promoter express more *TLR4* in the blood than those with low EP (Hanson et al., 2021). Consequently, we hypothesized here that sparrows with high EP in *TLR4* would express more *TLR4* in their gut tissue and this could associated with a greater propensity to take feeding risks considering these birds are presumably better protected against infection. To test this hypothesis, we queried how EP and *TLR4* expression were related to the propensity and sensitivity of individual sparrows to approach and consume a favoured food (i.e., birdseed) when that food was modestly contaminated with sterile domesticated chicken faeces. We chose to use this particularly approach to making feeding risky given that i) it is naturalistic given the tendency of sparrows to forage in groups on the ground in proximity to domesticated and wild species, ii) many sparrow pathogens are faecal-orally transmitted, and iii) it is highly standardizable.

An unexpected result in the previous study on EP and *TLR4* expression in house sparrow was that *TLR4* expression across tissues differed between sexes. Particularly, high EP females showed greater inducibility and reversibility in *TLR4* expression in the blood than males (Hanson et al., 2021). On the contrary, high EP females also tend to express less *TLR4* in the liver whereas males expressed *more TLR4* in the spleen at the end of the experiment (Hanson et al., 2021). Interestingly, sex differences have also been recorded in behavioural immunity in other species (Sarabian et al., 2018). Females of different primate species including humans, for instance, are more likely to avoid food contaminated with faeces (Müller-Graf et al., 1997; Poirotte et al., 2019; Sarabian and MacIntosh, 2015). One proposed reason for this sex difference is that females generally invest more in immunity than males (Rolff, 2002), including a more sensitive behavioural immune system. On the other hand, the directionality of aversion to contaminated food can vary between sexes in some species, and there are no detectable differences between males and females in others (Sarabian et al., 2018; Sarabian et al., 2017). As high EP female sparrows exhibited greater nimbleness in the control of *TLR4* expression in response to an infection in the previous study, we hypothesized that they should be better protected against infection and thus should show a greater willingness to take feeding risks here, particularly in the context of high EP or high *TLR4* expression.

2. Methods

2.1. Bird capture and husbandry

We captured house sparrows in the Tampa Bay region of Florida using mist nests from mid-April to the end of May 2021 between 06:30 and 09:30. The experiment included 37 adult birds (14 females, 23 males) split into 7 cohorts (cohort 1: 2 females, 5 males; cohort 2: 3 females, 5 males; cohort 3: 3 males; cohort 4: 2 females, 2 males; cohort 5: 1 female, 3 males; cohort 6: 3 females, 3 males; cohort 7: 3 females, 2 males). Captures of cohorts were on average separated by a week, which was necessary given the demanding nature of the behavioural tests and unavoidable given the low densities of sparrows in Tampa, FL. Upon transfer to the University of South Florida vivarium, birds were individually housed in 35.6 × 40.6 × 44.5 cm cages for three days within visual and auditory contact of each other. Light conditions were 13L:11D to match natural day lengths. Water and food (ABBA 1900 seed mix) were provided *ad libitum* at all-times except overnight from 30 min before the lights went off until approximately one hour after the lights were turned on the following morning when the behavioural tests occurred. Birds did not show any signs of extreme stress and lost on average 3g between capture and the end of the experiments, a change typical in studies resembling this one.

2.2. Characterizing risk-taking when foraging

To characterize risk-taking when foraging, birds were involved in

two behavioural tests. One assessed latency to approach food (seeds) spiked with sterile chicken faeces, and the other assessed the ability to discriminate and willingness to consume faeces-spiked from unspiked food. Spiked (risky) food consisted of the same seeds (ABBA 1900) that birds were typically provided throughout the study mixed with a small but consistently-sized amount of domesticated chicken faeces. Fresh chicken faeces were collected from a chicken coop overnight by placing foil at the bottom of the coop. To be able to standardize the amount of faeces added to the seeds for each test, faeces were mixed and then dried in an oven (60 °C) for about 24h until all water evaporated, then kept at −20 °C until mixed with seeds for behavioural tests. A fresh stock of spiked food was prepared each morning, just before the beginning of each behavioural test, by mixing seeds and dried faeces in a 2:1 mass ratio. Then, water was sprayed on the seed/faeces mix so that seeds were homogenously covered and so that birds could not eat seeds without also risking ingestion of faeces. Unspiked food was also sprayed with water.

Behavioural tests started the day following capture and spanned two consecutive days with half of the birds performing the latency test first and the other half performing the discrimination test first, alternating which test was performed first across cohorts (16 birds (7 females and 9 males) performed the discrimination test first, 21 (7 females and 14 males) performed the latency test first). Regular food was removed from each cage and the bottom of the cages cleared of all seed 30 min before lights out the night before behavioural tests. Birds were then tested the following morning between 8:00 and 9:00 when they were motivated to forage. Before starting each test each morning, cages were visually separated with opaque panels to keep birds from seeing each other. These visual separations were removed as soon as tests were finished, then normal food was returned to all cages. For both tests (discrimination and latency), the same feeders were used as those that were already in cages to avoid object neophobia towards feeders.

