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RESEARCH ARTICLE

Captivity induces hyper-inflammation in the house sparrow (*Passer domesticus*)

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SUMMARY

Some species thrive in captivity but others exhibit extensive psychological and physiological deficits, which can be a challenge to animal husbandry and conservation as well as wild immunology. Here, we investigated whether captivity duration impacted the regulation of a key innate immune response, inflammation, of a common wild bird species, the house sparrow (*Passer domesticus*). Inflammation is one of the most commonly induced and fast-acting immune responses animals mount upon exposure to a parasite. However, attenuation and resolution of inflammatory responses are partly coordinated by glucocorticoid hormones, hormones that can be disregulated in captivity. Here, we tested whether captivity duration alters corticosterone regulation and hence the inflammatory response by comparing the following responses to lipopolysaccharide (LPS; a Gram-negative bacteria component that induces inflammation) of birds caught wild and injected immediately *versus* those held for 2 or 4 weeks in standard conditions: (1) the magnitude of leukocyte immune gene expression [the cytokines, interleukin 1β and interleukin 6, and Toll-like receptor 4 (*TLR4*)], (2) the rate of clearance of endotoxin, and (3) the release of corticosterone (CORT) in response to endotoxin (LPS). We predicted that captivity duration would increase baseline CORT and thus suppress gene expression and endotoxin clearance rate. However, our predictions were not supported: *TLR4* expression increased with time in captivity irrespective of LPS, and cytokine expression to LPS was stronger the longer birds remained captive. Baseline CORT was not affected by captivity duration, but CORT release post-LPS occurred only in wild birds. Lastly, sparrows held captive for 4 weeks maintained significantly higher levels of circulating endotoxin than other groups, perhaps due to leakage of microbes from the gut, but exogenous LPS did not increase circulating levels over the time scale samples were collected. Altogether, captivity appears to have induced a hyper-inflammatory state in house sparrows, perhaps due to disregulation of glucocorticoids, natural microflora or both.

Key words: animal welfare, captivity, disease, health, immune, stress, trade-off.

INTRODUCTION

Rarely is one immune measure at one point in time sufficient for predicting how an individual or species will maintain fitness in light of parasite exposure or infection (Graham et al., 2011; Hawley and Altizer, 2011). However, to obtain multiple samples, most animals must be held in captivity because repeat capture in the wild is difficult if not impossible. Although such approaches can be experimentally powerful, allowing controlled common-garden experiments to disentangle genetic from environmental effects (Adelman et al., 2010a; Martin et al., 2004), captivity itself may have unintended consequences (Buehler et al., 2008a; Buehler et al., 2008b; Ewenson et al., 2003; Ewenson et al., 2001; Kuhlman and Martin, 2010; Matson et al., 2006). For domesticated species, minimal effects of human handling and husbandry are expected because animals would have already been selected for docility (Kunzl and Sachser, 1999). However, for wild species, organisms may perceive the 'controlled' conditions of captivity as threatening (Kunzl et al., 2003) and hence respond poorly. Indeed, although many wild species respond well to captivity, quite a few do not (Mason, 2010). Although predators are absent, food is plentiful, climate is benign and parasites and competitors are rare, some animals experience reproductive, behavioral and physiological deficits in captivity (Morgan and Tromborg, 2007). Thus if species, strains, breeds or individuals respond differently to captivity, insight from comparisons between domesticated and wild organisms and success of captive breeding and conservation programs may be limited.

One of the most-studied effects of captivity on vertebrates is glucocorticoid (GC) regulation. GCs are steroids that foster gluconeogenesis and generally help organisms cope with or overcome stressors (Sapolsky et al., 2000). However, effects of captivity on GCs are varied. In some species, baseline GCs (i.e. circulating levels in the absence of stressors) are elevated, and stress responses (i.e. release of additional GCs in response to a stressor) are small to absent for the first few days. However, after an acclimation period, baseline GCs and stress responses recover to wild-like values (Davidson et al., 1997; Dickens et al., 2009a; Dickens et al., 2009b; Romero and Wingfield, 1999). In other species, acclimation is very slow if not absent (Mason, 2010). In some captivity-averse species, GC receptor expression in the brain and negative feedback mechanisms regulated by these receptors can also be altered even after a month in what are commonly thought to be acceptable housing conditions (L.B.M., A. Urban, C.A.C.C. and A.L.L., submitted). In some zoo animals, GC effects may endure for very long periods, which may explain why some species are prone to opportunistic infections when captive (Mason, 2010).

