Costs of immunity and their role in the range expansion of the house sparrow in Kenya

Lynn B. Martin¹, Holly J. Kilvitis¹, Amber J. Brace¹, Laken Cooper², Mark F. Haussmann³, Alex Mutati⁴, Vincent Fasanello³,⁶, Sara O’Brien², and Daniel R. Ardia⁵

¹ University of South Florida, Department of Integrative Biology, Tampa FL 33620
² Radford University, Department of Biology, Radford, VA 24142
³ Bucknell University, Department of Biology, Lewisburg, PA
⁴ National Museums of Kenya, Department of Ornithology, Nairobi, Kenya
⁵ Franklin and Marshall College, Department of Biology, Lancaster, PA
⁶ Washington University in Saint Louis, Department of Biology, Saint Louis, MO

Corresponding author:
Lynn B. Martin: phone - 813-974-0157; email: lbmartin@usf.edu

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Summary statement:
During the range expansions, hosts will encounter comparatively few familiar parasites and many novel parasites. This scenario might require adjustments in the nature of immune responses. Here, we investigated the costs of inflammation in sparrows invading Kenya. Birds at the range edge had high resource but low self-damage costs; at the level of individuals, costs were related to the expression of a microbial surveillance gene, Toll-like receptor 4.
Abstract:
There are at least two reasons to study traits that mediate successful range expansions. First, dispersers will found new populations and thus impact the distribution and evolution of species. Second, organisms moving into new areas will influence the fate of resident communities, directly competing with or indirectly affecting residents by spreading non-native or spilling-back native parasites. The success of invaders in new areas is likely mediated by a counterbalancing of costly traits. In new areas where threats are comparatively rare, individuals that grow rapidly and breed prolifically should be at an advantage. High investment in defenses should thus be disfavored. In the present study, we compared the energetic, nutritional and collateral damage costs of an inflammatory response among Kenyan house sparrow (*Passer domesticus*) populations of different ages, asking whether costs were related to traits of individuals from three different capture sites. Kenya is among the world’s most recent range expansions for this species, and we recently found that the expression of *Toll-like receptors* (TLRs), leukocyte receptors that instigate inflammatory responses when bound to microbial elements, was related to the range expansion across the country. Here, we found (contrary to our expectations) that energetic and nutritional costs of inflammation were higher, but damage costs were lower, in range-edge compared to core birds. Moreover, at the individual level, *TLR-4* expression was negatively related to commodity costs (energy and a critical amino acid) of inflammation. Our data thus suggest that costs of inflammation, perhaps mediated by *TLR* expression, might mitigate successful range expansions.
Introduction

Many species are innocuous when introduced outside their native ranges, barely maintaining viable populations. However, some invaders become extremely damaging (Parker et al. 2013), with damage occurring directly through predation or competition or indirectly through the transmission of parasites (Raffel, Martin & Rohr 2008; Kelly et al. 2009). Surprisingly, we still know little about what traits comprise successful invaders, especially for vertebrates. Perhaps the best-studied introduced vertebrate species is the cane toad (*Rhinella marina*), which spread across Australia in ~80 years (Phillips et al. 2006; Kolbe, Kearney & Shine 2009; Brown & Shine 2014; Brown et al. 2015a; Brown et al. 2015b; Rollins, Richardson & Shine 2015). Other non-native vertebrates, particularly rodents and songbirds (Losos, Warheit & Schoener 1997; Kolbe et al. 2004; Lee, Martin & Wikelski 2004; Lee, Martin & Wikelski 2005; Fassbinder-Orth, Barak & Brown 2013; Vilcinskas et al. 2013; White et al. 2013; Morand et al. 2015; Tian et al. 2015), have gained some recent attention, but the diversity of research approaches makes generalizations about facilitators of range expansions premature.