For the latency test, 3 g of spiked food were added to each feeder, and the feeder was placed in the cage of each bird. As soon as all feeders were in all cages, the observer (CZ) moved behind a visual separation equipped with a one-way mirror, which allowed him to see all birds in all cages without being seen by the birds. The observer then recorded over a period of 20 min the latency of each bird to eat (bird picked up a seed with its beak and ingested it) from its feeder and the number of feeding bouts. At the end of the test, the feeders were removed from cages and the remaining spiked food (in the feeder and cage bottom) was weighed to determine the amount of food eaten.

For the discrimination test, methods resembled the latency test except two identical feeders were added to each cage, one with 3 g of spiked food and one with 3 g of unspiked food. Food type was alternated with feeder position (left or right side of the cage) across cages. For this test, feeders were placed in the cages by a different person than the observer. Thus, the observer was blind to the treatment, as it was not possible to determine whether a feeder contained spiked or control food from the observation position. The observer then recorded the latency to eat from each feeder and the number of feeding bouts in each feeder for 20 min. At the end of the tests, feeders were removed from the cages and the remaining food in feeders and the cage bottom was weighed to determine the amount of food eaten. On the third day, the day after the second behavioural test, all birds were euthanatized via isoflurane overdose and rapid decapitation. The whole gut and a section of the liver were immediately collected from each bird using RNA-free tools, placed in a tube with RNAlater in dry ice, then stored at −80 °C until further processing.

2.3. Characterizing epigenetic potential in toll-like receptor 4

DNA was extracted from ~0.1 g of liver tissue using a DNAEasy Blood and Tissue kit (Qiagen). We developed primers that spanned the putative *TLR4* promoter region (726 to 1228 nucleotides upstream of the transcription start site), which likely includes regulatory regions and CpG sites that affect gene expression (Kilvitis et al., 2019, Hanson et al., 2021). Each PCR reaction to amplify this region contained 12.5 μl of 2× PCR Master Mix (Promega), 1 μl forward primer, 1 μl reverse primer, 8.5 μl of nuclease-free water, and 2 μl of DNA. Cycling conditions included an initial denaturation step 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 in one batch.

Resulting chromatograms from DNA sequences were analysed manually on Unipro UGENE (Okonechnikov et al., 2012). All single nucleotide polymorphisms (SNPs) and CpG sites in the putative *TLR4* promoter were examined across all individuals, counting CpG sites on each chromosome separately (Hanson et al., 2020; Hanson et al., 2021). Previously in this population, we classified individuals into low or high EP categories, as most birds had either 7 or 8 CpG sites in the focal sequence, and this binary form of EP was also the best predictor of *TLR4* expression and resistance to *Salmonella* infection (Hanson et al., 2021). In that previous work, in addition to also analysing 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; Sheldon et al., 2023). Consequently after checking that models with EP as continuous variable yielded statistically analogous results and as all individuals had either 7 or 8 CpG sites in this region, we also used this binary approach for EP here.

2.4. TLR4 expression in the gut

Whole gut samples were 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 were washed out with distilled water. We then separated the small intestine into three sections: proximal, medial, and distal. From the middle of each intestinal section, we collected a transverse piece of tissue (about 1 mm wide) and immediately placed it into a microtube on dry ice. We also collected a section from a caecal segment and processed it the same way. Samples were then stored at −80 °C until RNA extraction. RNA from each gut sample was extracted using a TRI-reagent extraction method; each extract was then diluted to 25 ng μl^{-1} (Hanson et al., 2021; Zimmer et al., 2021). We measured *TLR4* mRNA expression using one step qRT-PCR. All qRT-PCR reactions (20 μl) were run in duplicate alongside i) non-template controls (NTC) and ii) 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, 0.3 μl of reverse primer, 0.25 μl of SCRIPT, 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. Thermocycling 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 15s at 95 °C then 30s at 60 °C. Melt-curve analyses were performed from 65 to 95 °C with 0.5 °C increment step every 3s to confirm single-product specificity of each sample. A calibrator (i.e., a mix of RNA from a homogenate of the four different gut samples from four different birds) and an internal reference gene (*HMBS* that was previously validated in this population (Hanson et al., 2021; Zimmer et al., 2021) were run on all plates to calculate mRNA abundance using the comparative Ct method ($2^{\Delta\Delta Ct}$). This method returns mRNA abundance for *TLR4* as the fold-change in expression compared to the calibrator sample, normalized to the reference gene (Livak and Schmittgen, 2001).