This last observation, that opportunistic infections often co-occur with poor responsiveness to captivity, was a major motivation for the present study. Stress hormones, particularly GCs, are integral to immune regulation (Sternberg, 2006), although interactions are

complex and dynamic (Martin, 2009). One immune response that is particularly sensitive to GCs is inflammation (Glaser and Kiecolt-Glaser, 2005). Inflammatory responses can take many forms (Sears et al., 2011). Often, inflammation is localized and resolves quickly as pathogens are controlled and damage is healed. In other cases, pathogens replicate at high rates and/or move to body areas where local inflammation alone is ineffective (Kopp and Medzhitov, 2009; Nathan, 2002). In these cases, an escalation of immune defenses is necessary (Medzhitov, 2008), and it is these cases that acute phase responses (APRs) occur. APRs are whole-body inflammatory responses that entail fever, lethargy, anorexia, anhedonia and a host of other behavioral and physiological changes (Dantzer et al., 2008). APRs and inflammation generally are among the fastest-acting and general immune defenses available to animals (Klasing, 2004), capable of controlling viruses, bacteria, protists and even some macroparasites (Medzhitov, 2008). APRs come with high costs, however: calorie and amino acid turnover can be high for individuals mounting fevers and synthesizing anti-microbial molecules (Klasing, 2004). Collateral damage and opportunity costs (e.g., reduced reproductive efforts) can also emerge (Adelman and Martin, 2009; Graham et al., 2005; Graham et al., 2011), making inflammation a double-edged sword (Kopp and Medzhitov, 2009; Sorci and Faivre, 2009).

At the molecular level, inflammation is regulated (among other factors) by Toll-like receptors (TLRs) and cytokines (Medzhitov, 2008; Nathan, 2002). TLR-4 is particularly important in inflammation, as it is one of the most important sensors of Gram-negative bacterial components including lipopolysaccharide (LPS). LPS is a conserved element of bacteria that is integral for replication and structure. Thus, unlike other parasite-associated molecules, LPS cannot undergo extensive structural changes, a constraint that provides hosts a relatively fixed target for attack (Medzhitov and Janeway, 2002). After LPS is detected, macrophages and other leukocytes possessing TLR-4 induce transcription of pro-inflammatory cytokines including interleukin 1β (*IL1β*) and interleukin 6 (*IL6*) (Sternberg, 2006). These cytokines are the primary coordinators of inflammatory responses, and both instigate the release of GCs (Glaser and Kiecolt-Glaser, 2005). It is these same GCs that eventually lead to the downregulation of pro-inflammatory cytokine expression and hence resolution (Sternberg, 2006). In individuals in which GCs are elevated for prolonged periods, inflammatory responses are often disregulated (Glaser and Kiecolt-Glaser, 2005), increasing the risk of both infectious (e.g. influenza and herpes) and non-infectious (e.g. arthritis and depression) disease.

Our goal in the present study was to determine whether and how captivity impacted the regulation of inflammation in the house sparrow, *Passer domesticus* (Linnaeus 1758). We chose the house sparrow for several reasons. First, captivity had previously been found to alter multiple immune functions and GC regulation in this species (Kuhlman and Martin, 2010) (L.B.M., A. Urban, C.A.C.C. and A.L.L., submitted). Second, if captivity affected APR regulation in house sparrows, a close commensal of humans, other avian species that avoid humans or human-modified landscapes might be even more sensitive to captivity (or other chronic stressors). To determine whether captivity impacted inflammation regulation in house sparrows, we compared responses to LPS in terms of: (1) leukocyte gene expression, (2) corticosterone (the predominant avian GC), and (3) circulating endotoxin among wild birds and birds held for 2 or 4 weeks in captivity. By measuring the latter parameter, we hoped to obtain a functional readout of quantitative variation in gene expression (Boughton et al., 2011); quicker clearance of endotoxin should be proportional to cytokine and/or *TLR4* expression and presumably favorable because inflammation could subside more rapidly (Graham et al., 2005).