One pattern that is emerging involves immune system architecture. For instance, variation in the regulation of inflammation appears important to the invasion success of the house sparrow (*Passer domesticus*) (Liebl & Martin 2012; Liebl & Martin 2013; Liebl et al. 2013; Coon et al. 2014; Coon & Martin 2014; Liebl & Martin 2014; Martin et al. 2014; Martin & Liebl 2014; Schrey et al. 2014), one of the world’s most common birds. Compared to a less successful congener, the tree sparrow (*Passer montanus*), the house sparrow invests little energy in inflammatory immune responses (Lee, Martin & Wikelski 2005) at one site in its introduced range, biasing its defenses towards adaptive responses (Lee et al. 2006). In Kenya, where house sparrows were introduced to Mombasa in approximately 1950 (Lewis & Pomeroy 1989), inflammation seems to have mitigated spread over the last 60 years. Range-edge birds have become quite distinct from birds at the core (Liebl & Martin 2012; Liebl & Martin 2013; Coon et al. 2014; Coon & Martin 2014; Liebl & Martin 2014; Martin et al. 2014; Martin & Liebl 2014), in particular in terms of Toll-like receptor (TLR) expression. Indeed, twice previously (Martin et al. 2014; Martin, Liebl & Kilvitis 2014), we found that *TLR-4* and *TLR-2* expression from leukocytes (blood samples) was higher in range-edge than core Kenyan house sparrows. TLRs are pattern-recognition receptors (PRRs), meaning that they serve an important role in host surveillance for infectious disease threats (Medzhitov & Janeway 1998). For instance, TLRs 2 and 4 are predominantly responsible for detecting Gram-
positive and Gram-negative threats, respectively, whereas TLR-3 detects some viral threats (Alcaide & Edwards 2011). TLRs perform these functions by binding constituents of pathogens, namely molecules such as lipopolysaccharide, peptidoglycan, and others that do not exist in hosts but play an integral role in the physiology and structural integrity of the infecting organism.

Because of these particular functional roles for TLRs in immune defense, we proposed that population differences in TLR expression might represent adaptations useful at the range edge and/or disadvantageous traits selected against at the range core. In terms of adaptation, more TLR could help range-edge birds control their parasites better (Martin et al. 2014; Martin, Liebl & Kivitis 2014). On the range-edge, where enemies are probably rarer yet comparatively more novel than at the core, greater TLR expression might be an especially critical aspect of broadly effective, fast-acting inflammation, providing sufficient defense against vital threats. In terms of disadvantages at the core, inflammation is among the most expensive and self-damaging immune defenses available to vertebrates. So, absent strong selection for its persistence, core individuals might express little TLR to minimize the costs of parasite exposures, which can be handled effectively through other mechanisms (e.g., B or T-cell mediated immune memory).

Here, we investigated aspects of the second possibility, asking whether TLR-4 expression was associated with the costs of inflammation (Klasing 2004) among sparrows living at three sites across Kenya differing in time since colonization. First, we compared TLR-4 expression in circulating leukocytes to determine whether previously observed among-population patterns were reproducible. Then, we compared the relative impacts of TLR-4 expression and site of capture on three costs of inflammation: energetic, nutritional and collateral damage. Each cost type has different ramifications for hosts and hence the ecology and evolution of hosts and parasites (Cressler, Graham & Day 2015). Energetic costs entail increased calorie turnover, which could impose trade-offs with other host life functions. Nutritional costs involve, among other things, the assimilation of critical amino acids, which could also impose trade-offs with other life functions. Unlike calories though, critical amino acid costs cannot be compensated by reductions in other activities; additional consumption is mandatory to avoid trade-offs. Collateral damage costs, particularly for inflammation (Raberg, Graham and Read 2009), include DNA and cellular damage, and are a common consequence of the activity of effectors (i.e., oxidative burst) of this broadly
protective defense mechanism. Once inflammation costs were measured, we asked whether variation in costs was predicted by TLR-4 expression at the individual level and site of capture at the population level. We expected that birds from the range edge would exhibit larger costs of inflammation than core birds and that these costs would be positively related to TLR-4 expression at the individual level (Raberg, Graham & Read 2009).

Methods:

Sparrow capture and husbandry: House sparrows were caught using mist nets between the hours of 0600 and 1200 from the cities of Mombasa (n = 12), Nairobi (n = 14), and Nakuru (n = 14) in early July 2013. Mombasa is likely the point of introduction of house sparrows to Kenya (Martin et al. 2014). Nairobi, the capital city located ~500km from Mombasa, was probably colonized around 1980 and is thus presumed to be of intermediate age. Nakuru (650km from Mombasa) is one of the most recently colonized cities in Kenya (~1990), yet also possesses a population dense enough to enable capture of sufficient individuals for study. To attempt to avoid confounds in site comparisons associated with timing of sampling, we conducted the project first in Nakuru followed by Mombasa and finally Nairobi.

We well recognize that the inclusion of only three populations in our study limits inference (because factors such as climate, elevation, parasite communities, and many other factors that differentiate sites could also impact immune costs and/or TLR-4 expression). Nevertheless, we could not include additional populations and also ensure all measurements were made in under three weeks (to minimize any seasonal/climatic influences on results). The USF IACUC as well as the Kenya Wildlife Service approved all methods in advance; all sample transport, export and storage were in compliance with USDA-APHIS and Kenyan governmental requirements.

