Surveillance for microbes and range expansion in house sparrows

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Interactions between hosts and parasites influence the success of host introductions and range expansions post-introduction. However, the physiological mechanisms mediating these outcomes are little known. In some vertebrates, variation in the regulation of inflammation has been implicated, perhaps because inflammation imparts excessive costs, including high resource demands and collateral damage upon encounter with novel parasites. Here, we tested the hypothesis that variation in the regulation of inflammation contributed to the spread of house sparrows (Passer domesticus) across Kenya, one of the world’s most recent invasions of this species. Specifically, we asked whether inflammatory gene expression declines with population age (i.e. distance from Mombasa (dfM), the site of introduction around 1950). We compared expression of two microbe surveillance molecules (Toll-like receptors, TLRs-2 and 4) and a proinflammatory cytokine (interleukin-6, IL-6) before and after an injection of an immunogenic component of Gram-negative bacteria (lipopolysaccharide, LPS) among six sparrow populations. We then used a best-subset model selection approach to determine whether population age (dfM) or other factors (e.g. malaria or coccidian infection, sparrow density or genetic group membership) best explained gene expression. For baseline expression of TLR-2 and TLR-4, population age tended to be the best predictor with expression decreasing with population age, although other factors were also important. Induced expression of TLRs was affected by LPS treatment alone. For induced IL-6, only LPS treatment reliably predicted expression; baseline expression was not explained by any factor. These data suggest that changes in microbe surveillance, more so than downstream control of inflammation via cytokines, might have been important to the house sparrow invasion of Kenya.

1. Introduction

Organismal mechanisms underlying range expansions are poorly known in vertebrates [1]. Behavioural traits such as aggression [2] and innovation [3] can be important, as can morphological [4] and physiological traits such as reproductive proclivity [5], regulation of stress hormones [6,7] and immune defences [8]. For instance, in cane toads (Bufo marinus) spreading across Australia, changes in morphology, behaviour and physiology have arisen even though the species arrived in 1935 [4]. Here, our interest was to determine whether regulation of a critical immune defence, inflammation, differed among populations of a recent invader in a manner that would implicate inflammation as a mediator of range expansion. Of all the immune processes that could facilitate range expansions [8], inflammation might be among the most important.

Inflammation can occur on the tissue level and be resolved rapidly, but it can also be a whole body response involving prolonged increases in hepatic synthesis of acute phase proteins, alterations of body temperature and the induction of sickness behaviours [9]. Such acute phase responses (APRs) are among the most expensive immune defences available to animals [10], so inflammation should be damped during invasions for two reasons. First, if enemy release (i.e. a reduction in parasite burden or diversity) also occurs, then individuals that allocate resources to traits conducive to competition in new areas (i.e. growth rate,
body size, reproductive output) should fare best. Second, if parasites in new areas elicit strong, damaging immune responses, as many novel parasites do when they infect novel hosts [11], individuals with dampened inflammation should suffer the least collateral damage upon parasite exposure. In our study species, the house sparrow (Passer domesticus) [12], both enemy release [13] and a dampening of inflammation [14–16] seem to explain its broad distribution.

In this study, we asked whether range expansions themselves (i.e. spread from the point of introduction) are influenced by inflammatory responses. Tests of such ideas are inherently rare, because one can rarely introduce organisms outside their native range. However, one can study species that are expanding their ranges naturally or owing to human actions. Our approach here involved the latter, in the form of theongoing house sparrow range expansion across Kenya. We asked whether distance from Mombasa (dDM), the site of introduction around 1950 [12,17], could predict baseline (constitutive) or induced expression of key mediators of inflammatory responses: Toll-like receptors (TLRs) and a pro-inflammatory cytokine (see below). Three lines of evidence supported the use of dDM as a surrogate for population age. First, house sparrows are reliant on human resources, and northern Kenya is predominantly desert with sparse human settlement. Second, anecdotal observations (National Museums of Kenya 2010, unpublished data; [17]) indicate that the Kenyan house sparrow range expansion took place recently along the main road connecting Mombasa to the Ugandan capital, Kampala. Third, genetic characteristics of Kenyan house sparrows are indicative of a recent bottleneck, one that has not been replenished across the entire Kenyan range [18]. Given these traits and a lack of evidence of anthropogenic movements of other introduced African house sparrows (e.g. Tanzania, South Africa, multiple west African countries), Kenyan birds likely come from one or few introductions.