MATERIALS AND METHODS

Bird capture

Sparrows were caught in mist nets from September to December 2009 at two different sites in Tampa, FL, USA. Birds were removed from the nets within 3 min of initial impact. Mass (to the nearest 0.1 g), age (mature *versus* questionable) and sex (both based on plumage) were noted. Birds were then separated randomly into three cohorts (with sexes and ages distributed evenly as possible among them): wild (N=8), 2 week captives (N=10) and 4 week captives (N=10). Birds in the wild cohort received the experimental treatment (listed below) immediately upon extraction from nets. The remaining birds were temporarily housed in cotton bags for transport to the University of South Florida (College of Public Health vivarium), where they were kept in individual cages with two perches and *ad libitum* access to seed and water for the project duration. Ambient photoperiod was simulated (12 h:12 h light:dark), and room temperature and humidity were held constant. The only disturbance birds experienced (besides experimental procedures) was a few minutes of human intrusion as animal care staff replaced food and water daily. After the final samples were collected, birds were released to their site of capture. All procedures were approved by the USF-IACUC (W3202) prior to the studies, and all procedures are in compliance with US animal welfare regulations.

Experimental treatment

Birds were removed from nets (or cages) and immediately the inner surface of one wing was swabbed with 100% ethanol. The brachial vein was then pricked with a 26-gauge needle, and blood samples were collected into heparinized (~50 µl) and non-heparinized (~100 µl) capillary tubes, which were immediately transferred into sterile 1.5 ml collection tubes. An aliquot of 5 µl of fresh blood was then collected from the heparinized aliquot into 200 µl of RNAlater® (Ambion) in an autoclaved 1.5 ml tube. To minimize researcher impacts on all parameters under captive conditions, and because multiple birds were housed in each room, blood sample collection was performed by a group of individuals, enabling all blood samples to be collected under 3 min of entering rooms. All samples were kept on ice until centrifugation to collect plasma. Immediately after each (baseline) blood sample was taken, 100 µl of 1 mg ml^{-1} LPS (from *E. coli* 055:B5; Fisher L4005) was injected subcutaneously over the breast muscle (Coon et al., 2011). Four hours later, birds were bled again in the same fashion, but this time only ~100 µl of heparinized blood was collected, with a 5 µl fraction being added immediately into RNAlater®. Another blood sample was taken 8 h after the injection, but this time only ~50 µl of heparinized blood was taken. Although additional blood samples may have been informative, they were not collected so as to prevent birds from becoming anemic. Plasma and RNA were stored at –40°C until assays were performed.

Sequencing house sparrow genes

RNA was extracted from spleens and livers of house sparrows caught in Tampa, FL (a separate cohort, N=5) that were injected with Complete Freund's adjuvant (100 µl, 0.5 mg ml^{-1}; Fisher ICN55828). Tissues were stored in RNAlater® and frozen at –40°C until RNA processing. Total RNA was extracted from ~30 mg of tissue using a rotor-stator homogenizer and an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized

using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) following manufacturer's instructions, using up to 0.5 μg μl^{-1} total RNA; RNA and cDNA concentrations were determined using a spectrophotometer. Semi-degenerate primers were then developed based on conserved regions of other avian gene sequences extracted from GenBank's BLAST (Altschul et al., 1997) using PrimerExpress software (Applied Biosystems). PCRs were performed using 1 μl cDNA (100–200 ng μl^{-1}) with TaqMan® PCR Master Mix (Promega) in a thermocycler using annealing temperatures of 43–50°C. The following PCR program was used: 95°C for 5 min; 40 cycles at 95°C for 30 s, the annealing temperature for 60 s, 72°C for 60 s; followed by an extension step of 72°C for 5 min. Appropriate gene amplification was confirmed on a Tris-acetate and ETDA (TAE) agarose (0.7%) gel containing 0.05% ethidium bromide. PCR products were sequenced at the Genome Center at Washington University (St Louis, MO, USA) on an Applied Biosystems 3730 96-capillary DNA sequencer. Primers and probes (MGB, non-fluorescent quencher on 5′ end) for subsequent qPCR were synthesized as follows (IDT Technologies): *TLR4* (GenBank: GU229789.1), forward TGCCTATTGGCCACTTGCA, reverse AATGAAGTAATGCTTATGTGGCCTAA, FAM-tagged probe ACTCTGCAGGAGCTG; *IL1*β (GenBank: GU229790.1) forward CCGTCCTCGGTCGTCTCA, reverse AGCCCTTGATGCCCAATG, VIC-tagged probe TGCTCCAGGGTCTG; *IL6* (GenBank: GU229791.1), forward AACCAAAACGTTGAATCGCTATC, reverse GGATTGATCACCATCTGTCTTATGG, VIC-tagged probe ACAGAGCACCTGGCAC.