2.5. Ethical note

Our research adhered to the ASAB/ABS Guidelines for use of animals in behavioural research and teaching and to the guidelines outlined by the National Research Council. House sparrow densities are low in Tampa, FL, but as an introduced species, populations are not protected. Regardless, house sparrow populations across much of the US including other parts of Florida are relatively healthy. The broad geographic distribution of the species, its high populations densities in some parts of its range, and its disposition to be moved by humans during commerce mean that our study was very unlikely to have had any population-level impacts. Birds in this study were kept only for three days, limiting any negative effects of captivity. Food was removed 30 min before lights out the night before behavioural tests each night. Birds were then tested the following mornings between 8:00 and 9:00. As house sparrow do not feed at night, birds were food deprived for a maximum of 1.5h. All procedures were approved by USF IACUC (number IS00007628).

Our study design was generally guided by maximizing scientific inference, but also minimizing individual-level discomfort and stress. While house sparrows are quite social, we housed bird individually because group housing during breeding season (period we conducted the study) could have led to harassment and harm in the groups. This issue plus the very large challenge of tracking foraging behaviour of individuals while in groups led us to our study design. We also minimized the length of experimental timelines and human contact with birds, except for imperative interactions during feeding trials.

Regarding any potential negative effects of providing faeces-spiked food to birds' health, there are several important factors to consider. First, exposure to pathogens and thus risk of infection is very unlikely as the experimental period was very short (2 days) and all experimental faeces were dried in an oven, which should have killed most microbes. Second. house sparrows are opportunistic ground feeders and human commensals and thrive in areas where the foods they eat are largely from waste feed or intermingled with animal dung (Gavett and Wakeley, 1986). Thus, faecal contamination of food is probably very common during natural foraging. Altogether, our experimental design represents a balance of natural realism and ethically-motivated research.

2.6. Statistical analysis

First, to determine whether EP differed between male and female sparrows, we ran a generalized linear model (GLM) fitted with a Poisson distribution with sex as a fixed factor. To determine whether *TLR4* expression was affected by sex, EP, and/or gut region, we ran a generalized linear mixed model (GLMM) fitted with a Gamma distribution with sex, EP, gut region and their interactions as fixed factors and bird identity as a random factor.

To investigate whether risk-taking behaviours were influenced by sex, EP, and/or *TLR4* expression, we ran separate models where the dependent variables were: i) the latency to eat from the feeders, ii) the number of times a bird ate from the feeders, and iii) the amount of food eaten from the feeder(s) for the latency test (3 models) and the discrimination test (3 models). As sex and EP effects on *TLR4* expression were not dependent on gut region and to simplify models, we used average *TLR4* expression across all 4 gut regions in all subsequent models. Average *TLR4* expression and EP were not colinear, as the variance inflation factor (1.04) and the condition number (1.23) were both <10 and tolerance (0.96) was well above 0.2. For each dependent variable in the latency test, we ran a GLM with sex, EP, *TLR4* expression alone as well as and their 2 -way interactions as independent variables. For each dependent variable in the discrimination test in which there were two food types (spiked food and unspiked food), we ran a GLMM with sex, EP, *TLR4* expression, food type, and all their 2-way and the 3-way interactions including food type (as it is the main experimental factor) as fixed effects. Bird identity was added as random effect. Models for latency and amount of food eaten were fitted a Gamma distribution and models for number of feeding bouts were fitted with a negative binomial distribution to take into account zero overinflation.

GLMs were run using proc GENMOD and GLMMs using proc GLIMMIX in SAS OnDemand (SAS Institute Inc.). Post-hoc comparisons for categorical variables and interactions between categorical variables were performed using Tukey-Kramer multiple comparison adjustments to obtain corrected p-values. For 2 and 3-way interactions including categorical and continuous independent variables, we used proc PLM and used the regression information stored from the GLMM to calculate the estimates of the relationships between the continuous independent variable and the dependent variable within each category of the categorical variable or set of categories of the categorical variables. This approach also allowed us to test whether estimates significantly differed from 0. Results below are presented as means ± SE.

3. Results

3.1. EP and TLR4 expression

Across all birds (*N*=37), average EP was 7.50 CpG sites (range 7–8, SD = 0.51) but EP did not differ between females (7.43 ± 0.14) and males (7.48 ± 0.11) ($X^2_{1,35} = 0.00$, $p = 0.96$). *TLR4* relative expression was higher in high EP (8 CpGs) birds (1.14 ± 0.11) than in low EP (7 CpGs) birds (0.87 ± 0.08) ($F_{1,36.93} = 4.31$, $p = 0.045$; Table A1). *TLR4* expression did not differ between females (0.94 ± 0.08) and males (1.02 ± 0.09) ($F_{1,36.93} = 0.00$, $p = 0.99$) but differed among gut regions ($F_{1,109.2} = 15.96$, $p < 0.0001$; Table A1). Expression in the proximal region (0.64 ± 0.08) was significantly lower than in the other three regions (medial: 1.00 ± 0.11, distal: 1.15 ± 0.19, caecum: 1.19 ± 0.11; $t \geq 4.36$, $p \leq 0.0002$). However, this difference among gut regions did not depend on sex and/or EP (2 and 3-way interactions were not significant: $F_{3,103.9} \leq 0.97$, $p \geq 0.45$; Table A1).

