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Immune Redistribution to Skin in Wild and Domesticated Songbirds

Joshua Ryan Kuhlman
University of South Florida

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by

Joshua Ryan Kuhlman

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Major Professor: Lynn B Martin, Ph.D.
Earl McCoy, Ph.D.
Jason Rohr, Ph.D.
Toru Shimizu, Ph.D.

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ABSTRACT

Implantation of dental sponges under the skin of lab rodents has been used to evaluate whether acute stress enhances leukocyte infiltration to a surgical site. First, I replicated this technique in house sparrows to test whether transient stressors cause similar immunoredistribution (i.e., movement of immune cells out of circulation and to the periphery) in a wild animal. As placement into captivity alone may serve as a stressor to wild animals, I compared sponge infiltration over different periods of captivity. Second, I compared how domestication affects immunoredistribution by comparing results of wild sparrows to domesticated zebra finches. Zebra finches were chosen because they are widely used for evolutionary ecology research, and they share a similar diet and comparable body size and lifestyle to house sparrows. Birds were randomly assigned to treatment groups of either a restraint stressor or no restraint stressor treatment prior to implantation. In the first chapter birds were also divided into one of three groups: sponge implantation at capture, after short duration captivity (1 or 2 days), or long duration captivity (1 month). Total leukocyte infiltration into the sponge varied among captive groups. Birds implanted at capture had greater leukocyte infiltration to the sponge compared to birds kept in captivity 1 or 2 days before implantation. Birds placed into captivity for 1 month before implantation

showed similar sponge infiltration relative to the immediate implant group. However, time in captivity altered the dominant type of leukocytes present in the sponge at explant with lymphocytes decreasing with time in captivity and granulocytes increasing. Domestication affected cell infiltrates with domesticated species exhibiting more infiltration of heterophils and monocytes while wild house sparrows exhibited more infiltration of lymphocytes and basophils. My data indicates that in house sparrows, time in captivity affects the magnitude and character of immune responses to surgery and more importantly data are suggestive of immunoredistribution. My data also indicate that domestication has an impact on infiltrating cell types.

Preface

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Thesis Summary

The immune system plays an important role for all organisms in the fight against disease and infection by helping in the defense against foreign pathogens. However, stressors experienced by the individual may have an impact on the immune system's performance by either hindering or helping the immune response. Until the last couple of decades, most research has focused on what has become known as chronic stress (long duration stressor) which has a suppressive impact on the immune system. Research has now shifted to the study of short duration stressors, known as acute stress, which have an immunoenhancing effect on the immune system. Transient stressors seem to prime the organism for defense against pathogens by inducing a rapid influx of white blood cells from the blood and to the periphery (i.e. skin). Study of this phenomenon, termed immunoredistribution, is becoming increasingly popular in bio-medical research with the use of laboratory rodents; however, little is known how this phenomenon responds in the wild. Knowing how immunoredistribution works in the wild will make it possible to study and understand the ultimate forces that have shaped immunoredistribution. To test immunoredistribution, I examined how transient stress effected the redirection of immune cells out of the blood and to the skin in wild house sparrow (*Passer domesticus*). Later domesticated zebra finches (*Peophilia guttata*) were used in comparison with wild house sparrows to test whether there was a difference between responses in wild and domesticated songbird species.

Examining immunoredistribution will provide novel understanding of how acute stressors affect the vertebrate immune system in naturally relevant contexts. Evolutionarily, the stress response has evolved to help individuals survive by releasing neurotransmitters and hormones at the onset of a perceived stressor that aid in the individual's ability to escape and/or fight off infection. This is seen in the wild when an animal sees a predator: neurotransmitters, epinephrine and norepinephrine, are released and the animal undergoes the fight or flight response in order to escape predation. If the animal should be bitten but escapes predation, released glucocorticoid hormones aid in the redistribution of immune cells to the site of wounding or infection. This inflammatory response has evolved with the overall purpose of removing disturbances and restoring homeostasis. Thus, leukocyte trafficking to the skin has evolved as an important defense mechanism during stressful situations.

In the first chapter of this thesis, I set the ground work by investigating whether wild house sparrows exhibit leukocyte infiltration to skin in response to a transient stressor (restraint) as seen in laboratory rodents. Second, I examined effects of captivity on leukocyte infiltration of wild sparrows, expecting long-duration captivity to impose chronic stress and thus reduce leukocyte infiltrations. In particular, I expected continuous elevation of the avian stress hormone, corticosterone, to dampen skin infiltration of leukocytes in birds kept in captivity for short-term (1-2 days) and more so long-term (1 month) periods compared to wild-caught individuals. I found that restraint of wild house sparrows did not influence the magnitude of leukocyte immunoredistribution to skin. I found that

placement of wild sparrows into captivity affected the leukocyte infiltration response with short-term captive birds (1-2 days) exhibiting less leukocyte influx than long-term (30 days) and wild individuals, and that captivity altered the profile of cellular infiltrates to skin with a profound shift from lymphocytes to heterophils.

In the second and final chapter, I addressed whether restraint could exhibit increased immunoredistribution by incorporating in a domesticated avian species of zebra finches. I found that neither avian species demonstrated an increase in immunoredistribution with restrain stress. However, I found that the infiltration response differed between the two species with wild house sparrow skin infiltration being dominated by lymphocytes and basophils whereas domesticated zebra finch responses were dominated by heterophils and macrophages.

In sum, my thesis shows that immunoredistribution to skin can be measured in birds, though without any impact from transient stress. Investigating the ecological and evolutionary implications of immunoredistribution will be challenging in wild birds, however, given the impacts of captivity on the leukocyte profile that comprises the response. It is possible that longer duration captivity or different housing conditions (open aviaries instead of cages) may provide more naturalistic responses. Also, my thesis shows that wild and domesticated avian species varied in the character and intensity of infiltration responses. Nevertheless, my thesis should give background for future research to be able to test evolutionary aspects of immunoredistribution, as well as evaluate how

natural context stressors in the wild (e.g. predators, conflicts with conspecifics, or severe weather) may affect immune cell trafficking to the skin.

This thesis will provide the groundwork necessary to be able to test how natural context stressors affect immune cell trafficking to the skin. Future studies will have the ability to evaluate whether the immunoenhancing capability of acute stressors is consistent for any stressor, or if it is context specific for each type of stressor. For instance, sight of a predator alone induces a stress response in an individual, so an experiment should be conducted to see if a stress response to a predator better prepares an individual for an immune response against infection when compared to a novel stressor, such as restraint. How other natural context stressors, such as low temperatures and food restriction, affect immunoredistribution should be examined as well. These experiments will help give the immunoredistribution hypothesis ecological and evolutionary relevance.

Chapter 1

Captivity affects immune redistribution to skin in a wild bird

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Summary

1. Effects of stressors on immune functions have long been studied, but most have focused on chronic stressors, which tend to be immunosuppressive. More recently, an emphasis has been placed on identifying and understanding effects of transient, unpredictable stressors, especially whether and how they affect movement of immune cells to bodily areas in need of rapid protection. These latter effects have been termed, immunoredistribution.

2. In the present study, we surgically implanted small gelatin sponges subcutaneously to measure leukocyte infiltration to skin in house sparrows, *Passer domesticus*. First, we evaluated whether wild birds exhibit leukocyte infiltration to skin in response to a transient stressor (restraint) as seen in laboratory rodents. Second, we examined effects of captivity on leukocyte infiltration of wild sparrows, expecting long-duration captivity to impose chronic stress and thus reduce leukocyte infiltrations. In particular, we expected continuous elevation of the avian stress hormone, corticosterone, to dampen skin infiltration of leukocytes in birds

kept in captivity for short-term (1-2 days) and more so long-term (1 month) periods compared to wild-caught individuals.

3. As in lab rodents, house sparrows exhibited cutaneous leukocyte infiltration, but one hour of restraint prior to surgery did not influence the magnitude of cellular infiltration. The leukocyte infiltration response was influenced by captivity, however, with short-term captive birds (1-2 days) exhibiting less leukocyte influx than long-term (30 days) and wild individuals. Interestingly, the profile of cellular infiltrates changed with time in captivity with a dramatic shift away from lymphocytes and towards heterophils in long-term captive birds. Placement in captivity did not affect total circulating leukocytes; however, lymphocytes decreased in numbers in wild birds in short-term captivity. Corticosterone followed the expected pattern with the highest baseline levels in short-term captives, intermediate levels in long-term captives and lowest levels in wild-caught birds. Stress responses were not affected by time in captivity.
4. These results indicate that an integral aspect of immunoredistribution occurs in wild birds but future efforts to understand the ecological and evolutionary forces that have shaped this immune response must account for the confounding effects of captivity and attempt to understand the proximate and ultimate basis for heterophillia and lymphopenia with chronic stress.

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Key Words: Immunoredistribution, corticosterone, glucocorticoids, stress, immunocompetence

Introduction

Stress is traditionally thought of as immunosuppressive, but this perception likely stems from the changes in the immune system that arise in response to long term (chronic) stressors (Dhabhar & McEwen 1997; Sapolsky 1992). Such long-term stressors cause a sustained interruption to homeostasis and are coincident with prolonged elevation of glucocorticoids (GCs, McEwen & Wingfield 2003). Prolonged elevation of GCs tends to suppress immune functions (Leung & Bloom 2003). In European starlings for instance, prolonged exposure to a rotating series of stressors reduced the cutaneous immune response to phytohemagglutinin (Cyr & Romero 2007). Social separation, which elevates GCs in some species, also slowed wound healing in rodents (Glasper & DeVries 2005), and likewise, prolonged artificial administration of GCs slowed wound healing by attenuating the expression of pro-inflammatory cytokines (Hubner et al 1996) and heat shock proteins (Gordon et al 1994), the latter of which play an important role in the cellular response to stress (Carper, Duffy, & Gerner 1987).

Stressors are not always immunosuppressive, however, especially if they arise and resolve rapidly. Acute stressors, or stimuli that cause short duration interruptions to homeostasis, enhance certain immune responses (Dhabhar and McEwen 1997; Dhabhar and McEwen 1999). Restraint and shaking for 6 hours

a day for a few weeks (chronic stress) significantly suppressed cell-mediated inflammatory immune responses, however, a one-time exposure to the same stressor (acute stress) increased the same immune response (Dhabhar & McEwen 1997). These results suggest that organisms may prime their immune system in response to acute stressors, in particular increasing surveillance in regions of the body most likely to be wounded while coping with a stressor (Viswanathan & Dhabhar 2005). This phenomenon, termed immunoredistribution, is partly orchestrated by GCs and entails rapid deployment of immune cells out of the blood and into the periphery (Braude, Tang-Martinez, & Taylor 1999; Sprent & Tough 1994; Dhabhar & McEwen 1999). Adrenalectomy, which eliminates the source of endogenous GCs, prevents stressor-induced immune enhancement while treatment with artificial GCs in adrenalectomized animals restores it (Dhabhar and McEwen 1999).