Quantitative real-time PCR

Pre-LPS and 4 h post-LPS RNA samples were used in this analysis. Total RNA was extracted from blood using an RNeasy Mini-Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) following manufacturer's instructions; both RNA and cDNA concentrations were determined using a spectrophotometer. Gene expression was then measured using quantitative real-time PCR (qPCR; StepOne, Applied Biosystems). Briefly, ~100 ng μl^{-1} cDNA was added to PCR MasterMix (Applied Biosystems) and a four-step standard curve was made (100, 33.3, 11.1 and 3.7 ng μl^{-1}) using a homogenate of post-LPS house sparrow liver and spleen cDNA (from birds in 'Sequencing house sparrow genes' section above). A negative control was also used, containing no cDNA but an equivalent of ultra-pure water, with PCR conditions as follows: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples contained 2.5 μl ultra-pure water (except the negative control, which was 5 μl ultra-pure water), 12.5 μl of PCR MasterMix (Applied Biosystems), 7.5 μl of primers (900 nmol l^{-1} each) and probe (250 nmol l^{-1}), and 2.5 μl of sample cDNA (at 100 ng μl^{-1}). All samples, standards and controls were run in duplicate, as well as 18S RNA expression for each sample (TaqMan® 18S rRNA, Applied Biosystems, 4308329), to which expression of each target gene was adjusted ($\Delta\Delta C_T$).

Limulus amoebocyte lysate assay

A limulus amoebocyte lysate (LAL) kit (Lonza QCL-1000; Walkersville, MD, USA) was used per manufacturer's instructions; the kit uses LAL and a synthetic chromogen to detect Gram-negative endotoxin. Briefly, 50 μl of diluted samples (1:10) and standards were dispensed into a sterile 96-well plate in duplicate with 50 μl of LAL added to each well. Ten minutes later, 100 μl of substrate solution was added; 6 min after that, 50 μl of stop reagent (25% glacial acetic acid in ultrapure water) was added and the plate was read at 405 nm. All incubations were performed at 37°C. Only pre- and 8 h post-LPS samples were used for this assay because of the restrictively high volume of plasma necessary.

Corticosterone assay

A commercially available enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA; catalog no. 900-097) was used to measure plasma CORT (Breuner et al., 2006). Steroid displacement reagent (10%; 5 μl) was added to 5 μl of plasma and, 5 min later, assay buffer (240 μl) was added to each sample, vortexed and aliquoted in duplicate (100 μl per well) to assay plates (Kuhlman and Martin, 2010). In addition, a standard curve (ranging from 32 to 200,000 pg ml^{-1}) was measured in duplicate. Samples and standards were then incubated with conjugated corticosterone and antibody for 2 h at room temperature while being shaken. Wells were washed before substrate was added then plates were incubated 1 h at room temperature without shaking. Stop solution was then added, and each plate was read at 405 nm (corrected at 610 nm to minimize background absorbance per manufacturer's recommendations). Plasma samples pre- and 4 h post-LPS were used for these assays, and inter- and intra-assay variation were both <10%.

Data analysis

Data were tested to fit assumptions of parametric statistics, and when assumptions were not met, transformations were performed ($\log_{10}$ for gene expression), which were successful at normalizing distributions or eliminating variance heteroscedasticity (except for endotoxin, see below). Our experimental design was unbalanced and utilized repeated measures (Bolker et al., 2009), so we used mixed linear models (with restricted maximum likelihood estimation) to assess how fixed factors (time in captivity, LPS injections and their interaction) affected response variables (CORT, gene expression and endotoxin clearance); individuals were included as random factors in all models and degrees of freedom were estimated using the Satterthwaite method. *P*-values for significant fixed effects, including interactions, are reported in the results, and *post hoc* pairwise differences between fixed-effect groups were identified using Bonferroni tests and are depicted in figures. When significant LPS × captivity duration interactions were indicated, separate univariate ANOVAs of post-LPS dependent variables were performed (corticosterone and *IL6*), followed by Bonferroni tests to identify significant differences between groups. Endotoxin data were non-normal; this could not be rectified *via* transformation. Thus, Kruskal–Wallis tests (for time in captivity) and Mann-Whitney *U*-tests (for LPS effects) were used to determine what experimental factors affected this variable. All analyses were performed using SPSS v19 (IBM, Somers, NY, USA). We did not have enough statistical power to determine whether sex affected dependent variables.