Study timeline: Immediately upon capture in the field, birds were weighed (to 0.1g) and a small (~100ul) blood sample was taken from the brachial vein. This blood sample was later used to quantify TLR-4 expression, as captivity can affect expression of this gene in house sparrows (Martin et al. 2011). Within a few hours thereafter, birds were placed individually into cages (~33 cm³) where they were held for the duration of the study. Throughout captivity, animals were left undisturbed except at the time of measurements, and they were provided with ad libitum access to tap water (treated with an anti-coccidial medication) and a mix of sorghum and red and white millet, which they consumed readily. Climate
conditions and lighting tracked ambient conditions. Trials began the night of capture (~2100h). Birds were randomly assigned to two cohorts due to limited respirometry equipment; one group was measured from 2100h until 0200h and the second was measured from 0200h until 0700h. For each cohort, an individual was placed singly into a respirometry chamber where it remained for 5h to obtain resting metabolic rate (RMR) estimates. In exploratory data analyses, we queried whether metabolic rate differed between cohorts, given that it was measured at a different time of night between cohorts. As there was no detectable impact of cohort on MR, it was not included in the below analyses. After RMR measurements, birds were removed from chambers, injected with lipopolysaccharide (LPS), gavaged with $^{13}$C-labeled leucine, weighed as above, and returned to their home cage. LPS was derived from Escherichia coli 055:B5 (Fisher L4005), maintained in silanized vials at ambient temperature prior to all injections, and was injected subcutaneously over the breast muscle (1 mg kg$^{-1}$ in 100ul saline vehicle). Labeled leucine (20 mg of $^{13}$C leucine, 99%; Cambridge Isotopes) was administered orally in 200ul peanut oil (McCue et al. 2011; Coon et al. 2014) within seconds of LPS injections. The next day, measurements occurred again following the same schedule, yet unlike the prior night, no new treatments (LPS or leucine) were made. The same approach was taken on a third night, and the morning of the fourth day, all birds were euthanatized via inhalation of isoflurane followed by rapid decapitation. Organs and trunk blood were collected and stored in a liquid-nitrogen charged dry-shipper until they reached the Martin lab in the US, at which time they were stored at -40°C. Sham control individuals (those given only vehicle, not LPS) were not included in the study due to equipment/time limitations.

**Quantitative PCR (qPCR) for TLR-4 expression:** We first extracted RNA from whole blood samples using the SurePrep Leukocyte RNA Purification Kit (Fisher) (Liebl & Martin 2013; Martin, Liebl & Kilvitis 2014). Using a spectrophotometer, these RNA extracts were then diluted to a concentration of 25-50 ng ul$^{-1}$ before being stored at -40°C until qPCR. Reverse transcription and quantitative PCR was conducted using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad). Each reaction contained 10ul iTaq Universal SYBR Green reaction mix (2x), 0.25ul iScript reverse transcriptase, 600nM each of forward and reverse primers, 1ul RNA (25-50ng ul$^{-1}$) and nuclease-free water to a final volume of 20ul. Cycling conditions and melt curve analyses were programmed according to manufacturer’s instructions specific for the StepOne Plus qPCR platform (Applied Biosystems). The TLR-4 forward (5’-GCTCCTGTGTGTACCTGGAC -3’) and reverse (5’- ACAACACAAACCACCTGGGGAG -3’)
qPCR primers were designed using the zebra finch (*Taeniopygia guttata*) TLR-4 mRNA sequence in Genbank (Accession: NM_001142454.1). A six-point standard curve made from a homogenate of zebra finch splenic RNA (728, 227, 76, 24, 7, 2 ng ul⁻¹) was included on each plate, and all samples were run in duplicate.

**Respirometry:** We used an open-flow push-through respirometry system to quantify the energetic costs of inflammation. First, we estimated baseline RMR on the first night after capture (prior to LPS exposure) and RMR again on the 2 subsequent nights, measuring consumption of O₂ and production of CO₂ each time. Birds were measured singly, and each chamber housing a bird was measured for a 20-min period followed by a 2-min pause to clear the system before shifting measurement to a different chamber. Each respirometry chamber received 600 ml min⁻¹ ambient air allowing for constant flushing of chambers; a multiplexer (TR-RM8, Sable Systems, Las Vegas, NV) controlled the chamber measured by the O₂ and CO₂ analyzer (FoxBox, Sable Systems, Las Vegas, NV). Exiting air from chambers passed through a water vapor analyzer (RH-300, Sable Systems) before being scrubbed of water in a column of drierite. After scrubbing, CO₂ concentration was measured. Following CO₂ measurement, air was scrubbed of CO₂ using ascarite and dried using drierite before passing through the O₂ analyzer. A baseline chamber was analyzed for three minutes at the start and end of trials and between sampling of chambers 2 and 3, and 4 and 5. RMR was calculated as the minimum 10-min mean of O₂ consumption for each individual. All birds were post-absorptive and inactive during measurements. Air temperature in each room was measured using two dataloggers (Hobo U-14) and varied between 29 and 31°C, within thermoneutral conditions for this species.