The goals of the present study were to develop a methodology amenable to studying immunoredistribution in wild birds and to assess whether captivity impinged on the magnitude and character of the immune response because captivity impacts GC regulation in some species (Dickens, Earle, & Romero 2009; Kock et al. 1999). Also, all examples of immunoredistribution to date come from laboratory rodents bred for docility and thus weak stress responses. Because many neuroendocrine processes in domesticated organisms are unlikely representative of their free-living relatives (Calisi and Bentley 2009), the absence of a technique to assess immunoredistribution for wild organisms will make it difficult to understand the ultimate forces that have shaped

immunoredistribution. However, as recapture of most free-living animals is difficult, especially when samples must be collected within specific time frames, it is important to determine whether immunoredistribution can be studied in a captive, wild animal.

To this end, we modified a technique used in laboratory rodents (Viswanathan & Dhabhar 2005) for a wild bird species, the house sparrow, *Passer domesticus*. In rodents, this technique entails implantation of a small gelatin sponge under the skin followed by excision hours later and subsequent quantification of leukocyte infiltration. Few leukocytes are present in skin absent a wound (pre-surgery). In house sparrows, we asked i) whether infiltration was influenced by restraint just prior to implantation, and ii) whether skin infiltration and circulating leukocyte numbers and corticosterone varied with time in captivity. We also tested whether corticosterone or circulating leukocytes were correlated with leukocyte densities in sponges. We quantified all parameters in six groups of birds: i) individuals caught wild and implanted immediately, ii) individuals held for a short period in captivity (1-2 days) then implanted, and iii) individuals held for long period in captivity (1 month) then implanted. Approximately half of the individuals in each group were also restrained for one hour prior to surgery and the other half were implanted immediately upon capture (from mist nets in the wild or from cages in captivity). We chose these periods of captivity because GCs are elevated shortly after capture (Dickens et al 2009; Rich and Romero 2005) but tend to return to natural levels after ~1 month in captivity (Dickens et al. 2009; Romero & Remage-Healey 2000; Rich & Romero

2005). We hypothesized that captive birds would show attenuated leukocyte infiltration to skin as compared to the wild groups since transfer into captivity may represent a chronic stressor (Morgan & Tromborg 2007). However, we expected recovery of infiltration after 1 month captivity given that circulating glucocorticoids recover to wild levels within this period. We also expected that skin influx of leukocytes would be proportional to efflux of leukocytes from circulation, indicating genuine redistribution, and we expected individual variation in the magnitude of leukocyte movements to be correlated with corticosterone either at the time of sponge implant or explant. Last, we expected a shift to heterophilia and lymphopenia in blood and sponges with time in captivity, as this shift is often observed in response to chronic stress in birds (Gross and Siegel 1983).

Materials and Methods

Study Animals

Forty-six adult house sparrows were caught in Tampa, FL, USA (27^{o} N, 82^{o} W) in April and early May 2008. This period is pre-breeding for this species, and was chosen because immune responses are influenced by breeding conditions in this and other species (Greenman, Martin, & Hau 2005; Martin, Weil, & Nelson 2008). All females were checked to verify the absence of active brood patches (featherless, highly vascularized skin on the abdomen); no females had active patches. At capture, birds were bled from the alar wing vein (50ul) within 3 minutes to ensure that corticosterone (CORT) was at its lowest point (Wingfield, Smith, & Farner 1982). Then individuals were marked with

numbered aluminum leg bands and randomly assigned to a treatment group. Some birds were implanted in the field (see below) but all were returned to a room in a vivarium in the College of Public Health at the University of South Florida and single-housed in cages until explant or implant (Fig. 1). The room was maintained at 22 $\pm$ 2 ^{o}C and relative humidity around 45%; photoperiod was 12h light: 12h dark with lights on at 0700. Birds were given mixed seeds and water *ad libitum,* and remained in the cages for the duration of the study except when undergoing surgeries (<5 minutes). After explants, birds were released back to sites of capture. Experimental procedures were in accordance with the University of South Florida's Institutional Animal Care and Use Committee (IACUC protocol # W3202).

Experimental Groups

Birds were randomly assigned to one of six groups (Fig. 1). Group 1 (n = 10) consisted of 60 minutes of restraint in cloth bags just after capture in mist nets followed by surgical sponge implantation in the field. This restraint procedure effectively increases circulating glucocorticoids in wild birds (Canoine et al 2002). Group 2 (n = 10) was also implanted in the field but received no restraint treatment. Groups 3 and 4 (n = 10 in each) were kept in captivity for 1 or 2 days, respectively, before restraint treatment and sponge implantation. Group 5 (n = 3) and group 6 (n = 4) were held in captivity for one month prior to restraint and implantation. For all individuals, additional blood samples (50 μl) were taken post restraint and at the time of explants. For those individuals

brought back into captivity, a second baseline blood sample (50 μl) was taken within 3 minutes of entering the room in which birds were held. For all birds at all sampling points, blood smears were made for later leukocyte quantification.

Sponge Implantation and Retrieval

Birds were lightly anesthetized with isoflurane vapors (Butler Animal Health Supply, Dublin, OH), and their right flanks were swabbed with 100% ethanol. Squares of Gelfoam absorbable gelatin sponge (10 mm x 10 mm, Pharmacia & Upjohn Co, Division of Pfizer Inc, NY, NY) were pre-soaked in 0.9% sterile saline then implanted subcutaneously (s.c.) on the right flank. Wounds were sealed with Vetbond (3M, St. Paul, MN). Gelatin sponges were explanted 12h post-implantation as maximal leukocyte infiltration occurred in rodents at this time (Buchanan & Murphy 1997, Viswanathan & Dhabhar 2005). For explants, birds were again anesthetized with isoflurane vapors; sponges were collected by opening the Vetbond seal with surgical scissors and excised with sterile forceps. Then, wounds were sealed again with Vetbond and gelatin sponges were immediately placed into 10% formalin until they were processed for later quantification (embedded in paraffin, sectioned at 5um thickness, mounted on glass slides and H&E stained, Histology Core, Moffitt Cancer Center, University of South Florida). As sponge orientation could not be discerned (which surface had been in contact with skin or muscle), two random sections of sponge were collected for all birds. Persons preparing and scoring the slides were blind to treatments.

Leukocyte Quantification

Leukocytes were differentiated into lymphocytes, heterophils and eosinophils, basophils, and monocytes (Campbell and Ellis 2007). Heterophils could not be distinguished from eosinophils, but as heterophils comprise the largest fraction of circulating granulocytes, all such cells were identified as 'heterophils' (Martin et al 2006). To quantification leukocyte infiltration to surgical sponges, slides were observed under a microscope (Leica DME) at 5x magnification to identify a path traversing the sponge section such that the maximum possible fields of view could be quantified. Fields of view consisted of a 100 mm^2 grid mounted within the eyepiece of the microscope that was moved across the section. All leukocytes falling within or in contact with the grid were then quantified for each slide at 1000x. Leukocyte counts were then adjusted according to the fields of view observed. Occasionally, sponge sections were saturated with erythrocytes, which made them unquantifiable. These samples (n = 9) were eliminated from analysis.

Blood smears were fixed in 100% ethanol then left to air dry until they were stained using a Protocol HEMA 3 staining kit (Fisher Scientific Company L.L.C., Kalamazoo, MI, USA). Differential leukocyte counts were then obtained using a microscope (at 1000x) by counting the number of each cell type per 10,000 erythrocytes (using a modification of the grid-based survey above). All slides were quantified, blind to treatments, by the same observer.

Corticosterone

Plasma was removed from blood samples after centrifugation and stored at -40^{o}C. A commercially available Corticosterone Enzyme Immunoassay Kit (Correlate-EIA, Assay Designs, Ann Arbor, MI, USA) was used for CORT analysis following a previously established protocol (Breuner et al. 2006). We validated the assay for house sparrow plasma by a stripping (with Norit-activated charcoal) and spiking (with standard concentrations of exogenous CORT) process (Breuner et al. 2006), finding that a 1:50 plasma dilution with 10% steroid displacement buffer (SDB) was most effective. For each sample in the study, 5 μl of 10% SDB was added to 5 μl of plasma. After 5 minutes, 240 μl of Assay Buffer 15 was added to each sample, vortexed and aliquoted in duplicate (100 μl each) to wells of 96-well plate. Standard curves were produced using assay standards (32 – 20,000 pg) and samples were agitated or incubated according to manufacturer's guidelines. After the final incubation, stop solution was added and each plate was read in a plate reader (Biotek EL808 with Gen5 software) at 405nm (corrected at 595nm). Samples were randomly allocated among plates and both inter- (3.7%) and intra-(3.6%) plate variation were low.

Data Analysis

Absolute leukocyte cell counts (differential and total cells) were tested for normality using one-sample Kolmogrov-Smirnov tests. Whereas variable distributions were not significantly non-normal, variance heterogeneity was

indicated in some cases, so counts were ln-transformed. Then univariate analysis of variance (ANOVA), with time in captivity and restraint as fixed factors and total and differential cell counts or corticosterone as dependent variables, were performed. Potential relationships among variables were assessed using Spearman's rank correlation analyses. For all analyses, SPSS v. 17 was used with $\alpha \leq 0.05$. Figures were produced using GraphPad Prism v. 5.

Results

Restraint effects on leukocytes in wild birds

Restraint induced a significant reduction in total circulating leukocytes in wild birds ($F_{1, 15} = 6.70$, $P = 0.023$, Fig. 2a). This result was also consistent for lymphocytes ($F_{1, 15} = 8.07$, $P = 0.014$, Fig. 2b), and monocytes ($F_{1, 15} = 4.92$, $P = 0.045$, Fig. 2c), but not heterophils ($F_{1, 15} = 0.541$, $P = 0.475$, Fig. 2d) or basophils ($F_{1, 15} = 0.678$, $P = 0.425$, not shown). Restraint had no effect on sponge infiltration however ($F_{1, 14} = 0.004$, $P = 0.951$, Fig. 3); birds restrained for 60 minutes prior to implant immediately after capture in the wild exhibited a similar level of leukocytes in sponges as wild birds that were not restrained. This result also held when leukocytes were differentiated (data not shown). As restraint did not affect sponge infiltration in wild birds, both restrained and unrestrained birds were combined into one group for all further analyses.