We tested the hypothesis that inflammation impacted the Kenyan range expansion by investigating what factors affect expression of two TLRs (TLR-2 and 4) and the cytokine, interleukin-6 (IL-6), before and after injection with lipopolysaccharide (LPS) from Escherichia coli [30]. We chose TLRs because they are critical genome-encoded receptors for conserved microbial elements [19]. In birds, TLRs are most abundant on macrophages, dendritic cells and heterophils [20], but they also occur on lymphocytes [21]. We targeted TLR-2 and TLR-4 because they are strong instigators of APRs [22] and recognize components of bacteria, common pathogens of wild birds [23]. Avian TLR-4 is directed predominantly towards LPS, whereas TLR-2 predominantly detects peptidoglycans from Gram-positive bacteria (but can also mediate responses to LPS [24] and other parasites such as malaria [25]). Once activated, TLRs instigate intracellular molecular cascades, including cytokine expression such as IL-6 [26]. IL-6 has many effects, including the coordination of fever [27] and some sickness behaviours [28].

We used a model selection approach to determine whether population age (dDM) was a better predictor of gene expression than other factors known or expected to impact expression of these genes. We investigated malaria [29] and coccidia [29] infections, because each can affect or be affected by TLR and IL-6. We included local population density, because density might be a surrogate for disease transmission risk and/or induce immune prophylaxis [30]. We included body mass, because immunity can be condition-dependent [31]. Finally, we evaluated genetic structure using data from seven microsatellites [18]; this information allowed us to assign individuals to genetic groups and hence probe coarsely whether selection or other population genetic processes affected geographical gene expression patterns. We expected population age (dDM) to have comparable or stronger effects as the above factors, and we expected that expression of one or more inflammatory genes would be inversely related to population age.

2. Material and methods

(a) Estimation of density of Kenyan house sparrows

In February–May 2011, we conducted fixed-radius (50 m) point counts [32] in eight Kenyan cities in which an earlier survey indicated that birds were sufficiently dense for study. At 6–10 sites in each of the eight cities (with number of count sites determined by city size and the availability of house sparrow habitat), we counted all house sparrows seen and heard in two 5 min periods between sunrise and 10.00 and calculated sparrow densities (birds per hectare) by quantifying city area with Google maps.

(b) Bird capture and care

In July 2010, we captured adult house sparrows in mist nets from sunrise to 11.00 in six Kenyan cities; time constraints prevented the inclusion of the other two cities. In less than 30 min of capture (typically, approx. 10 min) 50 μl of blood was collected from the brachial vein, and body mass was assessed to 0.1 g. An aliquot of 5 μl of fresh blood was then added to 200 μl of RNAlater (Ambion) in an autoclaved 1.5 ml tube. Immediately thereafter, birds were injected with 100 μl of 1 mg ml⁻¹ LPS (from E. coli 055:B5; Fisher L4005) in sterile saline subcutaneously over the breast muscle [33]. LPS solutions were stored in silanized bottles to minimize LPS adherence to storage vessel walls; all solutions were kept at approximately 4°C for the study duration and mixed well prior to each use. Each individual received a small aluminium leg band with a unique identification number (obtained from Kenya Wildlife Service). Four hours after LPS injection [33], another blood sample was collected as above and stored in RNAlater. Birds were released at sites of capture thereafter. All blood samples in RNAlater were stored at room temperature for approximately four weeks, after which they were frozen at −40°C until mRNA extraction. In a pilot study, this sample storage approach resulted in non-significant degradation of mRNA (data not shown). All procedures met guidelines for the use of animals in research and were approved by the USF IACUC (W3877) and the Kenyan Ministry of Science and Technology.

(c) House sparrow population genetic variation

We characterized the spatial genetic characteristics of Kenyan house sparrows from the six cities by Bayesian clustering with the software TESS [34,35] of microsatellite genotypes at seven loci [18] collected from blood samples. Four genetic groups (k) were detected among cities and this information was used to analyse gene expression.