**¹³C quantification:** We measured the nutritional costs of inflammation by assessing assimilation of a stable isotope-labeled amino acid, leucine, into livers and spleens. As in a previous study (Coon et al., 2014), we interpreted high assimilation values as high nutritional costs of inflammation. Isotope levels in both tissue types were measured at the University of South Florida Stable Isotope Lab with a Thermo Fisher Scientific (Finnigan) Delta V 3 keV isotope ratio mass spectrometer (Thermo Scientific, Waltham, MA, USA). Measurements were made following previously developed and validated methods for house sparrows (Coon *et al.* 2014). Measured ¹³C values were recorded as δ¹³CVPDB, which is the difference between the sample and an industry ¹³C standard (Coon *et al.* 2014). Background (pre-capture, natural diet-associated) levels of ¹³C in birds unexposed to
isotopically-labeled leucine were also measured; the mean value for all unexposed individuals from a site was then subtracted from all treated individuals at that site. All such unexposed individuals were adults in visibly good health. We also captured and analyzed unexposed individuals from each study site in case background isotope conditions varied among them.

*Reactive oxygen metabolites:* We measured collateral damage costs of inflammation in the form of reactive oxygen metabolites (ROMs) from blood using the d-ROMs test (Diacron International, Grosseto, Italy). This assay quantifies hydroperoxides, compounds that signal lipid and protein oxidative damage (Treidel *et al.* 2013); more ROMs are indicative of more oxidative activity in tissues and thus greater costs of inflammation. We optimized the assay by running dilution series on 10 individual house sparrow plasma samples and chose a dilution that fell at the midpoint between the calibrator and blank samples. Briefly, we diluted 1 μl of plasma in 200 μl of the provided acidic buffer solution, and then read the plate (at 490nm on a BioTek ELx800, VT, USA) kinetically for 30 minutes (1x min⁻¹). We then calculated the rates of reaction for the calibrator (provided by the manufacturer), a blank, and each sample. ROMs concentration (in mM of H₂O₂ equivalents) was calculated by subtracting the blank from both the calibrator and sample values and then dividing the sample by the calibrator and multiplying by the concentration of the calibrator. All analyses were run in duplicate, and the intra- and inter-assay coefficients of variation were 6.5% and 7.8%, respectively.

*Total antioxidant capacity:* We also measured a potential mitigator of collateral damage, total plasma antioxidant capacity (TAC), using the OXY-Adsorbent test (Diacron International, Grosseto, Italy). This assay measures the effectiveness of the blood antioxidant barrier to cope with oxidant action of hypochlorous acid (HClO) (Treidel *et al.* 2013); more TAC was interpreted to indicate more protection against collateral damage. We optimized the assay for Kenyan house sparrows by running dilution series on 10 individual Kenyan house sparrow plasma samples, choosing a dilution for subsequent assays that fell at the midpoint between the calibrator and blank samples. We diluted 10 μl plasma in 990 μl of distilled water and mixed 5 μl of this diluted plasma with 195 μl of the provided HClO solution. Absorbance was measured at 490nm (BioTek ELx800), and we calculated the rates of reaction for the calibrator (which is provided by the manufacturer), a
blank, and each sample. TAC concentration (in mM of HClO neutralized) was calculated by subtracting the blank from both the calibrator and sample values and then dividing the sample by the calibrator and multiplying by the concentration of the calibrator. All analyses were run in duplicate, and the intra- and inter-assay coefficients of variation were 3.4% and 1.5%, respectively. We failed to collect 48h plasma samples for TAC and ROM assays, and thus report only pre- and 24h-post LPS exposure values.