Effects of captivity on circulating and sponge infiltrating leukocytes

Leukocytes infiltrating sponges in birds housed in captivity for one or two days did not differ ($F_{1, 16}$ = 0.001, P = 0.977) nor were there differences for one month captivity groups ($F_{1, 7}$ = 0.061, P = 0.815), so these groups were combined into either a short-term or long-term captivity group. Total circulating leukocytes did not differ among wild and captive birds ($F_{2, 38}$ = 0.16, P = 0.856, Fig. 4a), nor did heterophils, monocytes, or basophils when compared separately. Lymphocytes were significantly decreased in captive compared to wild-caught birds though ($F_{2, 38}$ = 3.64, P = 0.037, Fig. 4b), although longer time in captivity did not further depress lymphocyte counts.

In contrast to circulating leukocytes, duration in captivity had a marked effect on leukocytes infiltrating the skin. Birds housed for 1-2 days in captivity had the lowest infiltration whereas birds housed in captivity for 1 month had levels comparable to wild-caught individuals ($F_{2, 37}$ = 4.33, P = 0.021, Fig. 5a). When cells were differentiated by type, however, only lymphocytes ($F_{2, 37}$ = 4.81, P = 0.014, Fig. 5b) and heterophils ($F_{2, 37}$ = 11.41, $p < 0.001$, Fig. 5c) varied among groups; lymphocytes decreased whereas heterophils increased with time in captivity.

Negative correlations between circulating leukocyte densities and sponge leukocyte densities were expected if immunoredistribution from circulation to skin occurred in response to either captivity, restraint, or surgery, all of which might have been interpreted as stressors. However, in wild (R = -0.046, P = 0.876), short-term captive (R = -0.103, P = 0.704) and long-term captive (R < 0.001, P =

1.000) sparrows, there was no evidence of relationships between circulating and infiltrating leukocytes. When leukocytes were differentiated, correlations remained non-significant in all groups (data not shown).

Corticosterone

Baseline CORT was significantly affected by captivity duration ($F_{2, 38}$ = 3.44, P = 0.043, Fig. 6a); birds housed in captivity for 1-2 days had the highest CORT, birds held in captivity for 1 month were intermediate, and wild birds had the lowest CORT. There was no difference in CORT levels between the 1 and 2 day captivity groups ($F_{1, 16}$ = 2.03, P = 0.176), and CORT responses to restraint did not vary with time in captivity ($F_{1, 24}$ = 1.13, P = 0.341, Fig. 6b), although all groups elevated CORT when restrained ($F_{1, 24}$ = 24.13, P < 0.001).

Correlation analyses, between circulating leukocytes and CORT just prior to restraint (baseline), indicated CORT was correlated with circulating leukocytes (R = 0.456, P = 0.004, Fig. 7a) when all birds were analyzed together. When leukocytes were differentiated however, only circulating heterophils were correlated with CORT (R= 0.539, P < 0.001, Fig. 7b). This result held for heterophils (only) for wild (R = 0.693, P = 0.004), and was marginally non-significant for short-term captive (R = 0.438, P = 0.090) birds when groups were analyzed separately; no correlation was detected in long-term captive birds (R = 0.214, P = 0.645). However, correlation analysis indicated no relationships between leukocytes infiltrating skin and CORT at this time (R = -0.168, P = 0.319).

Discussion

Restraint just prior to implantation had no detectable effect on leukocyte infiltration to sponges, contrary to results observed in lab rodents (Viswanathan and Dhabhar 2005). This result was consistent, regardless of whether birds were wild-caught or kept in captivity. Time in captivity, however, had strong effects on a) CORT, b) circulating lymphocyte densities, and c) the repertoire of leukocytes infiltrating sponges. Baseline CORT was elevated after short periods in captivity but recovered after one month, but there was no indication that CORT release in response to restraint differed with time in captivity. In circulation, heterophils changed little whereas lymphocytes decreased initially (1-2 days captivity) but then recovered to wild-caught values after one month. Other leukocyte types exhibited little variation with captivity duration. After one month, sponge infiltration profiles became dominated by heterophils whereas initially they were dominated by lymphocytes; the same profile was not observed for blood however. There were no obvious relationships among circulating and sponge infiltrating leukocytes and corticosterone. However, missing relationships could be due to our inability to sample all parameters on appropriate time scales and perhaps because birds have other leukocyte reservoirs we did not characterize. Below, we provide several proximate and ultimate interpretations of these patterns and discuss their implications on understanding putative immune redistribution in wild animals.

Corticosterone

Corticosterone has profound effects on immune responses (Martin 2009). Acute stress induces an increase in GCs which enhances innate and adaptive immunity (Dhabhar 2002) and increases immune surveillance to skin (Dhabhar 2009; Dhabhar and McEwen 1996; Dhabhar and McEwen 1997; Dhabhar and McEwen 1999; Viswanathan and Dhabhar 2005; Viswanathan, Daugherty, and Dhabhar 2005). Prolonged elevations of GCs, however, have adverse effects on immune cell parameters (Sapolsky, Romero, and Munck 2000). GCs can initiate apoptosis of lymphocytes (Sapolsky et al. 2000), but eosinophils, basophils, and monocytes are also affected (Martin 2009). Stress induced immune suppression may be a mechanism to conserve resources and energy to be allocated elsewhere, however, only over long periods of time would resources be gained from immune suppression; in the short-term the costs of immune suppression would outweigh the benefits because immune suppression requires apoptosis and hence calories (Martin 2009). This perspective suggests two possible interpretations for the change in leukocyte profile we observed with time in captivity: either granulocytes provide an energy savings in that they are less costly to produce or use than lymphocytes, or 1 month captivity is beyond that window of time where reductions in immune function can be explained by energy savings. An additional possibility is that individuals enduring chronic stress may be unlikely to survive long periods anyway. Consequently, they may sacrifice those immune components with long-term protective values (Martin et al. 2007),

such as lymphocytes that encode antigen memory and/or lower the cost of future immune activation, and enhance, fast-acting, broadly effective, but immunopathological cellular defenses involving heterophils. Finally, the detected pattern may not be strategic inasmuch as it is the simple consequence of birds experiencing conditions that their ancestors would likely never have encountered.

Corticosterone coordination of redistribution

Our results provide little evidence for genuine immunoredistribution, as we detected no significant correlations between leukocyte numbers in circulation and sponges. However, there was a correlation between GC levels and the number of leukocytes, mainly heterophils, in circulation which is to be expected since heterophils proliferate in the presence of GCs (Jain 1993; Campbell & Ellis 2007; Davis et al. 2008). Immunoredistribution occurs when elevations in glucocorticoids induce circulating leukocytes to adhere more readily to blood vessels and migrate from circulation into other tissues such as lymph nodes and skin (Braude et al. 1999; Dhabhar & McEwen 1999; Dhabhar 2002; Dhabhar 2009). Perhaps over time in captivity, leukocytes, and especially heterophils, aggregated in lymphoid tissues. This possibility could explain the increase in heterophils to the sponge without an increase in blood in 1 month captive birds. Indeed, short duration stressors caused a significant increase in lymph node weight in rodents (Viswanathan et al. 2005).

Circulating leukocytes

Under homeostatic conditions, 80% of leukocytes in birds consist of lymphocytes and heterophils with lymphocytes being most common (Rupley 1997; Campbell & Ellis 2007; Davis et al. 2008). Our results indicated no effect of time in captivity on most circulating leukocytes (in the absence of restraint); however, lymphocyte numbers decreased in short-term captive birds but returned to intermediate values in long-term captive birds. It has been shown that after two months in captivity, lymphocytes actually increase in numbers compared to wild (Sepp, Sild, & Hõrak 2010). We expect that lymphocytes decreased in number because they are particularly sensitive to chronic elevation of GCs (Sapolsky 1992). It was unexpected, however, that circulating heterophils did not increase in circulation given that this cell type proliferates in response to stressors in other species (Jain 1993; Campbell & Ellis 2007; Davis et al. 2008), and administration of corticosterone increases circulating heterophils in chickens (Gross and Siegel 1983), neutrophils in cattle (Anderson, Watson, and Coditz 1999), bottlenose dolphins (Reidarson and McBain 1999), rhinoceroses (Kock et al. 1999) and humans (Dale et al. 1975), but see Buehler, Piersma, and Tieleman 2008 in which placement in captivity did not increase heterophil numbers in red knots. This result further indicates that heterophils may have been sequestered in another reservoir in captive sparrows.

Sponge infiltrating leukocytes

The majority of leukocytes infiltrating sponges in house sparrows were heterophils and lymphocytes but basophils and monocytes were also present. In mice, neutrophils dominated the response, but only T-cell infiltration was quantified so comparisons between this study and that one are inappropriate (Viswanathan & Dhabhar 2005). Nevertheless, captivity had a strong impact on the profile of leukocytes infiltrating the skin in sparrows. In wild-caught birds, the response was lymphocyte biased, but in captivity heterophils came to dominate the response. Because lymphocytes are involved in the recruitment of more leukocytes, activation of others, and the general coordination of immune responses (Parham 2000), the increase in heterophils and their broad efficacy might be compensation for the loss of lymphocyte-mediated immune coordination. Heterophils provide broad, rapid protection but at the cost of high collateral damage.

The reduction in lymphocytes infiltrating the sponge over time in captivity is not coincident with the return of circulating lymphocytes to levels in wild birds after 1 month in captivity. One possibility for this pattern is that lymphocytes developed GC resistance over time in captivity (Avitsur, Stark, and Sheridan 2001; Stark et al. 2001). Lymphocytes with high sensitivity to CORT may have been eliminated via apoptosis during early captivity when CORT was elevated for a prolonged period. All remaining lymphocytes would be minimally responsive to CORT. This reduced sensitivity may have altered the way that they responded to signaling molecules in the vasculature near the wound and hence prevented

most from colonizing the sponge, especially if the lymphocyte classes that are least sensitive to GCs have particular functions that GC-sensitive cells do not.