RESULTS

Gene expression

LPS injection did not affect *TLR4* expression ($F_{1,22.8}$=0.67, *P*=0.41); however, birds held for 4 weeks expressed significantly more *TLR4* than wild birds and birds held for 2 weeks ($F_{2,23.1}$=8.7, *P*=0.002; Fig. 1A). Unlike *TLR4*, LPS increased *IL1*β expression (LPS: $F_{1,42}$=4.02, *P*=0.05), but captivity did not further impact expression (LPS × weeks captive: $F_{2,42}$=2.1, *P*=0.14; Fig. 1B), although there was a tendency for captive birds to have larger *IL1*β responses to LPS. LPS increased *IL6* expression ($F_{1,19.1}$=4.2, *P*=0.05), and for this gene, captivity duration affected expression (LPS × weeks captive: $F_{2,19.1}$=4.4, *P*=0.03). Although 4 week

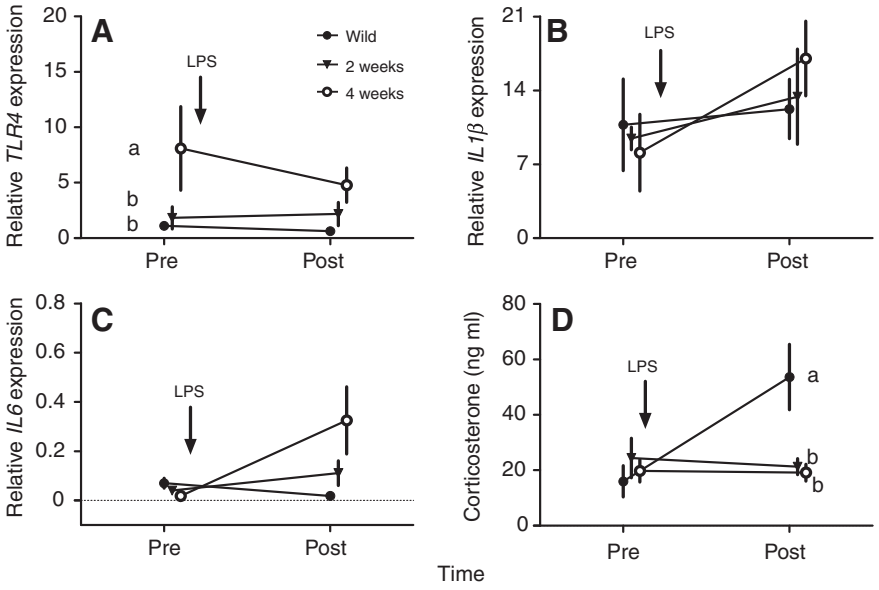

Fig. 1. Effects of captivity on house sparrow leukocyte expression of (A) *TLR4*, (B) *IL1*β and (C) *IL6*, and (D) circulating corticosterone before and 4 h after LPS. Symbols denote different periods of captivity; data are means ± 1 s.e.m. *Y*-axis for gene expression denotes fold-change of target genes relative to expression of 18S ribosomal mRNA. Letters denote significant differences among captive groups (Bonferonni *post hoc* comparisons).

captives tended to exhibit the greatest expression followed by 2 week captives and wild birds, these differences were not significant according to *post hoc* comparisons of post-LPS values (Fig. 1C).

Corticosterone

Baseline corticosterone levels did not differ among captive groups ($F_{2,23}$=0.54, P=0.59). However, LPS impacted corticosterone release ($F_{1,24.7}$=6.1, P=0.02), and this effect changed over time in captivity (LPS × weeks captivity: $F_{2,24.8}$=7.87, P=0.002): only wild birds released CORT in response to LPS whereas responses in captive birds were weak to absent (Fig. 1D).