Data Analysis: All dependent variables were checked for normal distributions; the following variables required log_{10} transformations: TLR-4 expression, RMR, TAC, and ROM. We first used a general linear model to determine whether TLR-4 expression varied among sites. In all models, dfM was always a continuous predictor, as it has been in all of our prior work in this system. This approach, however, precludes all pairwise comparisons and nesting of TLR-4 expression within sites. We then used linear mixed models (LMMs) to assess the impacts of TLR-4 expression and distance from Mombasa (dfM, our surrogate for population age), on inflammation costs. Also, several of our analyses involved repeated-measures of individuals, and linear mixed models allowed us to use individual as a random effect while simultaneously evaluating direct and interactive effects of predictors (e.g., dfM x time (pre vs. post LPS)) on cost metrics. Some models warranted inclusion of other predictors (e.g., organ mass for nutritional costs; baseline RMR for energetic costs). Note too that degrees of freedom sometimes varied among analyses because a) not all data were available for all individuals at all time points (i.e., qPCR failed upon multiple efforts until mRNA samples were exhausted for some individuals), and/or b) Satterthwaite approximations were sometimes required. As exploratory analyses did not reveal sex, metabolic rate cohort, or body mass as strong drivers of costs, none of these variables are mentioned in the below analyses.

We first used separate LMMs and/or GLMs to determine influences of population age (dfM) and TLR-4 expression on costs of inflammation (energetic (RMR), nutritional (leucine assimilation), and collateral damage (ROM)). We also used an LMM to investigate dfM and/or TLR-4 expression effects on TAC, a putative offset mechanism of collateral damage. We then performed separate GLMs on an integrated cost of inflammation generated via Principal Components Analysis. To simplify the PCA (i.e., account for the dynamics of physiological responses), the total cost of each cost type was calculated. For energetic costs, this value was (RMR 2 days post-LPS + RMR 1 day post-LPS) - RMR the day prior
to LPS). For nutritional costs, total leucine assimilation (spleen + liver) was used. For collateral damage, this total cost was (ROM 1 day post-LPS - ROM the day prior to LPS). TAC data (TAC 1 day post-LPS - TAC the day prior to LPS) were also incorporated into this PCA because i) TAC partly functions to ameliorate ROM and ii) TAC responses were inversely correlated to ROM responses (see below). Varimax rotation was used to maximize differences among PCs, and the Kaiser criterion (eigenvalue > 1) was used to identify meaningful PCs. PC scores were then treated as dependent variables in GLMs with TLR-4 expression and dfM as continuous predictors. We set $\alpha$ to $P < 0.05$, and performed all analyses with SPSS v23.0.

Results:

**TLR-4 expression:**

TLR-4 expression in blood samples at the time of capture (i.e., prior to LPS exposure) was not significantly related to distance from Mombasa (dfM: $F_{2,37} = 3.0, P = 0.06$). However, there was a tendency for a pattern reversed from what we have observed previously, with TLR-4 expression increasing with increasing population age (Fig. 1).

**Energetic costs of inflammation:**

Resting metabolic rate (RMR) prior to LPS exposure tracked population age (dfM: $F_{1,38} = 6.0, P = 0.02$) with RMR decreasing with increasing distance from Mombasa. However, TLR-4 expression did not affect RMR prior to LPS exposure ($F_{1,38} = 0.68, P = 0.42$). In response to LPS, changes in RMR initially appeared modest (time: $F_{1,76} = 0.18, P = 0.67$) and not predictable by dfM (time x dfM: $F_{1,76} = 1.2, P = 0.23$; dfM alone: $F_{1,106} = 1.9, P = 0.17$). However upon visual inspection of the time series data, RMR appeared to peak 24h after LPS exposure and return to pre-LPS values 48h after LPS exposure (Fig. 2A). Subsequently, the total energetic cost of LPS exposure was assessed with respect to TLR-4 expression, dfM, and RMR at the time of capture (given differences detected among sites). In that model, range-edge birds had higher energetic costs of LPS exposure than birds from the core (dfM: $F_{1,38} = 20.7, P < 0.001$; Fig. 2B). Moreover, individuals with the lowest RMR at capture also had the highest energetic costs of LPS exposure (pre-LPS RMR: $F_{1,38} = 38.6, P < 0.001$; Fig. 2C). TLR-4 expression was not a significant predictor of total energetic costs ($F_{1,38} = 0.87, P = 0.36$).
**Nutritional costs of inflammation:**

Exogenous, isotopically-labeled leucine significantly enriched organs beyond background levels (treated versus control sparrows: F\textsubscript{1,84} = 40.5 P = <0.001), but these effects did not differ by organ (organ x treatment: F\textsubscript{1,84} = 0.05 P = 0.82). In terms of nutritional costs of inflammation, dfM was positively predictive of leucine assimilation into lymphoid tissues (F\textsubscript{1,65} = 9.0 P = 0.004, Fig. 3). Neither TLR-4 expression (F\textsubscript{1,65} = 0.09 P = 0.76), nor organ mass (F\textsubscript{1,65} = 0.12 P = 0.29), nor organ type (F\textsubscript{1,65} = 0.86 P = 0.36), affected nutritional costs, however.

