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Abstract

An individual's investment in constitutive immune defenses depends on both intrinsic and extrinsic factors. We examined how Leucocytozoon parasite presence, body condition (scaled mass), heterophil-to-lymphocyte (H:L) ratio, sex, and age affected immune defenses in golden eagle (Aquila chrysaetos) nestlings from three regions: California, Oregon, and Idaho. We quantified hemolytic-complement activity and bacterial killing ability, two measures of constitutive immunity. Body condition and age did not affect immune defenses. However, eagles with lower H:L ratios had lower complement activity, corroborating other findings that animals in better condition sometimes invest less in constitutive immunity. In addition, eagles with Leucocytozoon infections had higher concentrations of circulating complement proteins but not elevated opsonizing proteins for all microbes, and eagles from Oregon had significantly higher constitutive immunity than those from California or Idaho. We posit that Oregon eagles might have elevated immune defenses because they are exposed to more endoparasites than eagles from California or Idaho, and our results confirmed that the OR region has the highest rate of Leucocytozoon infections. Our study examined immune function in a free-living, long-lived raptor species, whereas most avian ecoimmunological research focuses on passerines. Thus, our research informs a broad perspective regarding the evolutionary and environmental pressures on immune function in birds.

Keywords: allocation, bacteria-killing ability, complement activity, ecoimmunology, parasite, raptor, trade-off

Research highlights: Golden eagle nestlings from three regions differed in constitutive immunity, with those in better condition investing less in those defenses. This study of a long- lived raptor informs our understanding of evolutionary and ecological correlates of immunity.

Introduction

Constitutive innate immunity is a complex system of immune defense that is always present in an organism and therefore capable of immediate immunological defenses (Schmid- Hempel and Ebert 2003; Downs and Stewart 2014). Experimental evidence, theoretical ideas, and mechanistic pathways all suggest that a variety of intrinsic and extrinsic factors drive differences in immune defenses among geographic regions. These factors include broad geographic patterns, such as latitude gradients in life history patterns (Martin et al. 2004; Ardia 2005; Adelman et al. 2010a; Adelman et al. 2010b); parasite prevalence (Horrocks et al. 2012); population characteristics such as density (Ortego and Espada 2007; Downs et al. 2015); habitat (Ortego and Espada 2007; Schmitt et al. 2017); and weather (Lifjeld et al. 2002). Thus, there is substantial reason to expect differences in immune defenses among individuals from different regions. However, significant variation also exists among individuals within a region and how an individual invests in immune defenses at any given point depends on plastic trade-offs (Ardia et al. 2011; Downs et al. 2014). Regional differences may be driven by, or even obscured by, differences in an individual's physiological state, life stage, genetics, developmental conditions, and experiences that manifest at the individual level (Downs et al. 2014). Specifically, nutrient reserves, chronic stress, sex, age, and current parasite infections all affect an individual's current investment in constitutive immunity (Downs et al. 2015; Wilcoxen et al. 2015). Allocation theory and physiological network theory provide theoretical frameworks to understand how these factors may influence individual-level differences in immune defenses (Lochmiller and Deerenberg 2000; Lee 2006; Martin et al. 2011; Cohen et al. 2012).

From an energetics perspective, trade-offs may arise when resources are limited because energy must be allocated among costly function, including the immune system (Stearns '92; Lochmiller and Deerenberg 2000; Downs et al. 2014). In general, induced responses are more costly than constitutive responses (Armitage et al. 2003; Derting and Compton 2003), but logic dictates that production and maintenance of any constitutive cells and proteins costs energy, and studies show that caloric malnutrition is correlated with reduced constitutive immune defenses (Chandra '75). This leads to the prediction that individuals in poor body condition should invest less in immune defenses than those in better condition. Similarly, immune defenses continue to develop as individuals age, so younger individuals may not be capable of mounting the same intensity of response as older nestlings (Killpack et al. 2013). Mechanistically, this age pattern may arise because younger nestlings invest in structural growth over immune defenses.

The relationship between immune defenses and parasitism also can be viewed from the perspective of allocation theory. Typically, parasite infection causes a heightened immune response, though the intensity of the response depends on an organism's investment strategy (Caldwell et al. '58; Simms 2000; Houston et al. 2007; Råberg et al. 2009; Hawley and Altizer 2011). However, individuals with access to less energy on the landscape or internally may be immunosuppressed and unable to mount as intense of a response to a parasite infection and, thus, may have larger parasite loads (Christe et al. '98). This would lead to a negative relationship between parasite infection and immune defenses.

Physiological networks predict that stress mediates investment in immune defenses. Specifically, the release of glucocorticoids supports metabolically demanding activities and mediates immune responses, reproduction, and growth, among other traits (Sapolsky et al. 2000). However, chronic stress can (i) decrease immune function, thereby increasing susceptibility to infections and (ii) decrease investment in reproduction and growth (McEwen and Wingfield 2003; Martin 2009). Changes in glucocorticoids are reflected by changes in heterophil-to- lymphocyte (H:L) ratio, a commonly used measure of stress and well-being in avian ecological studies (Saks et al. 2003; Davis et al. 2008). Changes in H:L ratio are proportional to the level of glucocorticoid release during mild or moderate stress events or energetically demanding events (Maxwell '93; Davis et al. 2008). Generally, lower H:L ratios indicate a less stressed individual in good condition, whereas higher H:L ratios indicate the opposite (Saks et al. 2003).