Evolutionary perspective

One of the unresolved aspects of this study and others is why lymphocytes are reduced via elevated CORT but heterophils are increased. Apoptosis is activated in multiple lymphocyte types via CORT but the same molecule stimulates proliferation of heterophils. We propose that heterophilia during chronic stress is either an evolutionary adaptive mechanism to maintain defense at the skin, or an unavoidable consequence of chronic stress. Inflammation evolved to remove disturbances and restore homeostasis at the site of wounds or damage (Medzhitov 2008). Heterophils are phagocytic and the primary granulocyte in avian inflammation (Harmon 1998), so heterophilia may provide a broadly effective defense against host invasion by parasites regardless of the magnitude of stressors individuals experience. We expect that only individuals enduring chronic stressors would favor this strategy because whereas it may be quite protective (Campbell and Ellis 2007), it is also conducive to excessive collateral damage. Inflammation generally is disposed to pathology (Nathan 2002), so only individuals requiring a fast, effective immune defense but willing to endure self-damage would be expected to engage heterophilia. Alternatively or additionally, only individuals in minimally stressful conditions would utilize adaptive immune defenses that provide the greatest protection the second time a pathogen is encountered. Upon initial exposure to a parasite, the

adaptive immune system takes weeks or days to be effective (Parham 2000). There is convincing evidence to suggest the most important role of the adaptive immune system is to provide greater specificity in protection at a reduced cost upon subsequent encounters with parasites (Schmid-Hempel and Ebert 2002). Thus, in response to chronic stressors, adaptive immune functions might be sacrificed first because a) their greatest benefits would entail minimization of future immune activation and b) diminution could free resources for other more immediately important physiological processes (e.g., escape from a predator, winning a territorial conflict; Martin 2009).

Conclusion

Immunoredistribution to the skin can be measured in wild birds using a similar technique developed for lab rodents. However, investigating the ecological and evolutionary implications of this response will be challenging given the impacts of captivity on the leukocyte profile that comprises the response. It is possible that longer duration captivity or different housing conditions (open aviaries instead of cages) may provide more naturalistic responses. Nevertheless, future research to understand how natural context stressors (e.g. predators, conflicts with conspecifics, or severe weather) affect immune cell trafficking to the skin is possible.

Acknowledgments

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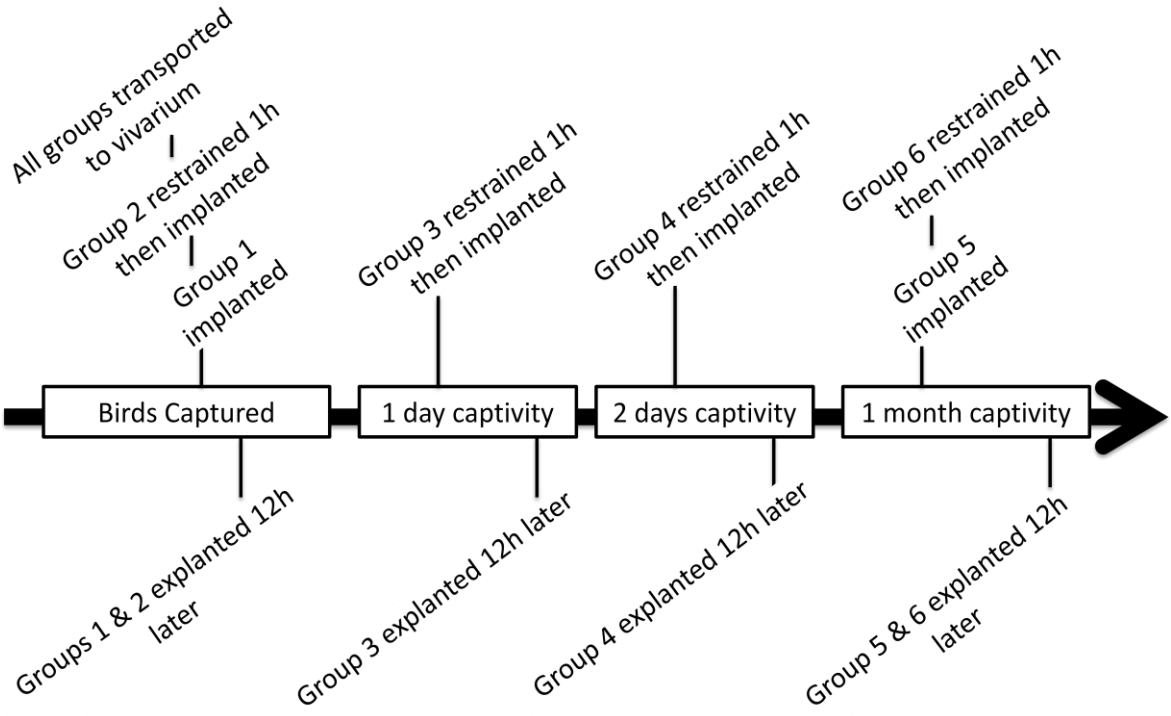

Fig. 1 Timeline for experimental design. Birds were randomly assigned to groups.

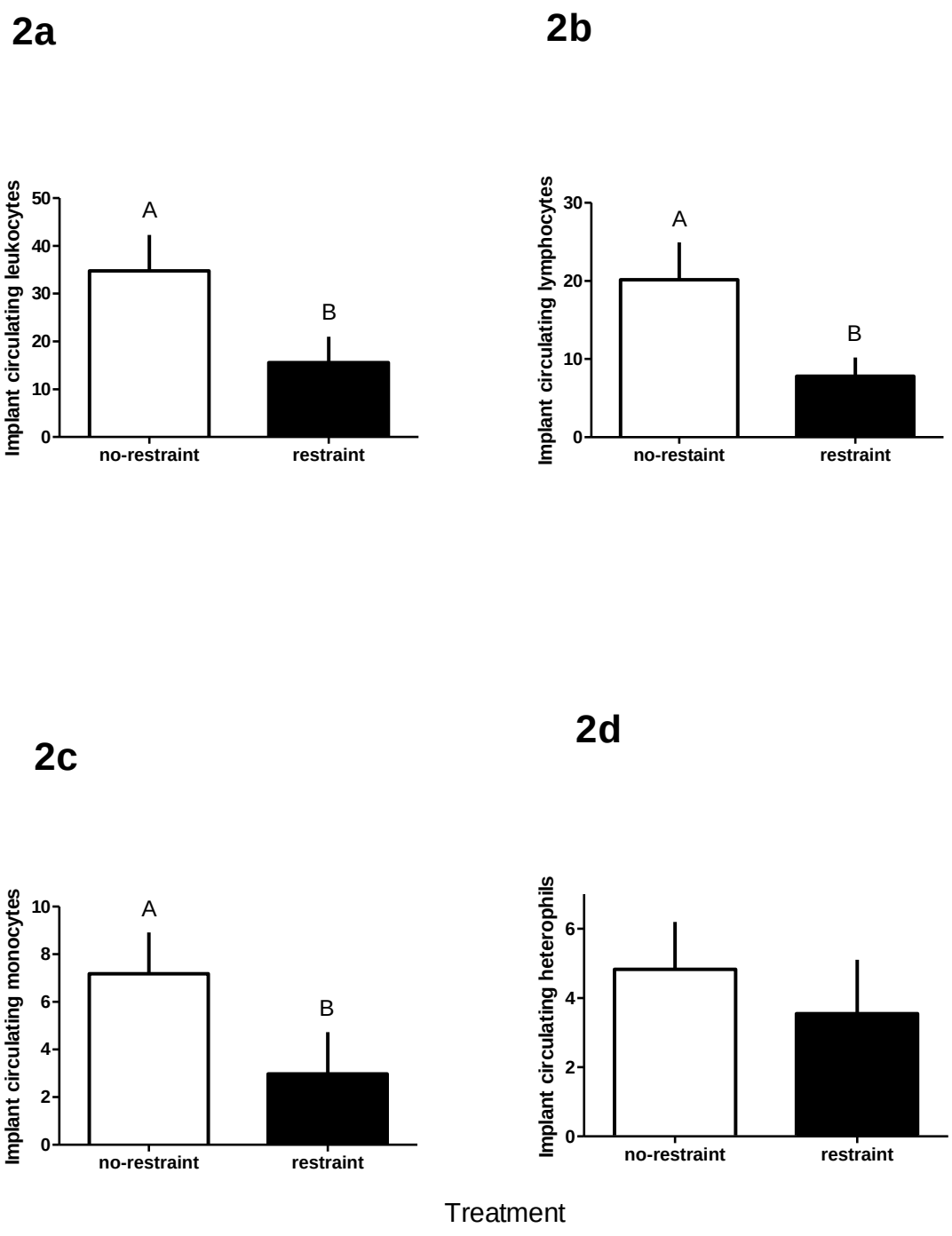

Fig. 2 Effects of restraint on circulating leukocytes in wild house sparrows: a) total leukocytes, b) lymphocytes, c) monocytes, and d) heterophils (per 10k

erythrocytes). Error bars represent + 1 standard error and letters denote significant differences.

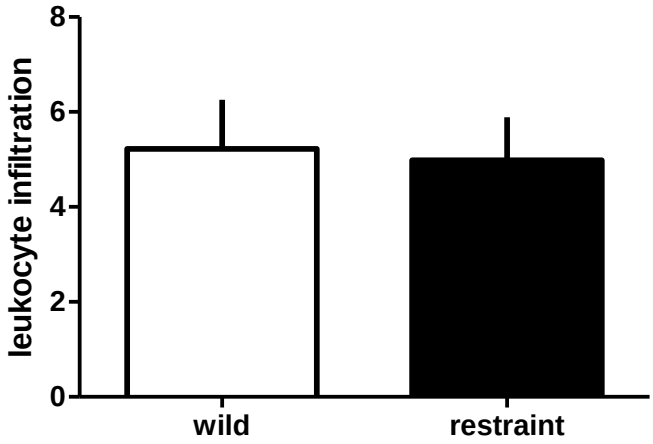

Fig. 3 No effect of restraint on total leukocytes infiltrating surgical sponges in wild house sparrows. Error bars represent + 1 standard error,