**Collateral damage costs (ROM) and mitigation (TAC):**

Our surrogate for collateral damage (ROM) increased in response to LPS exposure (β: 0.1 +/- 0.05; F\textsubscript{1,33.2} = 4.7, P = 0.04), but this effect varied among populations (dfM x time: F\textsubscript{1,32.8} = 6.3, P = 0.02). ROM declined in range-edge birds after LPS exposure whereas ROM tended to remain stable in the other two populations (Fig. 4A). Neither dfM alone (F\textsubscript{1,50.8} = 2.2 P = 0.15) nor TLR-4 expression (F\textsubscript{1,35.0} = 1.7 P = 0.21) affected ROM. Conversely, TAC decreased in response to LPS exposure (β: -0.06 +/- 0.02; F\textsubscript{1,36.1} = 7.4, P = 0.01), but dynamics varied with dfM (dfM x time: F\textsubscript{1,35.7} = 6.6, P = 0.02). TAC tended to decline in core birds but values remained static in the other two populations (Fig. 4B). Neither dfM alone (F\textsubscript{1,57.4} = 2.9, P = 0.10) nor TLR-4 expression (F\textsubscript{1,36.7} = 0.23, P = 0.64) affected TAC. Importantly, we detected a significant negative relationship (r = -0.55 P = 0.001) between delta TAC (TAC 1 day post-LPS - TAC the day prior to LPS) and delta ROM (ROM 1 day post-LPS - ROM the day prior to LPS; Fig. 4C).

**Integrated costs of inflammation:**

A PCA indicated that two principal components captured 63% of the total variation in inflammatory costs (or mitigators thereof) among individuals. PC1 explained 37.5% of this variation and was strongly related to collateral damage (delta ROM, r = -0.86) and mitigation (delta TAC, r = 0.78) factors. PC2 explained an additional 26.0% of the variation, mostly capturing energetic (r = 0.83) and nutritional (r = 0.56) costs. Individual variation in collateral damage costs (PC1 scores) were predicted by dfM (F\textsubscript{1,23} = 6.0, P = 0.02), but not TLR-4 expression (F\textsubscript{1,23} = 0.08 P = 0.79); birds from the newer populations were better at damage control than birds from the oldest one (Fig. 5A). dfM was marginally, but non-significantly (F\textsubscript{1,23} = 3.6, P = 0.07), predictive of variation in PC2 scores. TLR-4 expression,
however, predicted PC2 variation ($F_{1,23} = 4.5, P = 0.02$); more TLR-4 expression was related to lower energy and nutrient costs (Fig. 5B).

**Discussion:**

Inflammatory responses could be important in vertebrate range expansions for at least two reasons (Blossey & Notzold 1995). First, reductions could liberate resources for traits more conducive to pressing challenges in new areas (e.g. finding food and mates and securing shelter) (Brace, Sheikali & Martin 2015). Second, colonizers are more likely than core individuals to encounter novel threats (Dobson & Hudson 1986), which might require range-edge individuals to maintain more robust, broadly protective defenses or succumb to unfamiliar infections (Lee & Klasing 2004; Martin et al. 2010). At population cores, parasites should behave (relatively) as dear enemies (Prenter et al. 2004), such that generations of prior exposure would have selected hosts for low-cost types of defenses (Labbé, Vale & Little 2010), parasites for reduced virulence (all else being equal), or both. Here we found evidence for variation in costs of inflammation among Kenyan house sparrows. However, costs were linked to TLR-4 expression, the trait previously argued to have facilitated the range expansion, in a complex way.

*Population differences in inflammation costs:*

Only a handful of studies have evaluated the role of immunity costs in range expansions, and to date, there has been little consensus (White & Perkins 2012). In one comparison, songbird (*Passer*) congeners that were more or less successful at colonizing St. Louis, Missouri (USA) over a similar timespan also differed in how induced inflammation affected reproductive success. The more successful invader, the house sparrow, was unaffected by simulated microbial exposure but the same challenge halved reproductive output in the less successful species, the tree sparrow, *Passer montanus* (Lee, Martin & Wikelski 2005). An additional physiological study of the two species revealed that an inflammatory stimulus did not affect energy expenditure in the house sparrow, but it reduced it in the tree sparrow (*P. montanus*) (Lee, Martin & Wikelski 2005). Even within species, the roles of energetic costs of immunity in range expansion remain unresolved; at the southern edge of the North American range (Panama, where birds arrived in the 1980’s), the cost of an inflammatory response was 7x what it was from New Jersey house sparrows where they have occurred for >150 years (Martin, Hasselquist & Wikelski 2006). In cane toads spreading across Northern Australia, smaller energetic costs of inflammation were observed at the range-
edge (Llewellyn et al. 2012). One reason for the inconsistencies among studies might be the ability of hosts to compensate by reducing activity or otherwise adjusting their calorie budgets; there is some evidence that Kenyan house sparrows manage their body reserves differently when infected than a native *Passer* species (Martin et al., 2010a).