Hypotheses about immunological differences between sexes are rooted in both physiological networks and allocation theory. Males and females have different energy demands across stages of the life cycle and seasons and, therefore, allocate energy among activities differently (Lee 2006). These allocation choices may be mediated by sex hormones, which are integrative signaling molecules that coordinate functions across physiological systems, including the immune system (Ahmed et al. '85; Schuurs and Verheul '90; Martin et al. 2008; Demas and Nelson 2012). Generally, studies of passerines have found that nestling males have lower immune defenses than females (Tschirren et al. 2003; Dubiec et al. 2006).

Although innate immunity has been investigated in smaller bird species with short development periods in the nest (e.g. 2 to 3 weeks), many aspects of the immune response have not been evaluated in larger bird species such as eagles that spend up to 9-14 weeks in the nest before fledging, and therefore can be subject to a variety of immune challenges during development. Similarly, most raptors exhibit reverse sexual dimorphism, that is adult females are larger than adult males and nestling females grow faster than males (Snyder and Wiley '76; Newton '79; Collopy '83). Because of the extra energetic demand of faster growth in females, we might expect raptor nestlings to exhibit different sex-specific patterns of investment in immune defenses than passerines.

We investigated how location (region), parasite load, body condition, sex, and age affect investment in the constitutive immune response in nestling golden eagles (*Aquila chrysaetos)*. We tested plasma samples from golden eagle nestlings from three different study sites—Oregon, Idaho, and California (Fig. 1). We quantified bacterial killing ability and hemolytic-complement activity—two functional measures that primarily quantify aspects of the complement cascade (Mayer '48; French et al. 2010; Demas et al. 2011). We predicted that constitutive immunity of golden eagle nestlings would (i) vary among regions, (ii) increase with parasite load, (iii) decrease with decreasing body condition, (iv) increase with age, (v) decrease with stress (H:L ratio), and (vi) be higher in females.

Methods

Experimental Design

We sampled 96 golden eagle nestlings from a total of 59 nests between April and July, 2015 in three geographical regions (Fig. 1). We sampled 17 nests in the Idaho region, 31 in the Oregon region, and 11 in the California region (Fig. 1). The mean (± SD) number of nestlings in each nest was 1.6 (± 0.05). Volunteers associated with the project monitored golden eagle nesting territories for occupancy and nesting attempts by pairs. Using the observations of pair behavior and nesting phenology for golden eagles (Isaacs 2012), we entered selected sites when nestlings were estimated to be ~38 and ~50-days old. We attempted to sample each nestling at these two separate age points, but some nestlings were only sampled once due to inclement weather or time constraints that prevented a second nest entry. During the first nest entry, nestlings were banded by fitting a U.S. Geological Survey stainless steel band or uniquely

marked for subsequent identification. During each nest entry, we recorded morphometric data, estimated age based on feather development using photographs from Hoechlin ('76) and Driscoll (2010), and collected a blood sample from each nestling.

Sample Collection

After first sterilizing the area with an alcohol swab, we collected whole blood from the brachial vein using 25-27 gauge needles [sodium-heparinized (Oregon) or unheparinized (California, Idaho)]. We collected 3-5 ml of blood within 45 min of entering nests. Drops of whole blood were used immediately to make 2 blood smears for each bird. The remainder of the whole blood sample was aliquoted into lithium heparinized tubes and placed on ice until they could be processed in the lab or vehicle. Lithium tubes were centrifuged for 10 minutes and then the plasma was transferred to sterile cryovials. Blood samples were chilled in a cooler with ice in the field and frozen at -20 °C within 12 hours of collection. Plasma samples were transferred to a -80 °C freezer within one month of collection. Blood smears were fixed in methanol for 3 minutes, air dried, and stored for later use. Samples were collected under the appropriate state and federal permits. Sampling protocols were approved by Boise State University's Institutional Animal Care and Use Committee (permit #006-AC14-007), the Oregon Fish and Wildlife Office (Standard Operating Procedure F-004), or the appropriate agency. All procedures were in compliance with NIH guidelines.