3.2. Behavioural tests

Overall, we did not find any significant predictors of latency to feed except that females performed more feeding bouts than males ($X^2_{1,37} = 4.25$, $p = 0.040$; Tables A2, A3, A4).

3.2.1. Discrimination and consumption of food spiked with chicken faeces

When we offered birds a choice between spiked and unspiked food, both females and males fed more often on and ate more unspiked food than spiked food (food type × sex: number of feeding bouts $F_{1,74} = 8.18$, $p = 0.006$; Table A5; amount of food eaten $F_{1,37} = 8.75$, $p = 0.005$; Table A6; Fig. 1). There was no difference in food consumption between sexes within each food type (Fig. 1; Table A5, A6).

The amount of food eaten was dependent of *TLR4* expression ($F_{1,37} = 5.37$, $p = 0.026$; Table A6) and of the interaction between EP and *TLR4* expression, but independent of food type (unspiked vs. spiked) (EP × *TLR4*: $F_{1,37} = 4.36$, $p = 0.044$; Table A6). High EP birds that expressed high *TLR4* ate more food in total ($\beta = 0.41 \pm 0.17$, $t = 2.39$, $p = 0.019$; Fig. 2). For low EP birds, the total amount of food consumed was not associated with *TLR4* expression ($\beta = -0.13 \pm 0.18$, $t = -0.75$, $p = 0.456$; Fig. 2). More importantly with respect to the motivation of our study, the effects of *TLR4* expression on the amount of spiked and unspiked foot eaten was different between males and females (food type × sex × *TLR4*: $F_{1,37} = 3.99$, $p = 0.025$; Table A6; Fig. 3).; the amount of spiked food eaten significantly increased with increasing *TLR4* expression in females ($\beta = 0.73 \pm 0.31$, $t = 2.33$, $p = 0.0223$; Fig. 3c) whereas this relationship only approached statistical significance for unspiked food ($\beta = 0.63 \pm 0.31$, $t = 1.98$, $p = 0.0514$; Fig. 3a). However, in males, *TLR4* expression was unrelated to the amount of either food type eaten. The amount of food eaten for both food types (spiked and unspiked) was not associated with *TLR4* expression (normal food: $\beta = 0.22 \pm 0.18$, $t = 1.21$, $p = 0.230$; Fig. 3b; spiked food: $\beta = -0.17 \pm 0.18$, $t = -0.92$, $p = 0.358$; Fig. 3d).

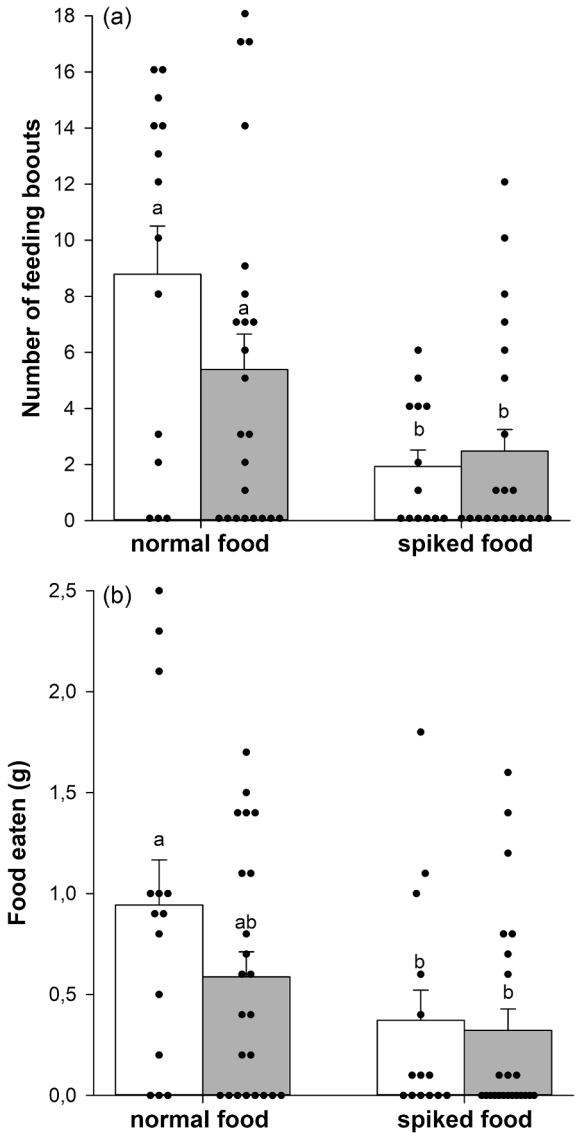

Fig. 1. Number of feeding bouts (a) and amount of food eaten (b) for normal (unspiked) and spiked (chicken faeces) food for female (white bars) and male house sparrows (grey bars). Different letters indicate significant differences by Tukey-Kramer post-hoc tests. Bars are means +/- 1se. Black dots represent individual data.