(d) Real-time quantitative polymerase chain reaction

We followed previously developed protocols to measure immune gene expression, including sequencing of house sparrow-specific partial mRNAs (see electronic supplementary material, table S1) for development of RT-qPCR primers [33]. Total RNA was extracted from blood using a SurePrep leucocyte RNA purification
kit (Fisher Bioreagents) based on the manufacturer’s instructions. The SuperScript III first-strand synthesis system for RT-qPCR (Invitrogen, Grand Island, New York, NY, USA) was used on extracted RNA (up to 0.5 μg μL−1) to synthesize cDNA following the manufacturer’s instructions; both RNA and cDNA concentrations were determined using a spectrophotometer, and all subsequent PCRs conducted with standard cDNA concentrations. To quantify immune gene expression, a StepOne (Applied Biosystems, Grand Island, New York, NY, USA) system was used. Briefly, approximately 250 ng cDNA was added to 12.5 μL qPCR MasterMix (Applied Biosystems), 2.5 μL ultrapure water and 7.5 μL of primers (900 nM each) and appropriate probe (250 nM; tagged with a VIC or FAM fluorescent label). TLR-2 and TLR-4 were measured in a multiplex reaction (replacing the ultrapure water with the second probe). Ultrapure water was used as a negative control and a four-step (100, 33.3, 11.1 and 3.7 ng μL−1) standard curve for each of the three genes was made using a homogenate of liver and spleen cDNA from LPS-treated house sparrows from Tampa, FL, USA [33]. Quantitative PCR conditions were 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles: 95 °C for 15 s and 60 °C for 1 min. All samples, standards and controls were run in duplicate.

(e) Malaria and coccidia infection status
To determine coccidia infection, faeces was collected from birds held alone in clean, cloth bags for approximately 10 min after capture (between capture and 1100). Fresh faecal samples were preserved at room temperature in an autoclaved, 2 ml screw-top tube which was then wrapped in parafilm until sugar flotations were performed (8–12 weeks; [36]). Individuals were defined as infected if oocysts were detected in samples by microscopic examination. To determine malaria infection, a portion of blood was stored in RNAlater (Life Technologies, Grand Island, New York, NY, USA) and kept at room temperature for up to one month, after which samples were frozen at −40 °C until DNA extraction. DNA was later extracted using a standard phenol : chloroform : isoamyl alcohol (25 : 24 : 1) protocol with a NaCl–tris purification. Extracted gDNA dissolved in Tris-EDTA (TE) buffer was kept frozen at −20 °C until PCR was performed. Our PCR protocol was based on Fallon et al. [37]. Briefly, we diluted primers 343F (5′GCTTACCCATGGCTTGT) and 496R (5′ACCAGTGCA TTTCTGGTG) to 10 μM and mixed 1 μL of each primer with 1 μL of 100 ng μL−1 template DNA, 9.5 μL of ultrapure water and 12.5 μL of PCR master mix (Promega, Madison, WI, USA) in a 25 μL reaction. Thermocycling conditions were an initial denaturing at 94 °C for 2 min, then 45 cycles of 94 °C for 50 s, 55 °C for 50 s and 72 °C for 25 s, and a final elongation period at 72 °C for 2 min. gDNA extracted from known malaria-positive birds was used as a positive control and ultrapure water and extracted E. coli DNA as negative controls. Controls were run between every 14 experimental samples [38]. PCR products were run on 1% agarose gels with 0.1% ethidium bromide and read with a UV light. Samples with bands at approximately 150 bp (exact amplicon size is 153 bp) were considered positive [37].

(f) Data analysis
We used regression to attempt to describe the relationship between distance from Mombasa (dM) and sparrow population density and individual body mass in Kenya. We used ANOVA to compare body mass among populations with dM as our independent predictor. Because our design was unbalanced and involved repeated-measures (with some randomly missing cells [39]), we used general linear-mixed models to investigate sources of variation in immune gene expression [40]. None of the three immune gene distributions was significantly non-normal (one-sample Kolmogorov–Smirnov (KS) tests). We used a model selection approach, which allowed us to determine the importance of dM effects versus other factors expected to influence gene expression. For baseline expression, we developed a global a priori model, including fixed effects such as body mass (an indicator of individual health), sparrow population density (an index of interindividual contact rates), individual infection with malaria or coccidia parasites (+ or −), genetic structure and distance from Mombasa (dM). As only one individual was assigned to k = 5 by TESS [18], we dropped that individual, so all remaining birds were assigned to k = 1–4. We could not include other predictor variables (e.g. altitude, precipitation, sex), as samples sizes were too small. We then took a best-subset approach starting from our global model (using corrected Akaike information criteria (AICc) scores) to identify the best models (based on evidence ratios (Δc, [41]). We then estimated the relative importance of predictors [41] across the top models (Δc, less than 2) and calculated model-averaged parameter estimates and standard errors for each predictor (see electronic supplementary material, table S3). For induced gene expression, we used a similar approach except that we included time (pre- or post-LPS) and two interactions (time×dM and time×genetic structure) as fixed effects and a random effect of individual to account for repeated-measures. Finally, to determine whether baseline gene expression was correlated within individuals, we used two-tailed Pearson tests. For all analyses, we used SPSS v 20. Gene expression data are available in Dryad (www.datadryad.org).