The second resource cost we considered was critical amino acids, and we found high deposition of leucine in lymphoid tissues in range-edge sparrows. As leucine must be obtained by foraging, cost compensation is much more challenging for critical amino acid costs than energetic costs (McCue et al. 2011). This pattern thus suggests that either range-edge birds value lymphoid tissues more than core birds, or range-edge birds have a greater propensity to shunt leucine to lymphoid tissues than core birds. Unfortunately, study design constraints prevented us from resolving a key assumption of leucine assimilation: whether greater deposition at range-edges is protective against infection. Future work could also better capture leucine metabolism dynamics, including assembly and export of defensive proteins (Iseri & Klasing 2013). In another species though, leucine assimilation scaled with parasite exposure (i.e., LPS dose), which partly supports our interpretation of more leucine assimilation as a more costly immune response (Brace, Sheikali & Martin 2015).

Our third cost estimate involved collateral damage, and these costs of inflammation too appeared to play a role in the distribution of house sparrows in Kenya. Birds from the range-edge mitigated reactive oxygen damage (ROM) without sacrificing antioxidant capacity (TAC), whereas core birds appeared to have to expend their antioxidant capacity to avoid damage (Haussmann et al. 2011). Collectively, these data indicate that populations might have diverged already in how they cope with collateral damage. On the other hand, the house sparrow, as a species, might be unique in terms of how it deals with oxidative damage during inflammation. Compared to domestic chickens (*Gallus gallus*) (Treidel et al. 2013) and prairie voles (*Microtus ochrogaster*) (Fletcher et al. 2015), two species measured in the same lab as the birds here, TAC was 10x higher and ROM was 2x lower prior to LPS challenge in Kenyan house sparrows. Thus, an alternative explanation for the pattern observed in Kenya is that there was strong selection for oxidative damage mitigation at the time of the initial introduction to Mombasa, and only those birds with robust TAC and/or modest ROM responses persist today. Compared to other species, Kenyan sparrows appear to maintain an exceptional collateral damage mitigation capacity.
The role of TLR-4 expression in the Kenya house sparrow expansion:

We expected birds at the range-edge to express high *TLR-4*, as we have observed twice previously (Martin *et al.* 2014; Martin, Liebl & Kilvitis 2014). However, *TLR-4* expression did not track population age significantly here; if anything, the trend was reversed from what we observed before. Several studies have found evidence of selection on *TLR* in wildlife over comparably small spatiotemporal scales (White *et al.* 2013). Most of that work involved variation in the sequence of leucine-rich repeat (LRR) regions of TLRs, not sequences influencing the regulation of expression, though (Sironi *et al.* 2015). Across birds, extensive evidence for episodic positive selection on TLR-4 LRR regions have been observed (Grueber, Wallis & Jamieson 2014). On a scale more comparable to our study, a genetic bottleneck at the time of introduction influenced variation in the TLR-4 exon sequence in a passerine introduced to New Zealand (Grueber, Wallis & Jamieson 2013). The only study so far to consider selection on drivers of gene expression in wild animals involved the mannan-binding lectin promoter in roe deer (*Capreolus capreolus*) (Quéméré *et al.* 2015). In that system, there was some evidence for selection over the course of a range expansion. An altogether different perspective for our data, then, is that neutral population-genetic processes might have lead to the accumulation of particular genotypes on range-edges (Shine, Brown & Phillips 2011). Thus, the gene expression patterns we observed now and previously might not be functional, but artifacts of how genes have combined as birds have moved (and been moved) across the country (Schrey *et al.* 2014).