3.2.2. *Latency to approach spiked food*

Overall, females (476.2 ± 101.5 s) took less time than males (593.3 ± 83.4 s) to feed, regardless of food type ($F_{1,37}$ = 11.39, p = 0.002; Table A7). Regarding our main interest, birds also took less time to feed on unspiked food (450.6 ± 87.6 s) than spiked food (647.3 ± 92.9 s) ($F_{1,37}$ = 11.63, p = 0.001). However, there was also a significant interaction among sex, food type, and EP ($F_{1,37}$ = 8.95, p = 0.0005; Table A7; Fig. 4): high EP females ate unspiked food faster than both low EP females and males (regardless of EP) (t ≥ 5.34, p < 0.0001). By contrast, latency to eat spiked food did not differ between high and low EP groups between and within sexes (Fig. 4).

The effect of sex and food type on latency to eat was also moderated by *TLR4* expression (food type × sex × *TLR4*: $F_{1,48.63}$ = 10.25, p = 0.0002; Table A7), but this effect was weak. Post-hoc comparisons showed that latency to eat was not significantly influenced by *TLR4* expression within each sex/group (females/normal food: β = 0.81 ± 0.72, t = 1.12, p = 0.26; females/spiked food: β = −0.19 ± 0.72, t = 0.27, p = 0.79; males/normal food: β = −0.57 ± 0.42, t = 1.36, p = 0.18; males/spiked food: β = 0.29 ± 0.42, t = 0.70, p = 0.49; figure A1).

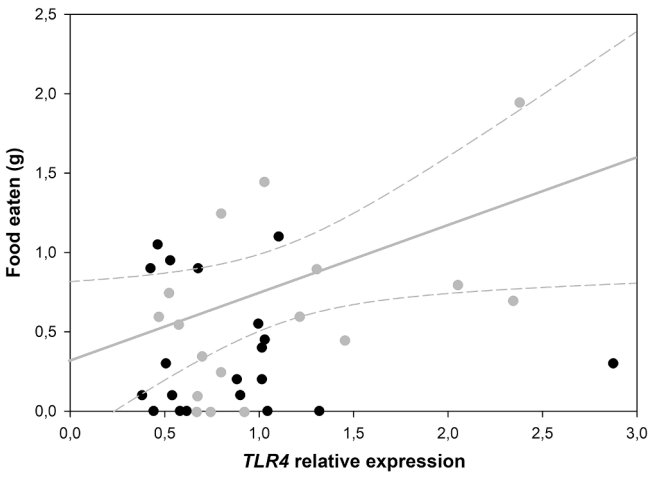

Fig. 2. Relationships between *TLR4* relative gene expression in the gut and the amount of food eaten (in grams) in low (black) and high EP house sparrows (grey). High EP birds that expressed high levels of TLR4 ate more food than low EP birds. The trend line and its 95 % confidence interval (dashed lines) depict the significant relationship in high EP birds based on the fitted model.

4. Discussion

A host's capacity to prevent infection is expected to map to its avoidance behaviour towards a parasite (Hutchings et al., 2000), but so far, this possibility has rarely been tested in host-parasite pairs. One instance of support comes from a study on honeybees (*Apis mellifera*) in which immune gene expression in forager bees, exposed to many environmental hazards, was much higher than nurse bees (Vannette et al., 2015). Another example from two salmonid species showed that individuals more resistant to infection by a natural parasite displayed low avoidance of the parasite (Klemme et al., 2020). Here, we investigated whether and how risk-taking behaviour in house sparrows (with respect to feeding) was related to the regulation of a key immune gene, *TLR4*. Whereas sparrows were quite adept at recognizing faecally spiked food, effects of *TLR4* expression and EP in *TLR4* on risk-taking were complex. Overall, birds that expressed a lot of *TLR4* simply consumed more food, irrespective of faecal spiking, and this effect was also stronger in birds with high EP. Bird sex was also related to how *TLR4* influenced food consumption: females expressing abundant *TLR4* consumed substantial spiked *and* unspiked food whereas males expressing high *TLR4* were more risk averse, consuming more unspiked than spiked food. Females with high EP in *TLR4* were also faster to approach and eat food of either type.

Collectively, these findings are consistent with the original framework of our study, but they are also nuanced in terms of relationships between behaviour and the epigenetic regulation of a key immune gene. Nevertheless, they hint that risk-taking behaviours, gut immunity, and the (epi) genomic regulatory architecture of immune gene expression in interconnected in a species that has colonized so much of the world. Below we discuss the implications of our results for house sparrows and other invaders, and we describe follow-up work necessary to reveal how such disparate processes could be coordinated at a physiological level. Indeed, one additional unexpected result of our study was that even unspiked food was perceived as risky at the beginning of the study; while birds took less time to eat unspiked food, most birds took a long time (more than 7 min) before starting to eat, despite being deprived of food since the previous evening. Going forward, sophisticated study designs with strong statistical power will be imperative to link risk-taking and immunity in this and other species.