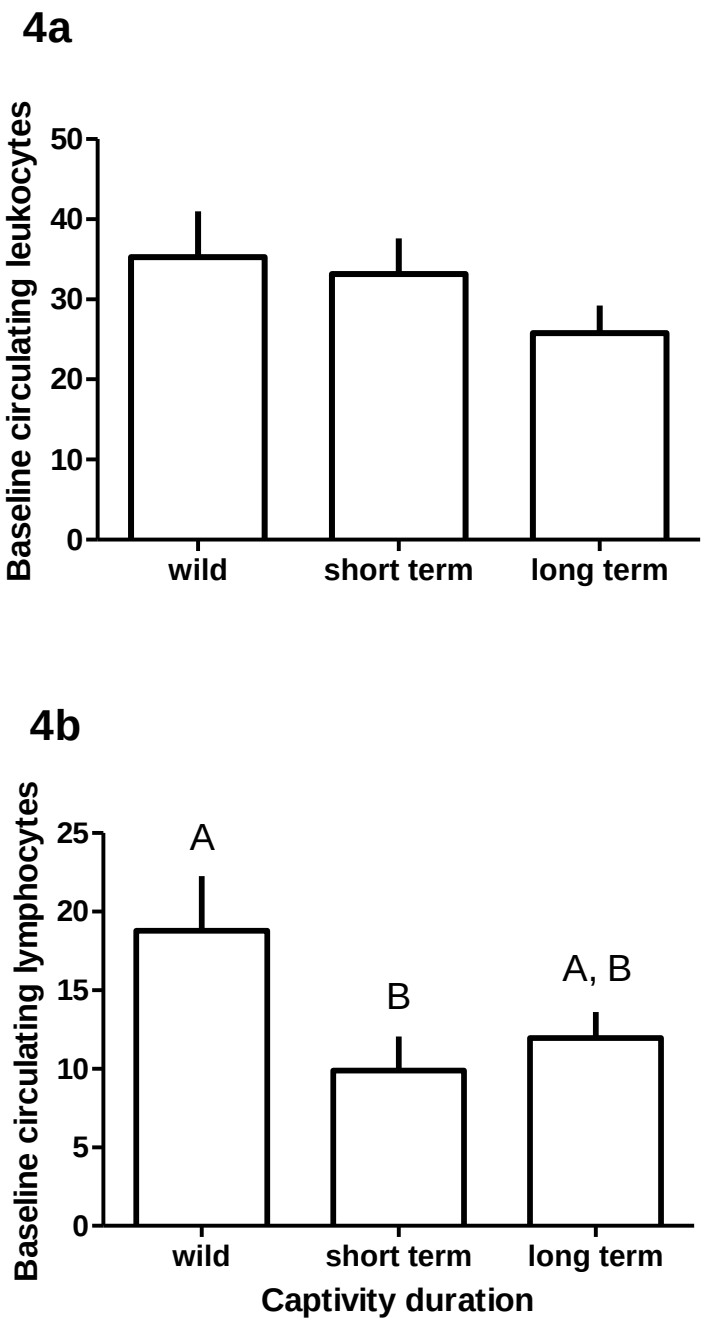

Fig. 4 Effects of captivity duration on a) total circulating leukocytes and b) circulating lymphocytes in house sparrows. Error bars represent + 1 standard error; and letters denote group membership by a simultaneous Bonferonni post-hoc test).

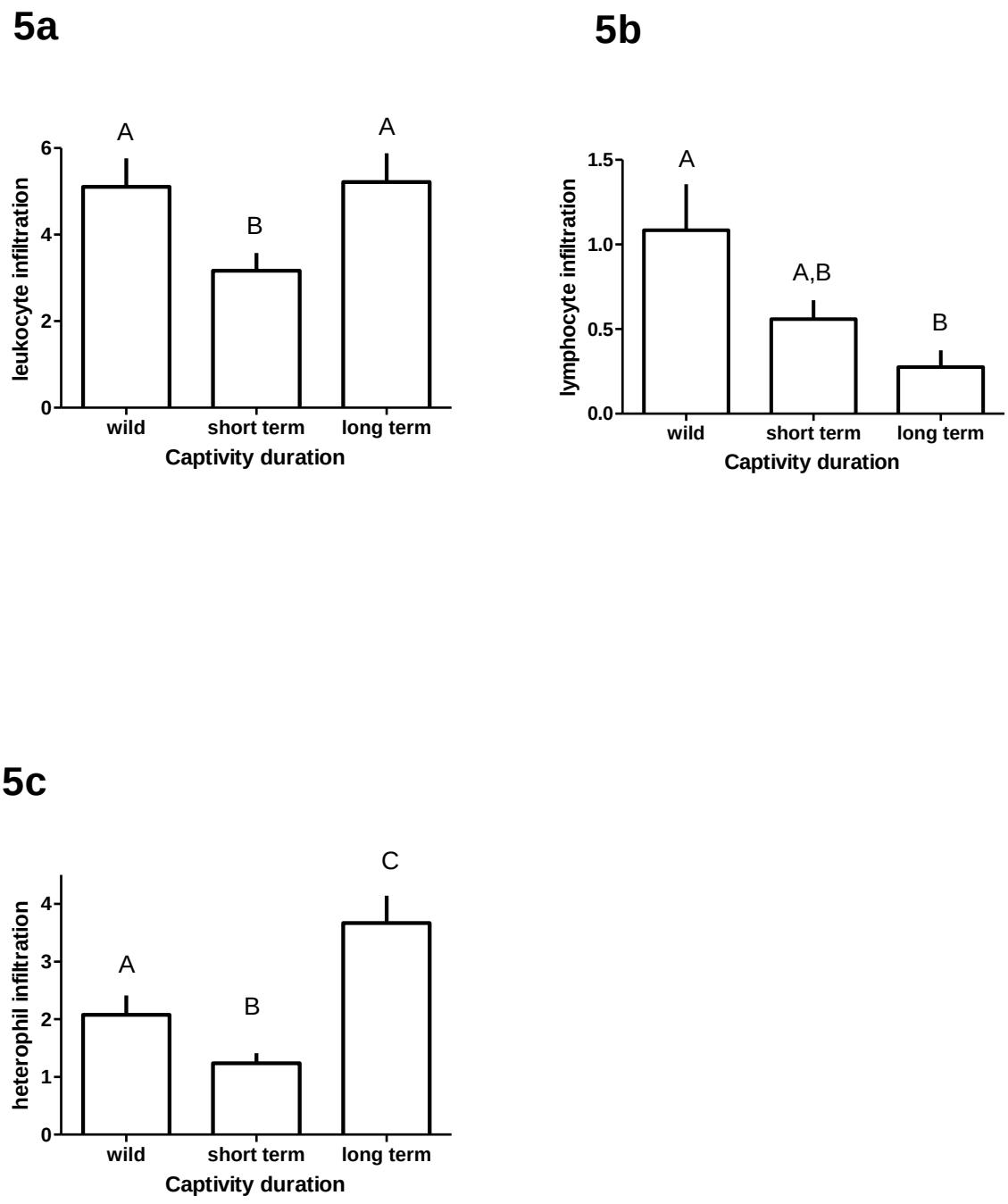

Fig. 5 Effects of captivity duration on leukocyte trafficking to surgical sponges in house sparrows: a) total leukocytes, b) lymphocytes, and c) heterophils. Error

bars represent + 1 standard error, and letters denote group membership by a simultaneous Bonferonni post-hoc test.

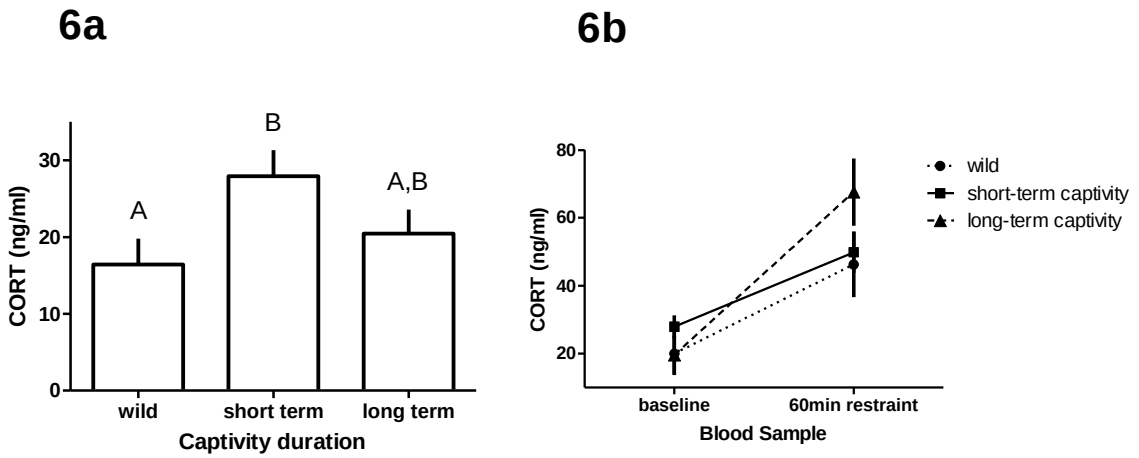

Fig. 6 Captivity affects a) baseline but not b) restraint induced elevations in corticosterone in house sparrows. In a), error bars represent + 1 standard error and letters denote group membership by a simultaneous Bonferonni post-hoc test; in b) all groups significantly increased from baseline post-restraint.

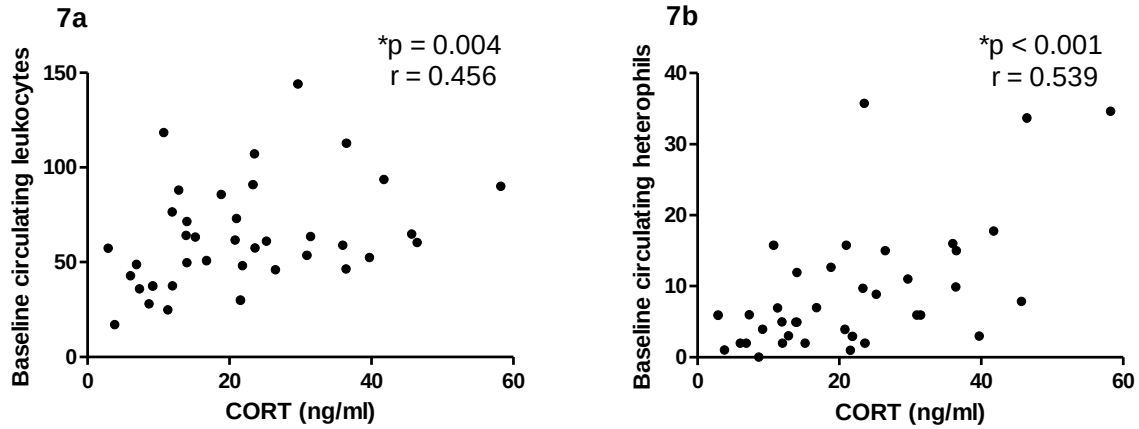

Fig. 7 Correlations of baseline CORT to circulating leukocytes. CORT prior to restraint (baseline) predicts circulating a) total leukocytes and b) heterophils.

Chapter 2

Immune surveillance of skin differs between domesticated and wild songbirds

Submitted as: Kuhlman, J.R., Martin, L.B., Ackerman, A.K., Fegely, J.L., Garringer, A., Rindfuss, E.M., and Ardia, D.R. in review.