Endotoxin

LPS injections did not further elevate or otherwise affect circulating endotoxin (Mann–Whitney U-test, P=0.61). However, circulating endotoxin was higher in 4 week captive birds than in wild or 2 week captive birds (Kruskal–Wallis test, P=0.04; Fig. 2). As exogenous LPS had no effect on circulating endotoxin, planned correlation analyses between gene expression and changes in circulating endotoxin were not possible.

DISCUSSION

Captive house sparrows exhibited more pro-inflammatory cytokine expression than wild-caught birds; generally, the longer birds were held in captivity, the stronger their inflammatory responses became. Expression of *IL6* post-LPS increased with time in captivity whereas corticosterone release to LPS decreased. Intriguingly, circulating endotoxin was higher in 4 week captive birds, which mirrored constitutive *TLR4* expression. These results are somewhat surprising, as inflammatory responses, specifically APRs, are weaker in house sparrows than other avian species (Coon et al., 2011; Lee et al., 2006; Lee et al., 2005; Martin et al., 2010), perhaps because of their status as an introduced species in some parts of their range (Lee and Klasing, 2004). Below, we interpret the putative captivity-induced hyperinflammation detected here in light of our initial hypothesis.

Stronger inflammation in captive birds: is more better?

Very high inflammatory gene expression is unlikely to be beneficial for house sparrows or any species (Glaser and Kiecolt-Glaser, 2005). Inflammation has both benefits and costs (Kopp and Medzhitov, 2009; Sears et al., 2011), so even if strong responses promote resistance of infection, they may be maladaptive in the evolutionary sense (Graham et al., 2011). For instance, wild house sparrows exhibit weaker APRs than most other birds (Owen-Ashley and Wingfield, 2007), and one experiment indicates that weak inflammation in house sparrows is adaptive, or at least consistent with its status as a successful introduced species. In a comparison of responses to simulated infection, house sparrows were able to maintain reproductive output when injected repeatedly with heat-killed bacteria whereas Eurasian tree sparrows (*P. montanus*) halved reproductive output (Lee et al., 2005). These differences are consistent with the much greater success the former species has achieved in terms of range expansion in the US, where both are non-native and were introduced to the US in approximately 1850.

Still, it is plausible that strong inflammatory gene expression could be interpreted as a protective immune response. In captivity, food availability is greater, more predictable and perhaps of higher quality than in the wild. Temperatures too are benign, and costs of other physiological and behavioral functions (e.g. competition, thermoregulation and infection with parasites) would be negligible. Most importantly, quantitative variation in cytokine expression is often predictive of resistance (Bradley and Jackson, 2008), so in many cases, more gene expression would truly be better. Our motivation for measuring circulating endotoxin was to test directly whether greater gene expression meant more rapid LPS clearance in house sparrows. However, we measured endoxtoxin at only two points, prior to exogenous administration and 8 h post-injection, because pilot studies had indicated persistent elevation of pro-inflammatory gene expression 8 h post-LPS injection (L.B.M., unpublished). However, a subsequent study (L.B.M., M. King and C.A.C.C., submitted), conducted concurrently with the present one, revealed that the dose of LPS used in the present study produced a surge in circulating endotoxin at 4 h post-LPS injection that by 8 h post-injection subsided to pre-challenge levels. Hence in the present study, the elevation in endotoxin that would have allowed us to determine whether gene expression was indeed functional was likely missed.

Based on other data from the present study, additional work in this species and results from domesticated animals (Martin and Kuhlman, 2010; Morgan and Tromborg, 2007; Mason, 2010)

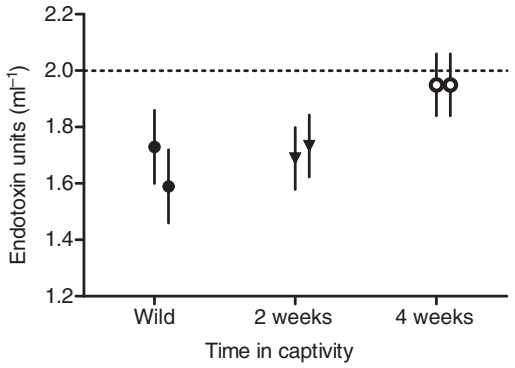

Fig. 2. Captivity duration, but not LPS injection, increased circulating endotoxin in house sparrows. For each captivity period, the first data point represents pre-LPS injection and the second represents post-LPS injection. Data are means ± 1 s.e.m.; dashed line denotes the upper detection limit of the assay.