3. Results
(a) Population density and genetic structure
Sparrow density was highest in Mombasa (approx. 80 birds ha−1), and all other populations tended to be at or below 40 birds ha−1 (see electronic supplementary material, figure S1). There was no significant relationship between dM and population density (see electronic supplementary material, table S2); linear (F1,7 = 2.6, p = 0.16), quadratic (F2,7 = 1.3, p = 0.35) and cubic (F3,7 = 3.4, p = 0.13) functions did not predict sparrow density. Body mass differed among populations though (see electronic supplementary material, figure S2), but not in a systematic manner with distance from Mombasa (linear regression: F1,57 = 5.9, p = 0.18, r = −0.31). Genetically, multiple waves of expansion likely occurred among the birds screened in this study. All cities but Mombasa were populated by individuals from multiple genetic groups (see electronic supplementary material, figure S3; [18]), suggesting anthropogenic admixture among Kenyan cities. Genetic differentiation among cities was not related to dM, and there was no pattern between individual membership in genetic group and dM (see electronic supplementary material, figure S3). This lack of a pattern suggests that the initial range expansion in Kenya did not follow a stepping-stone pattern; however, as estimates of genetic diversity correlate with dM [18], dM appears a reasonable surrogate for population age.

(b) Baseline gene expression
The best model describing baseline TLR-4 expression included distance from Mombasa (dM), malaria infection, population density and genetic structure (table 1). However, four other models provided appreciable support. The most important predictor across all five top models was dM, followed by genetic structure and malaria infection; population density, structure and body mass were of low importance, and coccidians were not included in any of the top models (table 2). Of the three top predictors, dM was positively related to TLR-4 expression; cities nearest the range edge expressed the
most TLR-4 prior to LPS treatment (figure 1a). Genetic structure also affected TLR-4 expression with the most common types (k = 1 and 2) expressing more TLR-4 than the least common type (k = 4; figure 1b). Finally, malaria-infected birds tended to express less TLR-4, although this effect was weak (figure 1c and table 2).

The best model for baseline TLR-2 expression included distance from Mombasa (dfM), malaria infection, coccidian infection, population density and body mass, but three other models were similarly supported (table 1). The most important predictors among the top models were dfM and malaria infection; density, mass and coccidian infection were less important, and genetic structure was not included in any of the top models (table 2). As with TLR-4, birds from cities near the range edge expressed the most TLR-2 prior to LPS treatment (figure 2a). Also as with TLR-4, malaria infection predicted reduced TLR-2 expression (figure 2b). Population density was inversely related to TLR-2 expression although this effect likely was driven by one exceptionally dense city, Mombasa (figure 2c). Baseline IL-6 expression was not influenced strongly by any predictor (table 1).

Table 1. Best-subset model results for immune gene expression among Kenyan house sparrows. Dist, distance from Mombasa (km); mal, infection with malaria (+); mass, body mass; density, sparrow population density; cocc, infection with coccidian parasites (+); k-value, genetic structure assignment.

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Table 2. Relative importance of predictors of baseline TLR expression in Kenyan house sparrows.

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4. Discussion

We predicted that TLR and/or IL-6 expression would decrease with distance from Mombasa (dfM), the site of introduction of house sparrows to Kenya. We found instead that baseline TLR-2 and TLR-4 expression increased with dfM, whereas induced expression did not vary systematically. Moreover, neither baseline nor induced IL-6 varied among populations, but as with TLRs, LPS elevated IL-6 expression. Although other factors (i.e. malaria infection, body mass, genetic structure) affected immune gene expression, a strong effect of population age on baseline TLR expression implicates microbial surveillance as a mediator of the Kenyan range expansion. Below, we discuss the implications of these findings as well as how one might disentangle their genetic, environmental and/or epigenetic basis.

(a) Microbe surveillance versus responsiveness at range edges

Birds expressing little TLR and/or IL-6 were expected to be more common at range edges, but for baseline TLR, the reverse was observed. One interpretation of this pattern is that high baseline TLR-2 and TLR-4 is a compensatory mechanism for other factors [42]. We find that stress hormone regulation is related to geographical distribution; birds near the range edge release more corticosterone in response to a stressor than birds near the site of introduction [6]. Glucocorticoids are immunosuppressive, inducing among other things apoptosis of leucocytes [43]. High TLR in new populations (i.e. more macrophages expressing more TLR) may enable birds to maintain microbial surveillance at comparable levels with old populations in which corticosterone responses are more modest.