Abstract

Stressors alter immune functions, with chronic stressors being suppressive and acute stressors being enhancive, but most of our understanding of such effects comes from studies of domesticated species. Wild animals, on average, experience greater frequency and intensity of stressors, so observations of stress-immune relationships in domesticated species may have limited evolutionary relevance. A form of immune enhancement that likely differs between wild and domesticated species is immune redistribution; the movement of immune cells to body areas in need of rapid protection. We compared immune cell influx to skin between wild house sparrows and domesticated zebra finches. Some individuals were subjected to restraint prior to implantation whereas others were implanted immediately upon capture. We predicted skin infiltration of leukocytes would be greater in house sparrows than zebra finches, as stressors are comparatively absent for domesticated species, but zebra finches, and not

house sparrows, would exhibit immunoenhancing effects of transient stressors. We found house sparrow skin infiltration was dominated by lymphocytes and basophils whereas zebra finch responses were dominated by heterophils and monocytes, but restraint had little impact on leukocyte influx in either species. These results suggest that domestication may have selected for altered immune processes in response to stressors in birds, but consideration of additional species will be critical to determining the importance of other factors.

Word count: 215

Key words: house sparrow, immunoredistribution, immunocompetence, stress, zebra finch

Introduction

The use of domesticated species has been valuable to biomedical science because many genetic and environmental factors (e.g., nourishment, prior infections or injuries) that can obscure accurate measurement of variables of interest can be controlled. Indeed, domesticated animals experience little to no fluctuations in weather, ambient temperature, food availability, or exposure to predators and parasites, all of which have strong impacts on physiology and behavior in wild animals (e.g., food quality or quantity ((Bourgeon *et al.* 2006; Raberg, Stjernman & Hasselquist 2003) and ambient temperatures (Dabbert, Lochmiller & Teeter 1997; Lifjeld, Dunn & Whittingham 2002). The extent to

which domestication impinges on physiological traits relative to free-living species is surprisingly little studied however. Patterns emerging from these studies suggest that directionality is due to the particular physiological variable being measured. For example, in some species for some physiological parameters responses are stronger in wild than domesticated variants, as seen with increased levels of glucocorticoids in response to acute stress (Cyr & Romero 2008; Kunzl & Sachser 1999), but for other variables, such as inflammation response, patterns can reverse with domesticated species exhibiting greater response (Ewenson, Zann & Flannery 2003; Ewenson, Zann & Flannery 2001). Thus, it is unclear whether and to what extent the physiological responses of domesticated animals have been altered by captivity, and what relevance does physiological variation in domesticated animals have for evolutionary or ecological topics (Calisi & Bentley 2009; Tschirren *et al.* 2009).

The focus of the present study was to determine whether domestication impacts coordination between two physiological processes in songbirds: immune and stress responses. Stress hormones and immunity have strong and many-faceted interactive feedback effects in animals (Martin 2009; Sternberg 2006; Dhabhar 2009). Prolonged elevation of stress hormones tends to suppress immune functions (Kusnecov & Rossi-George 2002; Leung & Bloom 2003) and is sometimes correlated with autoimmune disease (Nathan 2002). However, transient elevations of stress hormones enhance certain immune functions including leukocyte surveillance at the skin (Dhabhar & McEwen 1996; Dhabhar & McEwen 1997; Dhabhar & McEwen 1999; Viswanathan & Dhabhar 2005;

Viswanathan, Daugherty, & Dhabhar 2005). Both stress hormone regulation and immune functions have likely been modified through the reductions in the intensity or frequency of stressors associated with domestication. In this study, we sought to determine whether immune redistribution, or a shuttling of immune resources to the periphery in response to an acute stressor (Braude, Tang-Martinez & Taylor 1999; Dhabhar & McEwen 1999), has been impacted by domestication in birds. Immune redistribution is thought to occur to enable an organism to increase surveillance in the regions of the body that may become wounded while coping with a stressor (Viswanathan & Dhabhar 2005). Immunoenhancing effects of transient stressors have been studied almost exclusively in domesticated rodents (Dhabhar *et al.* 1996; Dhabhar & McEwen 1997; Dhabhar *et al.* 1999; Viswanathan *et al.* 2005, Viswanathan & Dhabhar 2005). In the only previous study, to our knowledge, that examined leukocyte redistribution in a free-living species, wild house sparrows, *Passer domesticus,* showed different leukocyte compositions relative to domesticated mice, suggesting a possible difference between either free-living vs. domesticated species and/or birds vs. mammals (Kuhlman & Martin, in press). Although mammals and birds have similarities in immune cell functions (Campbell & Ellis 2007), potential differences in leukocyte infiltration profile may be due to phylogeny, so a comparison of immunoenhancement between domesticated vs. wild species is warranted within a single vertebrate Class.

The goal of the present study was to compare leukocyte skin influx between a domesticated and a wild songbird species (Order Passeriformes).

Zebra finches, *Poephilia guttata,* are a domesticated species that is widely used for evolutionary ecology research (Ewenson *et al.* 2001; McGraw & Ardia 2003; Tschirren *et al.* 2009), and they share a similar diet and comparable body size and lifestyle to house sparrows (Zann 1996). House sparrows are a wild species for which more is known about immune function than most others (Martin *et al.* 2005; Martin, Hasselquist & Wikelski 2006; Bonneaud *et al.* 2003), and to which the sponge implantation technique used in this study has been adapted (Kuhlman & Martin, in press). We first tested for species differences in immunoenhancement in individuals implanted immediately upon capture because stress hormones are elevated shortly after capture (Dickens, Earle & Romero 2009; Rich & Romero 2005) and because captivity alone impacts physiological measures in this and other wild species (Cyr, Dickens & Romero 2009; Ewenson *et al.* 2001; Dickens *et al.* 2009). We predicted that overall skin infiltration of leukocytes would be greater in house sparrows than zebra finches. We also compared the effect of short-term restraint on infiltration to examine the effect of an acute stressor. We predicted that we would observe an effect of restraint on infiltration in zebra finches but be absent in house sparrows due to the continual stressor of translocation in the wild species (Dickens *et al.* 2009; Kuhlman & Martin, in press).

Materials and Methods

Study Animals

Sixteen adult male house sparrows were caught in Lancaster, PA, USA (40^{o} N, 76^{o} W) in December 2008. This period is non-breeding for this species, and was chosen because many immune responses tend to be elevated at this time of year in this and other songbird species (Martin, Weil & Nelson 2008; Greenman, Martin & Hau 2005). Birds were bled from the alar wing vein (50μl) within 3 minutes of capture (Romero & Reed 2005). Individuals were marked with numbered aluminum leg bands and randomly assigned to a treatment group. All house sparrows were implanted with sponges in the field (see below) but were translocated to an animal room on a natural photoperiod (12h light: 12h dark with lights on at 0700) until explant. In addition, 26 domesticated adult male zebra finches were purchased from a local breeder and kept under similar conditions in the same location for the duration of the study. Zebra finches too were bled (50μl) within 3 minutes of opening the cage door. Both species were housed in pairs from the time of implant until explant. The rooms were maintained at 22 $\pm$ 2 ^{o}C and relative humidity ~45%. Birds were given standard finch feed and water *ad libitum,* and remained in the cages for the duration of the study except when undergoing surgeries (<5 minutes each). After explants, house sparrows were released back to site of capture, and zebra finches were used in other studies. Experimental procedures were approved in advance by Franklin and Marshall College (IACUC protocol # 18-2008) and the University of South Florida (#W3202) Institutional Animal Care and Use Committees.

Experimental Groups

House sparrows were randomly assigned to one of two groups. Group 1 birds (n = 8) were implanted immediately at capture in the field whereas group 2 birds (n = 8) were restrained for 30 minutes in cloth bags after capture then implanted. Zebra finches were separated into similar groups. Group 3 consisted of zebra finches (n = 13) caught from cages and implanted immediately whereas group 4 (n = 13) consisted of 30 minutes of restraint followed by implantation. For all individuals, additional blood samples (50 μl) were taken post restraint and at the time of explants. This restraint procedure effectively increases circulating glucocorticoids in wild birds (Canoine *et al.* 2002) including wild house sparrows (Martin *et al.* 2005) and domesticated zebra finches (Evans *et al.*, 2006). For all birds at all sampling points, blood smears were made for later leukocyte quantification.

Sponge Implantation and Retrieval

Birds were lightly anesthetized with isoflurane vapors (Butler Animal Health Supply, Dublin, OH), and their right flanks were swabbed with 100% ethanol. Squares of Gelfoam absorbable gelatin sponge (10 mm x 10 mm, Pharmacia & Upjohn Co, Division of Pfizer Inc, NY, NY) were pre-soaked in 0.9% sterile saline then implanted subcutaneously on the right flank. Wounds were sealed with Vetbond (3M, St. Paul, MN). Gelatin sponges were explanted 12h post-implantation as maximal leukocyte infiltration occurred in rodents

(Viswanathan & Dhabhar 2005), and house sparrows (Kuhlman & Martin, in press) at this time. For explants, birds were again anesthetized with isoflurane vapors; sponges were collected by opening the Vetbond seal with surgical scissors and removing them with sterile forceps. Then, wounds were sealed again with Vetbond and gelatin sponges were immediately placed into 10% formalin until they were processed for later leukocyte quantification (embedded in paraffin, sectioned at 5um thickness, mounted on glass slides and H&E stained, Histology Core, Moffitt Cancer Center, University of South Florida). As sponge orientation could not be discerned (which surface had been in contact with skin or muscle), two random sections of sponge were collected for all birds.

Leukocyte Quantification

Leukocytes were differentiated into lymphocytes, heterophils and eosinophils, basophils, and monocytes/macrophages (Campbell & Ellis 2007). Heterophils could not be distinguished confidently from eosinophils, but as heterophils comprise the largest fraction of circulating granulocytes, all such cells were identified as 'heterophils' (Martin *et al.* 2006). To quantification leukocyte infiltration to surgical sponges, slides were observed under a microscope (Leica DME) at 5x magnification to identify a path traversing the sponge section such that the maximum possible fields of view could be quantified. Fields of view consisted of a 100 mm^2 grid mounted within the eyepiece of the microscope that was moved across the section. All leukocytes falling within or in contact with the grid were then quantified for each slide at 1000x. Leukocyte counts were then

adjusted according to the fields of view observed. In house sparrows but not zebra finches, some sponge sections (n = 2, No restraint; n = 6, Restraint) were saturated with erythrocytes, which made them unquantifiable. Blood smears were fixed in 100% ethanol then left to air dry until they were stained using a Protocol HEMA 3 staining kit (Fisher Scientific Company L.L.C., Kalamazoo, MI, USA). Differential leukocyte counts were then obtained using a microscope (at 1000x) by counting leukocytes per 10,000 erythrocytes (using a modification of the grid-based survey above). All slides were quantified blind to treatments.