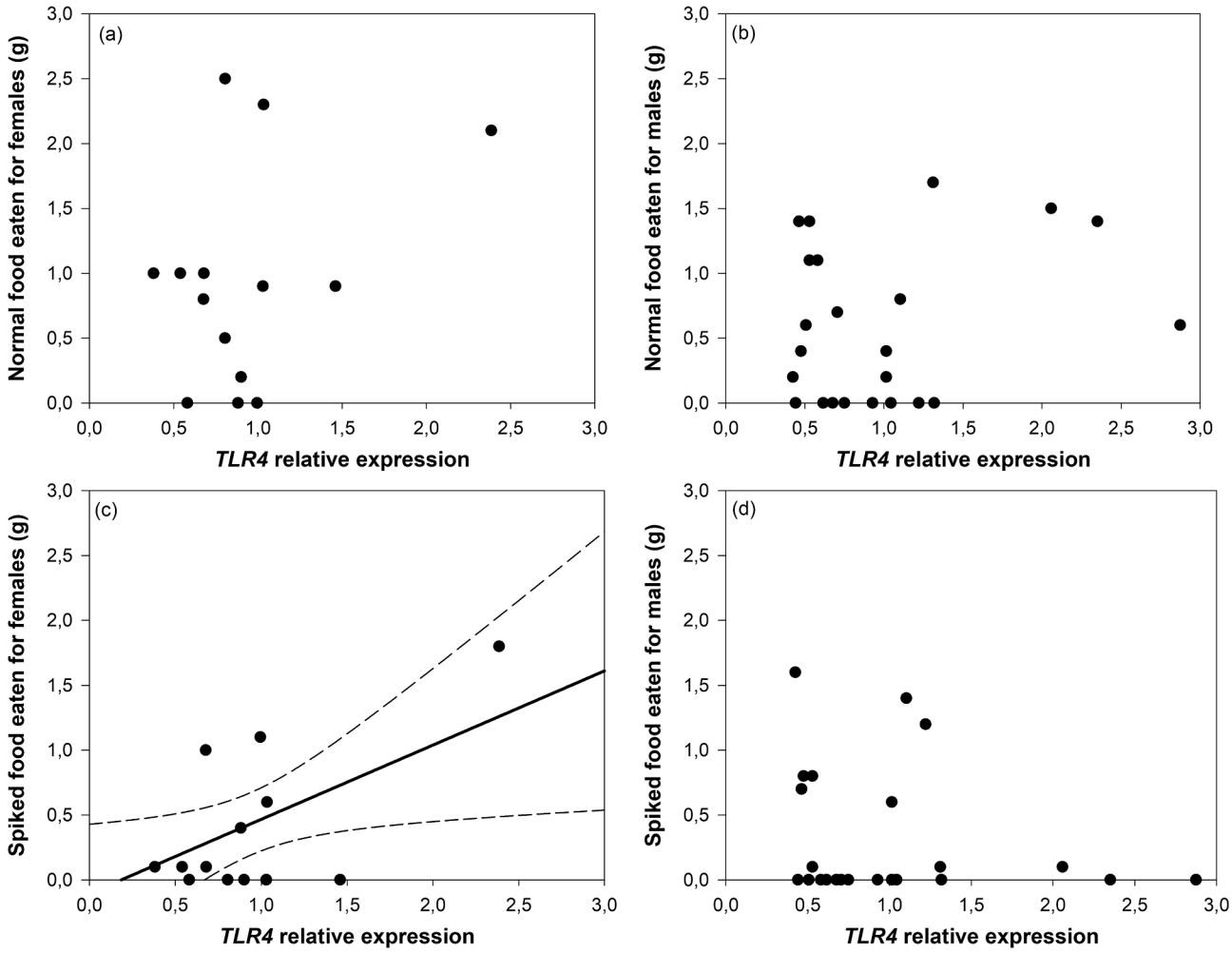

Fig. 3. Relationships between *TLR4* expression in the gut and the amount of normal and spiked food eaten (in grams) in female (a, c) and in male house sparrows (b, d). Overall, more food was eaten when *TLR4* expression was high, except in males for spiked food. However, the only significant relationship was for females eating spiked food. For females eating normal food, the result was marginally non-significant (see results for statistical details). Trend line and its 95 % confidence interval (dashed lines) depict the significant relationship for spiked food in females based on the fitted model.