A second possible explanation for inter-study inconsistency in *TLR-4* expression involves plasticity, either adaptive or maladaptive (Ghalambor *et al.* 2007). A similar argument was offered to explain the high incidence of spinal arthritis in cane toads at the margins of the Australian invasion (Brown *et al.* 2007). From this perspective, inconsistency in *TLR-4* expression among our studies might represent plastic changes in the defense portfolio of birds driven by differences in the local conditions they encounter (Gervasi *et al.*; Gervasi *et al.* 2015). One potentially strong driver could be a parasite that altered gene expression patterns (Charbonnel & Cosson 2012). Many domestic rodents alter *TLR* expression in response to microbes, viruses, or other stimuli (Gallic *et al.* 2009), and even in Kenyan house sparrows, *TLR-4* expression in circulating leukocytes can increase 3-fold in just 4h post-LPS (Martin *et al.* 2011). In three-spine sticklebacks (*Gasterosteus aculeatus*), immune gene expression profiles of fish transplanted from one lake to another converged
on the profile of the transplanted lake within a few months (Stutz et al. 2015). In the future, we plan to assess the relevance of lability of TLR-4 expression in Kenya, but samples here were not prepared in the manner necessary to identify the microbes most likely to affect variation in TLR-4 expression.

**Individual predictors of inflammation costs:**
Principal components analysis indicated that TLR-4 expression was related to commodity costs at the level of individuals, but in the opposite direction initially predicted: high TLR-4 expression was associated with low energetic and nutrient costs. Because we used LPS to incite inflammation, we cannot resolve whether/how these costs relate to infection control, nor can we determine whether measured costs translate to (fitness) costs of ecological significance. Nevertheless, our work hints that intermediate levels of resource expenditure might be most favorable for animals (Long & Graham 2011; Adelman 2014), enough of an investment to limit infection but not so much as to require trade-offs with other traits. Damage costs, which appear to mitigate movement distance of individual Australian cane toads at the range-edge (Brown & Shine 2014), were not predicted by TLR-4 expression, but an influence of site of capture suggests that damage costs warrant additional attention in future work.

Finally, an unexpected discovery offers additional insight to the importance of the energetic costs of inflammation at the level of individuals. Resting metabolic rate (RMR) was inversely correlated to changes in RMR after LPS exposure among individuals. Subsequently, the higher RMRs of range-edge birds might cap the magnitude of induced defenses or at least their costs. A similar pattern of high RMR at a range-edge was found before in Australian cane toads (Llewellyn et al. 2012) and was argued to arise because of an ‘Olympic village’ effect. At range-edges, strong selection for rapid maturation and prolific breeding may lead to dominance of individuals with particular life histories (Moreau et al. 2011). Associations between RMR and TAC responses to LPS at the individual level too suggest that life on the range-edge might have as much to do with general alleviation of oxidative damage as much as responses to parasites (Rollins, Richardson & Shine 2015).
Conclusion:

Our data suggest that costs of inflammation probably have affected the house sparrow range expansion in Kenya. Additional study involving common garden experiments would be insightful, especially if they included populations of different ages and from distinct introduction events (Dunn & Hatcher 2015). Our approach prevented us from determining whether other site traits better explained inflammatory costs. Likewise, the intriguing intra-individual correlations among costs and TLR-4 expression coupled with inconsistencies among TLR-4 expression patterns among years/studies leave unresolved the roles of TLR-4 expression as a driver of success in new areas (Ostfeld et al. 2014; Han et al. 2015).

Going forward, it will be useful to determine how the costs and benefits of inflammation in invaders work in concert (or conflict) to mitigate the eruption and spread of parasites in natural and modified systems (Zylberberg, Klasing & Hahn 2014; Barron et al. 2015).

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Fig. 1. Differences in TLR-4 expression in whole blood samples were marginally nonsignificantly different among house sparrows captured from three Kenyan locations. Bars are means +/- 1 SE.
Fig. 2. Effects of LPS exposure on resting metabolic rate (RMR) in Kenyan house sparrows. A) temporal changes in RMR before and after LPS exposure; B) relationship between distance from Mombasa (our surrogate of population age) and the integrated energetic cost of inflammation (i.e., area under the curves in A); C) relationship between RMR before LPS exposure and energetic cost of inflammation. Bars are means +/- 1 SE.
Fig. 3. Effects of distance from Mombasa (dfM) on leucine incorporation into organs in Kenyan house sparrows. Bars are means +/- 1 SE.
Fig. 4. LPS elicitation of collateral damage (reactive oxygen metabolites (ROM)) and mitigation (total antioxidant capacity (TAC)) in Kenyan house sparrows. A) ROM and B) TAC responses vary contingent on distance from Mombasa (dfM); C) TAC and ROM responses to LPS are inversely related among individuals. Bars are means +/- 1 SE.
Fig. 5. Significant associations among Principal Component (PC) scores capturing inflammatory cost variation in Kenyan house sparrows. A) distance from Mombasa predicted oxidative damage costs and mitigation thereof (PC 1 scores) whereas B) TLR-4 expression from whole blood predicted energetic and nutritional costs (PC 2 scores) among all sparrows.