Data analysis

One-sample Kolmogrov-Smirnov tests were used to test whether dependent variable distributions were significantly non-normal. No variable violated this assumption, but variance heterogeneity was indicated in some cases and subsequently those variables were ln-transformed. Univariate analysis of variance (ANOVA) was performed separately for each cell type, with species and restraint as fixed factors and total or differential cell counts as dependent variables. Species differences in changes over time in circulating leukocytes were tested using repeated measures ANOVAs. Potential relationships among variables were assessed using Spearman's rank correlation analyses. For all analyses, SPSS v. 17 was used with $\alpha \leq 0.05$. Figures were produced using GraphPad Prism v. 5.

Results

Species differences in sponge infiltrating leukocytes

Species did not differ in total leukocytes infiltrating sponges ($F_{1, 32} = 0.009$, $P = 0.927$, Fig. 1a), nor did restraint impact total infiltration in either species ($F_{1, 32} = 0.963$, $P = 0.335$, Fig. 1a). However, when leukocyte types were differentiated, infiltration responses were markedly distinct: lymphocyte ($F_{1, 32} = 6.916$, $P = 0.013$, Fig. 1b) and basophil ($F_{1, 32} = 15.370$, $P < 0.001$, Fig. 1e) infiltration was significantly greater in house sparrows than zebra finches, but heterophil ($F_{1, 32} = 3.493$, $P = 0.071$, Fig. 1c) and monocyte ($F_{1, 32} = 3.108$, $P = 0.088$, Fig. 1d) infiltration was marginally greater in zebra finches. Restraint did not impact any cell type in the skin more so in one species than another.

Effect of restraint on change in circulating leukocytes

Restraint induced a significant reduction in total circulating leukocytes ($F_{1, 19} = 16.384$, $P = 0.001$, Fig. 2a), but the magnitude of the reduction was larger in zebra finches than house sparrows (species x treatment: $F_{1, 19} = 5.155$, $P = 0.034$, Fig. 2a). Restraint also reduced circulating lymphocytes ($F_{1, 19} = 12.017$, $P = 0.003$, Fig. 2b), but not heterophils ($F_{1, 19} = 2.987$, $P = 0.100$, Fig. 2c), monocytes ($F_{1, 19} = 3.896$, $P = 0.063$, Fig. 2d), or basophils ($F_{1, 19} = 4.070$, $P = 0.058$, Fig. 2e). The effect of restraint was comparable between species for heterophils and basophils (species x treatment interactions: heterophils: $F_{1, 19} = 0.675$, $P = 0.422$, Fig. 2c; and basophils: $F_{1, 19} = 2.410$, $P = 0.137$, Fig. 2e) but tended to be stronger in zebra finches than house sparrows for lymphocytes and

monocytes (species x treatment interactions: lymphocytes: $F_{1, 19} = 4.164$, P = 0.055, Fig. 2b; monocytes: $F_{1, 19} = 3.876$, P = 0.064, Fig. 2d). For all cell types, a major influence on the stronger response in zebra finches was due to the higher pre-restraint values (Fig. 2).

Effect of captivity on circulating leukocytes differences

Total circulating leukocytes increased from just prior to implant to just after explant in both species ($F_{1, 40} = 27.719$, P < 0.001, Fig. 3a), but species did not differ ($F_{1, 40} = 1.005$, P = 0.322, Fig. 3a). The majority of this increase was due to increases in heterophils ($F_{1, 40} = 57.756$, P < 0.001, Fig. 3c), and house sparrow increases in heterophils were of larger magnitude than zebra finches (species x time: $F_{1, 40} = 6.421$, P = 0.015, Fig. 3c), but basophils decreased in house sparrows and not zebra finches ($F_{1, 40} = 7.080$, P = 0.011, Fig. 3e). Circulating lymphocytes ($F_{1, 40} < 0.001$, P = 0.990, Fig. 3b) and monocytes ($F_{1, 40} = 0.260$, P = 0.613, Fig. 3d) did not differ between species,

Correlations between circulating and skin infiltrating leukocytes

Negative correlations between circulating leukocyte densities just prior to implant and sponge leukocyte densities were expected if leukocytes were exiting circulation to increase surveillance of skin. However, in neither house sparrows (R = -0.148, P = 0.559) nor zebra finches (R = -0.247, P = 0.415) was there evidence that circulating leukocyte densities prior to implantation and skin

infiltrate densities were related. Even when leukocytes were differentiated, correlations remained non-significant for both species (data not shown).

Discussion

Here, we report differences between domesticated zebra finches and free-living house sparrows in the magnitude of leukocyte influx to skin in response to acute stress. We predicted that this immunoenchanement effect would be greater in house sparrows than zebra finches, but we found no difference in total leukocyte counts. However, we do report the novel finding that domesticated zebra finches responses were dominated by heterophils and monocytes whereas wild house sparrow responses were dominated by lymphocytes and basophils, suggesting different pathways of immunoenhancement. We also tested for the effects of restraint and predicted that zebra finches and not house sparrows, would exhibit immunoenhancing effects of transient stress. Restraint prior to implantation had no effect on leukocyte infiltration of skin in either bird species however, contrary to results for domesticated rodents (Viswanathan & Dhabhar 2005). Restraint just prior to implantation affected circulating leukocytes in both species though, but reductions occurring mainly due to changes in lymphocytes were larger in zebra finches than house sparrows. We had reduced power to detect an effect of restraint in house sparrows (n = 6, No Restraint; n = 2, Restraint); however, it was previously determined that restraint effects could not be shown in wild house sparrows (Kuhlman & Martin, in press) so lack of restraint effect here is probably genuine. Over the 12 hours of captivity prior to

sponge explantation, both species exhibited increases in circulating leukocytes, composed primarily of heterophils, with a slightly larger increase in sparrows than zebra finches. However, we observed no evidence of trade-offs between blood and skin surveillance for parasites, as the change in or absolute level of circulating leukocytes was not correlated to infiltrating leukocytes, Altogether, both species exhibited skin influx of leukocytes, but with differences in their specific responses. Below, we interpret the specific patterns we observed and identify important areas for follow-up studies.

Immunoredistribution: Leukocyte infiltration of skin

Domesticated zebra finch skin infiltration was dominated by heterophils and monocytes whereas wild house sparrow responses were dominated by lymphocytes and basophils. This pattern has parallels with observations in domesticated mice where responses in mice were dominated by neutrophils (the mammalian leukocyte most similar to avian heterophils (Maxwell & Robertson 1998)) (Viswanathan & Dhabhar 2005). However, only T-lymphocytes were quantified in that study, so whether domestication or vertebrate class better explains differences in the infiltration profile among species remains unreconciled. Basophils and monocytes have important antimicrobial functions in birds (Campbell & Ellis 2007; Davis, Maney, & Maerz 2008), but their relatively low numbers and/or small changes in densities in the present study make it difficult to discuss their relevance. Heterophils and lymphocytes, however, are the most abundant avian leukocytes (Maxwell & Robertson 1998), and levels

varied dramatically between and within house sparrows and zebra finches. Lymphocytes are comprised of diverse cell types (B and T cells, including helper and cytotoxic variants). These cells engage antibody production and provide antigen memory (B cells) as well as intracellular pathogen control (cytotoxic T cells) and coordination of other immune processes (helper T cells). If an individual has previously encountered a parasite (and thus encoded memory of it) lymphocytes can provide rapid, targeted protection against infectious organisms (Janeway *et al.*, 2004). However, lymphocyte efficacy is relatively poor in the absence of prior experience. Heterophils by contrast are rapidly released from bone marrow (within 30 minutes) post-infection or injury (Maxwell &Robertson 1998), and thus are typically the first leukocytes arriving at wounds. Heterophils are also the predominant phagocytic leukocyte (Maxwell 1993), but also contain many cationic antimicrobial peptides, various hydrolytic enzymes, defensins, and other anti-parasitic compounds. These substances are released at wounds (i.e. degranulation) and provide broad protection against Gram-negative and Gram-positive bacteria, fungi, protozoans, and even some viruses (Harmon 1998). Given this broad protection, one might expect that heterophils would dominate most immune responses in most species. However, heterophils effectors can cause collateral damage in the course of combating a parasite, thus enforcing a tradeoff. Indeed, local heterophilia in response to wounding or infection is a significant cause of morbidity and/or mortality in some species (Ficken & Barnes 1989; Campbell & Dein 1984), and thus may explain why heterophils and neutrophils are replaced by less aggressive cells later in

responses in most species (Maxwell 1993). However, responses in domesticated species may focus on heterophilia instead of a rapid response of lymphocytes simply due to the lack of exposure to pathogens needed for antigen memory encoding, whereas, wild species, having been exposed to many pathogens, may have an influx of memory B-cells causing greater lymphocyte infiltration to skin.

Species differences

Given the evolutionary conservative nature of the immune system, it is somewhat surprising that two closely related bird species with relatively similar ecologies and life histories would exhibit different immune responses when wounded. As a possible explanation several non-exclusive hypotheses warrant testing. The first relates to resource variability, as wild birds would be subject to fluctuations in resource availability of greater magnitude than domesticated ones, which can affect immune activities in wild birds (Bourgeon *et al*. 2006; Raberg *et al.* 2003). Under this variation, a lymphocyte dominated strategy is a more cost-effective one. A second possibility is that limited engagement of immune defenses due to domestication may select for reduced investment of costly immune defenses during development. Development of the adaptive immune system is thought to be the most costly component of immune investment in vertebrates (Klasing 2004). Therefore, if domestication reduces repeat rates of exposure to parasites, domesticated species may have evolved an increased reliance on low development cost and broadly effective general defenses, with a

corresponding reduction in the costly investment of acquired immunity. If this pattern is consistent for domesticated species generally, their use as models of the human immune system may be a biased one. A third possibility is that a diminution of heterophils and an increase in lymphocytes may be a consequence of house sparrows as invasive species. In a series of studies, it was discovered that house sparrows, one of the world's most successful invaders (Anderson 2006), biased their immune defenses away from innate immune responses and towards adaptive ones (Lee *et al.*, 2006; Lee, Martin & Wikelski 2005). Succesful introduced species are thought to be those that show reduced innate immune functions in response to allocation of resources towards other physiological processes more conducive to invasibility (Lee & Klasing 2004) or to avoid excessive morbidity or mortality when encountering novel parasites (Martin *et al.*, in press).