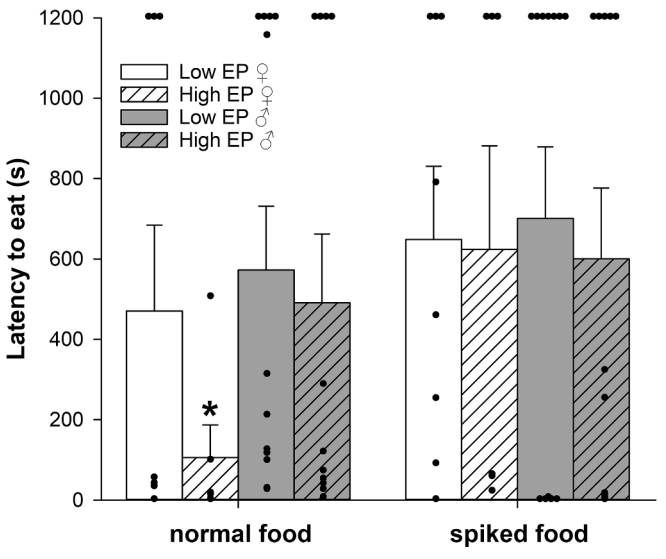

Fig. 4. Latency (in seconds) to eat normal and spiked food in low (plain bars) and high (hatched bars) EP female (white bars) and male (grey bars) house sparrows. High EP females ate normal food fastest. Asterisk indicates significant differences. Bars are means +/- 1se. Black dots represent individual data.

4.1. Interrelationships among risk-taking, TLR4 expression and EP

As TLR4 is one of the first points of contact between a pathogen and a host, EP in *TLR4* might provide a rapid and labile defence against parasites encountered when foraging on novel resources (Hanson et al., 2020). In another study, EP was protective against infection with a pathogenic *Salmonella* strain; house sparrows with high EP in *TLR4* promoter region were more resistant than birds with low EP (Sheldon et al., 2023). There, effects of EP on *TLR4* expression were also complex; high EP individuals expressed surprisingly low *TLR4* expression in the gut, but nevertheless high EP was related to greater protection against infection. Coupled with our previous work showing that EP is related to *TLR4* expression in the spleen, liver, gut and blood in distinct ways (Hanson et al., 2021; Sheldon et al., 2023; this study), it is impossible to yet link EP, *TLR4* expression and risk-aversiveness in a causal way. *TLR4* expression is quite likely protective based on extensive work in other vertebrates, but it is also dynamic within single tissues and heterogeneously expressed across tissues. Indeed, in the blood of sparrows, high EP was associated with a greater inducibility and range of *TLR4* expression, and only in females was reversibility in expression possible over the time-period of the study (Hanson et al., 2021). In the present study, we found that high EP individuals expressed more *TLR4* in the gut, but that both EP and *TLR4* expression were only associated with risk taking in females. The different relationships between EP and *TLR4*

expression among these three studies, involving sparrows caught from the same populations over just a few years, make clear that the role for *TLR4* as an intermediary of immune protection and food selectivity will take time to resolve. Indeed, in these different studies, *TLR4* expression was measured in different tissues at different time point and in the presence and absence of a pathogen. The studies were not designed to capturing the expected dynamic interplay between EP, gene expression, pathogen resistance and behaviour as it would require more intensive and elegant study designs that those we used, but these topics definitely warrant future attention.

Despite these challenges and as our results hint, we continue to expect that if higher *TLR4* expression in the gut provides greater defence against potential food-borne pathogens, individuals with higher *TLR4* expression and/or higher EP for nimble regulation thereof could take more foraging risks, as shown for females here. Usually, generalist foragers (including house sparrows) are less fearful of novelty and less wary towards new foods than specialists (Greenberg, 1983). Indeed, during range expansions house sparrows at the expanding edge of populations were more exploratory and showed higher propensity to eat novel foods than those from longer-established populations (Liebl and Martin, 2012; Martin et al., 2014; Martin and Fitzgerald, 2005). This pattern could arise as invading birds survive by obtaining resources quickly while increasing their resistance to exposure to pathogens (Canestrelli et al., 2016; Liebl and Martin, 2012; 2014; Martin and Fitzgerald, 2005). Coming full circle, introduced house sparrow populations have higher EP in the *TLR4* promoter compared to native populations (Hanson et al., 2020; Hanson et al., 2022).

4.2. Possible mechanistic links between risk-taking and TLR4 expression

EP was positively related to *TLR4* expression in the gut, and *TLR4* expression influenced the propensity and sensitivity of female sparrows to approach and consume risky food. We expect that these linkages evolved because high EP helps mitigate infection risk by providing a latently plastic yet heritable capacity for modulation of *TLR4* expression (Hanson et al., 2021; Sheldon et al., 2023). The suite of studies we have conducted on EP in *TLR4* in house sparrows suggests that it is selectively advantageous, probably for the above reasons. This ability could be crucial for invasion success and may be related to the success of house sparrows, generally, as invaders.