Another non-exclusive possibility invokes costs of inflammation, but in a subtle way. We expected that hosts at range edges would damp APRs outright to save resources for other processes [8] and/or suffer less from novel infections [44,45]. Given the complexity of the regulation of inflammation, the ubiquity of microbes and the propensity of TLRs to be flexible in regards to the effector mechanisms they elicit [46], our predictions should have been more nuanced. In the light of the data at hand, high constitutive TLR expression may enable house sparrows to avoid pathology in places where hosts and parasites will have had the least time to co-evolve [45]. Novel parasites are notorious for causing hosts damage via inflammatory over-exuberance [11,44], so the pressure to survey for microbes may be especially important at range edges [47]. The main function of TLRs is to detect and begin to coordinate responses to diverse parasites [9,19]. High constitutive TLR expression could thus enable sparrows to detect microbes quickly [48], tolerate their endotoxins more effectively [49] and/or initiate clearance processes before microbes incite pathology [50]. These hypotheses are consistent empirically with observations in rodents [48,51] and consistent theoretically with regards to the costs and benefits of immune defences [52]. The second proposed function is also consistent with the high endotoxin tolerance in house sparrows relative to mammals [33].

Although the hypothesis that elevated TLR promotes colonization success warrants testing, it does not address why sparrows from older sites expressed little TLR. One possibility is that constitutive TLR expression is itself costly.
House sparrows have been in Kenya for just a few generations [12], and such a short period of time would restrict but not rule out a role for evolution by natural selection. Resistance to a novel parasite (Mycoplasma gallisepticum) is thought to have evolved in just a few generations in house finches (Carpodacus mexicanus) [54], so perhaps Kenyan house sparrow populations possess distinct alleles that modify immune gene expression. Indeed, an influence of genetic structure [18] on TLR expression observed here implies a role for genetic factors in the invasion of Kenya. However, the small number of individuals in the most distinct group makes us reluctant to speculate much about this discovery. Moreover, for several reasons, it seems reasonable that phenotypic plasticity might also be important in population differentiation of gene expression. First, genetic variation is low in Kenya [55], but phenotypic variation is extensive [6]. Second, a growing body of evidence indicates that the configuration of the adult immune system is strongly impacted by early-life experiences, especially with respect to inflammation [56,57]. Third, molecular epigenetic variation in the form of methylation is high among individual house sparrows in their native and introduced ranges [58], and epigenetic variation at TLR-4 promoters in poultry is inducible and impactful of Salmonella infection outcomes [59]. In this study, individuals expressing little TLRs were more likely to be infected by malaria and coccidia, emphasizing the role of prior experience on gene expression. Finally, condition-dependence has been shown to mediate variation in immune gene expression in wild populations [31], and a role of condition in the Kenyan house sparrow invasion was indicated here by the inclusion of body mass in some models for baseline TLR expression.

Going forward, it will be critical to conduct controlled studies to address the many unanswered questions arising in a natural experiment such as ours. Perhaps the most important is to discern the roles of selection and plasticity in Kenyan house sparrow phenotypic variation. Another is whether mRNA expression represents protein expression. A third is covariance among gene expression; constitutive expression of all three genes was related within individuals, perhaps because of prior infections comparably sensitized measured elements of inflammatory responses [60]. However, it is surprising that TLR expression would covary so strongly, given the distinct microbes each TLR recognizes and the ancient divergence of TLR-2 and TLR-4 gene families [61]. A final issue will be to discern whether regulatory control at the level of TLRs [9] relates to microbial control [62]. The antimicrobial defences that are elicited upon TLR activation probably also influence how sparrows colonize new areas, but we did not test this possibility here. Altogether we have just scratched the surface in understanding the role of inflammation in range expansions, but we find it exciting to observe predictable, broad-scale patterns of immune gene expression in spite of the many environmental and genetic vagaries that exist in nature.

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(b) Genetic, environmental and epigenetic influences on immune gene expression

Figure 2. Best predictors of baseline Toll-like receptor 2 (TLR-2) expression in Kenyan house sparrows: (a) distance from Mombasa (dfM), a surrogate for population age (most distant = youngest), is positively related to gene expression; (b) malaria-positive birds expressed less TLR-2 than uninfected birds and (c) birds from high-density cities expressed less TLR-2 than birds from low-density cities. Bars are means ± 1 s.e.