A fourth possibility involves the differential sensitivity of leukocyte types to stress hormones (Maxwell 1993). Captivity, surgery and restraint elevate corticosterone in most vertebrates (Dickens *et al.* 2009; Romero 2004), so the increase in circulating heterophils at 12 hours may have resulted because this cell type (and neutrophils) proliferates in response to corticosterone (Gross & Siegel 1983). Why heterophils should proliferate in response to corticosterone, but lymphocytes experience apoptosis, and why house sparrows should respond more strongly than zebra finches, are as yet unclear. We have proposed previously that heterophilia may enable an individual to maintain a broadly effective defense against host infection even if this strategy is detrimental over

the long term (Kuhlman & Martin, in press). Likewise, lymphocytes may be sacrificed first because a) their greatest benefits would entail minimization of future immune activation (Schmid-Hempel & Ebert 2003) and b) diminution could free resources for other more immediately important physiological processes (e.g., escape from a predator, winning a territorial conflict; (Martin 2009)). We expect that the greater heterophilia in house sparrows is due to the greater duress it (as a free-living species in captivity and undergoing surgery and/or restraint) experienced over the course of the study, but this possibility, as well as the others above, are testable only through investigations of other wild and domesticated species (Garland & Adolph 1994).

Conclusion

In sum, we report immune redistribution in response to acute stress occurred in both species of songbirds examined, wild or domesticated. However, zebra finches and house sparrows varied in the character and intensity of responses, and additional study will be necessary to reconcile why. A major advance would entail a phylogenetically controlled comparative study that could identify ecological or life history factors that influence infiltration responses. Ideally, future studies would include pairs of domesticated and wild congeners to test directly the role of domestication on the infiltration response. At a more proximate level, it will be informative to assess the role of corticosterone via pharmacological manipulation of the hypothalamic-adrenal-pituitary axis. Further, it will be informative to determine whether quantitative differences in cell

infiltrates translate into functional differences in protection. A simple test of this possibility would entail mixing standard doses of bacteria with explanted sponges and subsequent quantification of bacterial killing capacity of the sponge exudate (Liebl & Martin 2009).

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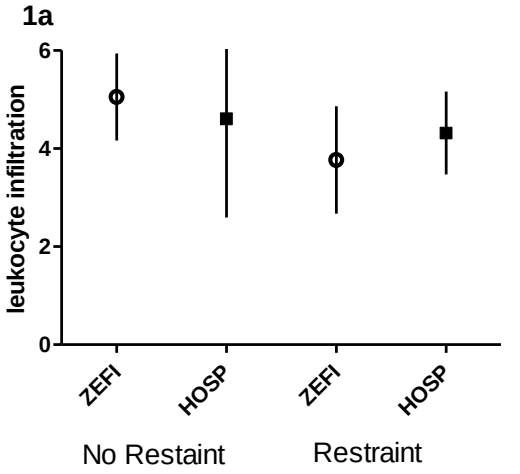
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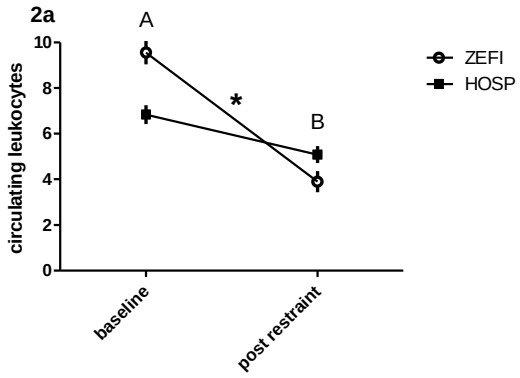
Fig. 1 Leukocyte infiltration to sponge in wild house sparrows and domestic zebra finches: a) total leukocytes, b) lymphocytes, c) heterophils, d) monocytes, and e) basophils (per 10k erythrocytes). Error bars represent + 1 standard error and letters denote significant differences between species.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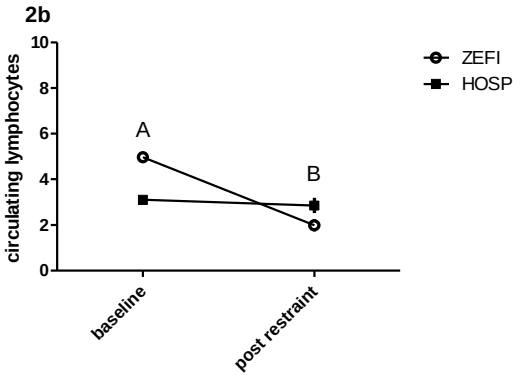
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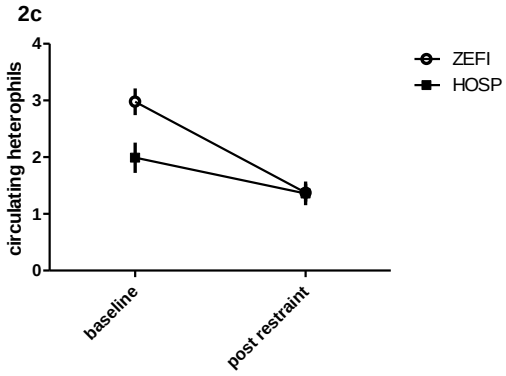
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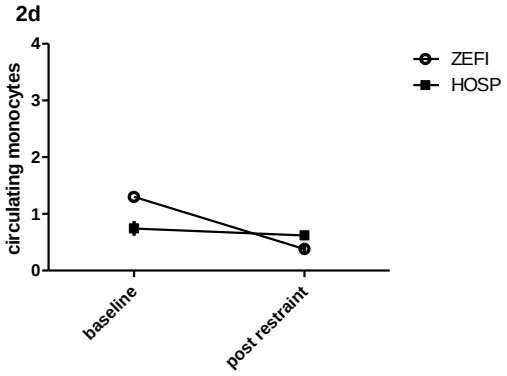
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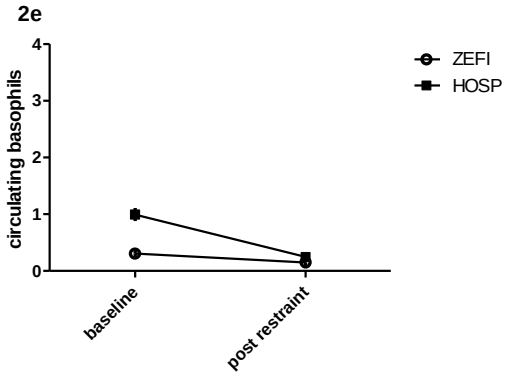
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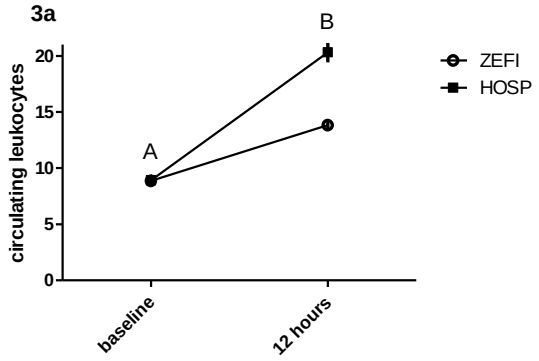
Fig. 2 Effects of restraint on circulating leukocytes in wild house sparrows and domestic zebra finches: a) total leukocytes, b) lymphocytes, c) heterophils, d) monocytes, and e) basophils (per 10k erythrocytes). Error bars represent + 1 standard error, letters denote significant differences between samples and asterisk denote significance between species.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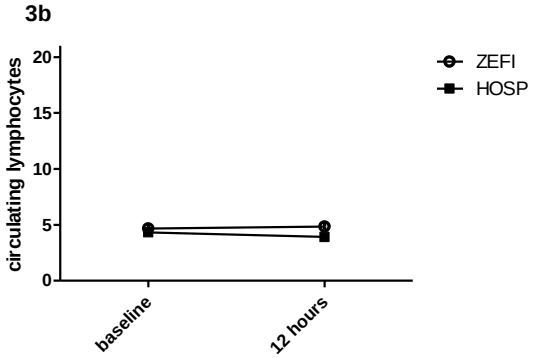
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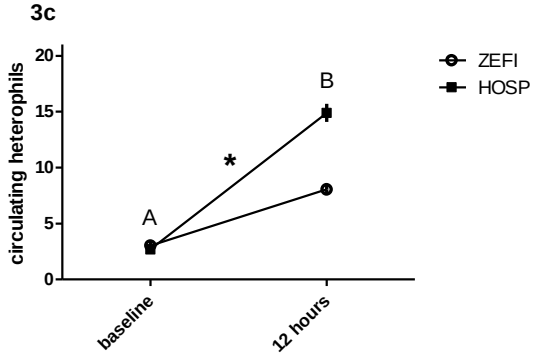
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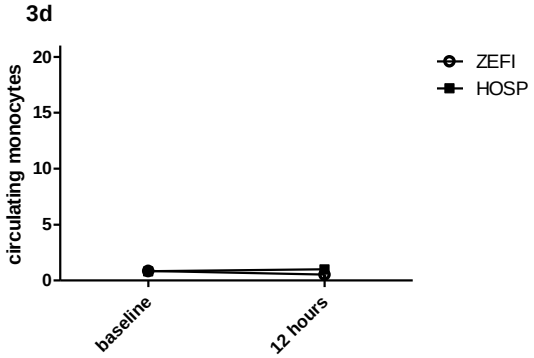
3d
circulating monocytes
20
15
10
5
0
baseline
12 hours
ZEFI
HOSP

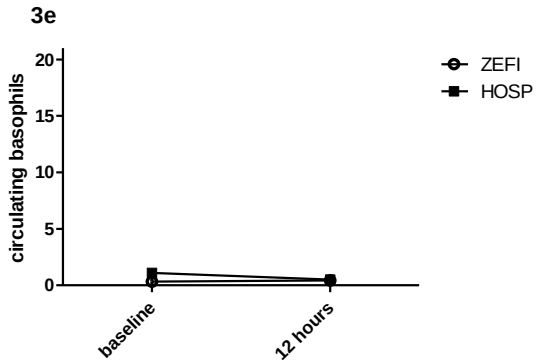
3e
circulating basophils
20
15
10
5
0
baseline
12 hours
ZEFI
HOSP

Fig. 3 Circulating leukocytes in wild house sparrows and domestic zebra finches 12h post sponge implantation: a) total leukocytes, b) lymphocytes, c) heterophils, d) monocytes, and e) basophils (per 10k erythrocytes). Error bars represent + 1 standard error, letters denote significant differences between samples and asterisk denote significance between species.