Mechanistically, the next enlightening steps would involve work on how *TLR4* expression in the gut is physiologically linked to behaviour: *how does the brain 'know' about TLR4 expression in the gut*? An interesting possibility is through the gut-microbiota-brain axis, as studies in human diseases have revealed. For Parkinson's and Alzheimer's disease, TLR signalling can influence neural circuits and immune processes in both the gut and the brain as well as communication between the host and its microbiota (Caputi and Giron, 2018; Lin et al., 2019). Perhaps similar connectivity underpins risk-aversiveness in house sparrows. Indeed, growing literature suggests a link between innate immune function particularly *TLR4* expression and behavioural changes in the context of motivation, reward, exploration, cognition, and stress-induced depressive-like behaviour and anxiety (Connolly et al., 2021; Fei et al., 2022; Femenia et al., 2018; Li et al., 2016; Li et al., 2021; Li et al., 2022; Okun et al., 2012; Potter et al., 2019; Quave et al., 2021). For example, male *TLR4*-knockout mice showed decreased novelty-associated exploratory behaviour and social interaction in a stressful environment but similar fear and anxiety level than wild-type mice (Li et al., 2016). In another study, male and female *TLR4* deficient mice were less anxious but socially-impaired in the absence of an experimental stressor with greater anxiety effects in males (Femenia et al., 2018). Furthermore, increased anxiety-like behaviour was observed in male mice with central administration of a *TLR4* antagonist (Okun et al., 2011). On the contrary, treatment with *TLR4* antagonist decreased anxiety in female mice (Connolly et al., 2021) and loss of *TLR4* in Tph2 neurons resulted in lower anxiety-like behaviours in males (Li et al., 2022). Altogether, not only activation of *TLR4* but just the presence of *TLR4* expression can affect spatial learning and memory in sex-specific ways (Connolly et al., 2021; Fei et al., 2022; Okun et al., 2012; Potter et al., 2019). These results suggest that the regulation of *TLR4* broadly could affect adaptative behavioural responses. One way to coordinate *TLR4* expression such that it complements behavioral priorities is through efficient epigenetic regulation and high EP. Whereas we did not measure central *TLR4* expression nor discern the cell types from which it was derived in any sample, the discrepancy in these results on the effects mostly of *TLR4* inactivation or deletion may explain the lack of clear direct link among behaviour, infection mitigation, EP and gene expression in the gut and/or the sex effects we observed in this and our previous studies.

4.3. Validating study elements

Several lines of evidence suggest that our study design was adequate to testing our hypotheses. First, as it has been shown in different vertebrates (Coulson et al., 2018; Poirotte et al., 2019; Sarabian et al., 2018), house sparrows can discriminate between unspiked and food spiked with faeces and show a preference for unspiked food. Birds ate normal food faster, fed at normal feeders more often, and ate more normal food than spiked food. Second, sex differences in foraging behaviour were detected and in the expected direction based on past studies and life history priority differences between the sexes. Females probably were faster to eat and ate more food in general than males because of the timing of our experiments. We caught birds during the breeding period when females likely had greater energetic needs. Moreover, the above effects of EP on *TLR4* expression and feeding behaviours were only evident in females. We previously found sex differences in *TLR4* expression across tissues and that high EP females showed greater inducibility and reversibility in *TLR4* expression in the blood (Hanson et al., 2021). In this study, high EP individuals showed higher *TLR4* expression in the gut, and females with higher *TLR4* expression ate more spiked food while there were no relationships in males. The greater nimbleness in the control of *TLR4* expression observed in females (Hanson et al., 2021) may provide them more effective or efficient gut immunity and thus protection against food-borne pathogens, which allow them to take more foraging risk.

4.4. Conclusion

Many species have evolved behavioural defences to facilitate detection and avoidance of potentially contaminated foods before they are consumed (Schaller and Park, 2011). The mechanisms mediating behavioural immunity much less its connections with physiological immunity are poorly understood (Sarabian et al., 2018). Here, we show that the regulation of a key immune gene, *TLR4*, may be involved. Whereas effects of *TLR4* expression and EP in *TLR4* were complex, if higher *TLR4* expression in the gut provides greater/faster defence against potential food-borne pathogens, individuals with higher expression and/or higher EP could take more foraging risks. This ability could be crucial in environments where food options are unfamiliar. It also suggests that the epigenetic regulation of key immune genes may be important drivers of behavioural decisions in the context of the trade-off between energy acquisition and infection avoidance, a subject deserving future research attention.

CRediT authorship contribution statement

Cedric Zimmer: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Haley E. Hanson:** Conceptualization, Investigation, Methodology, Validation, Writing – review & editing. **Marisa Garrison:** Investigation, Writing – review & editing. **Darrys Reese:** Investigation, Validation, Writing – review & editing. **Roi Dor:** Investigation, Writing –

review & editing. **Jørgen S. Søraker:** Investigation, Writing – review & editing. **Phuong Ho Thu:** Investigation, Writing – review & editing. **Elizabeth L. Sheldon:** Investigation, Validation, Writing – review & editing. **Lynn B. Martin:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be available in Figshare upon publication 10.6084/m9.figshare.21687971

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Appendix A. Supplementary data

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