(L.B.M., A. Urban, C.A.C.C. and A.L.L., submitted), the most reasonable explanation of the current results at present is that captivity induced chronic stress, leading to disregulation of inflammation. Importantly, only wild birds released CORT in response to LPS; captive birds did not. CORT is integral to the downregulation of inflammation (Shanks et al., 1998), and although baseline GCs were not elevated significantly in captive birds in the present study, in prior studies, captivity decreased expression of both glucocorticoid and mineralocorticoid receptors, increased baseline CORT and prevented further CORT release in response to a restraint stressor (Kuhlman and Martin, 2010) (L.B.M., A. Urban, C.A.C.C. and A.L.L., submitted). Furthermore, *IL6* expression was substantially higher in 4 week captives, and it is *IL6* that is predominantly responsible for sustaining inflammation and downregulated by GCs (Sternberg, 2006). Moreover, in rodents, chronic exposure to a social disruption stressor (SDR; a chronic stressor) made leukocytes resistant (i.e. apoptosis no longer occurred) to even very high GC concentrations (Bailey et al., 2004). SDR mice also upregulated *TLR4* expression (Bailey et al., 2007) and *IL1*β and tumor necrosis factor α (*TNF*α) from CD11b cells (Bailey et al., 2009). Whether the same molecular mechanisms are involved in house sparrow inflammation is unknown, although a previous study found that corticosterone implants have no effects on a skin inflammatory response in one population (Martin et al., 2005). When considered in their entirety, data suggest that captivity induced a state of chronic stress in house sparrows (although perhaps less so in this cohort than in previous ones) (Kuhlman and Martin, 2010) (L.B.M., A. Urban, C.A.C.C. and A.L.L., submitted), which led to inflammation disregulation. Future studies could evaluate whether the many ailments presently associated with chronic stress and/or disregulation of GCs in human populations (e.g. obesity, diabetes and rheumatoid arthritis) (Glaser and Kiecolt-Glaser, 2005; Shanks et al., 1998) occur in for animals in zoos, aquaria and captive breeding and translocation programs.

What about captivity might have induced hyper-inflammation?

Hyper-inflammation in captivity may have occurred because of altered GCs, but what about captivity caused these effects? Several factors are plausible, including: (1) climate and/or photoperiod; (2) psychological distress associated with restricted movement or social interactions; (3) differences in food quantity, quality or predictability; and (4) changes in the host microbiome driven by the above three factors. The first factor, climate/photoperiod, is unlikely as these were comparable to ambient conditions (photoperiod) or amenable to the physiological requirements of the species (i.e. temperature and humidity unthreatening to body temperature maintenance) throughout the study. The second, psychological distress, however, is plausible. Clearly, GC regulation can be altered by captivity in house sparrows (Kuhlman and Martin, 2010) (L.B.M., A. Urban, C.A.C.C. and A.L.L., submitted), and these effects would seem to be due to incarceration itself and/or limits on social interactions, as birds have *ad libitum* access to resources and shelter from inclement climate and other natural stressors. House sparrows are quite social, being territorial only in the breeding season and then only of the area immediately surrounding nest sites (Anderson, 2006). At other times of year, birds remain in sometimes very large groups comprised of all ages and both sexes. In other social species, isolation and/or separation can impact both GCs and immune functions (Glasper and Devries, 2005; Martin et al., 2006; Remage-Healey et al., 2003), but effects are as yet unknown for house sparrows.

A third possible cause of captivity effects is food. Wild house sparrows are predominantly granivorous, consuming insects and comparable high-protein sources only during the reproductive season (Anderson, 2006). As the present study was conducted during a short period in the non-breeding season, all birds were provided a simple seed diet and no food supplements (e.g. vitamins or calcium). Diet and especially micronutrients can have diverse and profound impacts on immune functions (Klasing, 2007), so diet effects deserve detailed consideration in the future. Indeed, a large and growing body of literature implicates a particular type of diet (low fiber, low short-chain fatty acids) in disposing inflammatory disorders in domesticated mammals and humans (Maslowski and Mackay, 2011).

Diet effects are especially intriguing in light of the fourth possibility listed above and a surprising outcome of this present study: significantly higher circulating endotoxin in month-long captive sparrows. A possible explanation for this outcome is that the chronic stress of captivity may have led to leakage of commensal microbes from the gut (Soderholm et al., 2002), subsequently increasing circulating endotoxin levels and inducing hyper-inflammation (Clarke et al., 2010). Such microflora spillover occurs in other taxa for at least three reasons: overgrowth of gut microbe populations, host immune deficiencies and damage to gut tissue (Berg, 1995). Moreover, spillover can be driven by physical stressors (e.g. burn, surgery and hemorrhagic shock) and, importantly, psychological stressors (Bailey et al., 2010). Some psychological stressors can drastically alter the natural microbiome of rodents, including large decreases in the abundance of certain microbial species, which can then increase host susceptibility to opportunistic pathogens (Bailey et al., 2010). Whether spillover is of commensals or pathogens, it typically follows a progression: bacteria colonize the lymph nodes first, then the liver and then the spleen (Ando et al., 2000). As all of these organs maintain large leukocyte populations, which would be exposed to endotoxins and other pathogen-associated molecular patterns, it is plausible that poor food constituency or the psychological stress of incarceration or lack of social stimulation could have increased constitutive expression of *TLR4* and hence expression of cytokines in response to exogenous LPS. Indeed in humans, diet can have strong impacts on the composition of the gut microflora, and perturbations of the climax microbial community can lead to translocation of gut microbes (Maslowski and Mackay, 2011).

One thing that remains to be reconciled is whether leakage of microbes is a directed or accidental process. In other words, are bacteria proliferating in response to host cues of stress, trying to propagate to another host if the one in which they presently reside dies (Freestone and Lyte, 2010)? Alternatively, are more virulent bacteria colonizing captive birds, or are bacterial translocations simply the consequence of a super-abundance of organisms occasionally getting out of host control? Although only recently has effort gone to discriminating these possibilities, bacteria do seem able to hedge their bets contingent on host condition. For instance, pigs shed more *Escherichia coli* when handled by humans (Callaway et al., 2006). Many bacteria can also detect and respond to host stress hormones, including catecholamines (Freestone and Lyte, 2010); however, evidence of responses to GCs is presently weaker (Kirimlioglu et al., 2006; Ünsal et al., 2008). In contrast, bacteria upregulate virulence factors to some stress hormones (Freestone and Lyte, 2010), including adrenocorticotrophic hormone (Mishra et al., 1994), the anterior pituitary-derived hormone that elicits adrenal CORT release in birds. Although we presently have no direct evidence that gut microflora in house sparrows impact or are impacted by captivity, such possibilities are important to pursue as microbes have intimate relationships with their hosts in other vertebrates (Maslowski and Mackay, 2011). For example, spleens from SDR mice tend to express more *IL6* and i-nitric oxide synthase mRNA and alter their gut microbiome towards microbial taxa that induce inflammation (Bailey et al., 2010). Importantly though, when SDR mice were given a broad spectrum antibiotic, SDR no longer affected *IL6* expression, suggesting that certain gut microbes were driving immune differences between groups.

In conclusion, captivity induced hyper-inflammation in house sparrows even though birds were kept in what many caretakers and researchers consider to be adequate conditions for controlled studies. These results call into question previous immunological results conducted solely on captive birds, and highlight the importance of efforts to discern how to mitigate captivity effects in future studies. In the short term, we advocate efforts to determine what about captivity causes stress to wild animals (Morgan and Tromborg, 2007). Unfortunately, this approach implies that wild-caught values be used as baselines to which comparisons are made. However, infections, food shortages or other stressors prior to or at capture may make such assumptions unviable. For this reason, we expect that a mix of field and laboratory studies will be most insightful (Adelman et al., 2010a; Adelman et al., 2010b), especially efforts to determine whether other immune components, including adaptive immune functions, are as affected by captivity as TLR and cytokine expression (Segerstrom and Miller, 2004). Fortunately, studies of captivity effects on immune functions will have many useful outcomes (Mason, 2010), including insight into the evolution of stress hormone–immune system interactions (Martin, 2009).

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