Sex

Sex was determined genetically from blood samples. Birds were genotyped by polymerase chain reaction and gel electrophoresis following the procedure described in Fridolfsson and Ellegren ('99) either at a commercial laboratory (Avian Biotech International, Tallahassee, FL 32312) or N. B. Fernandez's lab at Purdue University

Quantifying Blood Parasites and H:L Ratio

Presence or absence of haemosporidian parasites (i.e., *Leucocytozoon*, *Haemoproteus*, and *Plasmodium*) were determined from blood smears. Smears were stained with Diff-Quick (Richard Allan Scientific, San Diego, CA) (Müller et al. 2011). Each slide was scanned for 15 minutes at a magnification of 1000× with oil immersion to look for haemosporidian parasites. Parasites and blood cells were identified using standard guidelines (Campbell '95; Remple 2004; Zajac and Conboy 2012). If parasites were found, parasite intensity was calculated by the number of parasites per 10,000 red blood cells under 1000× objective (Appleby and Redpath '97). The number of red blood cells was estimated by comparing each field to a set of standardized photographs with known numbers of red blood cells (Ellis et al. 2014). H:L ratio was determined by counting the number of heterophils and lymphocytes per 100 fields. We consider our samples to be unbiased by the stress of nest entry and handling because our samples were collected within 45 min of entering nests and previous research has shown that stress from handling birds increases H:L ratios after 1 hr (Davis 2005; Davis et al. 2008). H:L ratio and parasite counts were done by a single individual without any knowledge of data collected in the field.

Immunocompetence Assays

We measured complement activity with two assays that focus on pathways ending in lysis: (i) a bacterial killing ability assay and (ii) a hemolytic-complement activity assay. In our bacterial killing ability assay, we quantified the ability of plasma from golden eagle nestlings to lyse E. coli strain ATCC 8739 via the complement pathway using methods adapted from French and Neuman-Lee (2012). Briefly, we prepared a 106 bacteria/mL stock solution of E. coli by dissolving one lyophilized pellet (Epower Microorganism, MicroBioLogics, St. Cloud, MN) in 10 mL of 37oC sterile phosphate-buffered saline (PBS) (Lonza 1M PBS). We used 20 µl of plasma sample per replicate and tested each sample in triplicate. We performed assays in round-bottom 96-well plates. We made positive controls containing 20 µl PBS and negative controls containing 23.5 µl PBS. Then, we made a working solution of 105 bacteria/mL E.coli and added 3.5 µl of it to each well, except for the negative controls, resulting in a final well volume of 23.5 µl for all wells. We agitated plates for 1 min at 700 rpm and incubated them for 30 minutes at 37 °C. Then, we agitated plates for another minute at 700 rpm, added 125 µl of tryptic soy broth to all wells, and agitated plates for 1 minute at 300 rpm. We read plates on a spectrophotometer (Synergy HTX multi-mode reader, BioTek, Winooski, VT, USA) at 300 nm to obtain a baseline absorbance. Next, we incubated the plates for 12 hours at 37 °C, agitated the plates for 1

minute at 300 rpm, and measured the absorbance a second time at 300nm. We subtracted the baseline optical density from the final optical density for each sample. Bacterial killing ability (% bacterais kill) was calculated as 1 – (mean of simple / mean of positive control) ×100 %. Samples were rerun if the replicates were not within 20% of one another.

The hemolytic-complement activity assay also measures complement activity using an integrated response (Sinclair and Lochmiller 2000). We adapted methods from Sinclair and Lochmiller (2000). Briefly, we washed sheep red blood cells (SRBCs) (Innovative Research, Inc., Novi, MI, IC10-0210) in PBS. In a round-bottom 96-well plate, we diluted golden eagle plasma 1:4 with dextrose-gelatin veronal buffer (DGVB) (BioWhittaker, Walkersville, MD) for a final volume of 40 µl per well. We made 0% and 100% lysis control wells in duplicate by adding 65 µl VB and 65 µl deionized water, respectively. Samples and controls were run in duplicate. We added 25 µl of 0.6% suspension of washed SRBCs in DGVB to all wells and 25 µl of 1:40 dilution of rabbit anti-SRBC antibody (S1389, Sigma-Aldrich, St. Louis, MO) to all wells, except for the controls. The plate was agitated for 5 minutes at 300 rpm and incubated at 37°C for 90 minutes. The plate was then centrifuged for 5 minutes at 500 rpm at room temperature. Next, 60 µl of supernatant was transferred from each well to respective wells in a new 96-well plate. The absorbance was measured at 405 nm on a microplate reader.

We calculated hemolytic-complement activity as a percentage of SRBCs lysed at a single dilution; this endpoint allowed us to capture the wide range of complement activity seen in our animals (Downs et al. 2015). A more established endpoint for this assay is the CH50, which integrates information about the slope of lysing activity with information about the dilution required to lyse 50% of the cells in the assay (Kabat and Mayer '61, Sinclair and Lochmiller 2000). We emphasize that our approach of using a single dilution as an end point does not summarize as much information as the well-established CH50 endpoint. However, our endpoint of percent of cells lyses at a single dilution does allow for a relative comparison of complement activity among individuals (Downs et al. 2015). We argue that this approach is analogous to comparing percent bacteria killed at a single bacteria concentration and plasma dilution as is used to quantify bacteria killing ability (French and Neuman-Lee 2012, Tieleman et al. 2005). Samples were rerun if the replicates were not within 20% of one another.

Body Condition

We evaluated body condition of golden eagles using structural size-corrected mass (Labocha and Hayes 2012). In our dataset, culmen length positivily correlated with body mass (correlation with mass: $r = 0.67$ in males, $r = 0.62$ in females), and we used this as our measure of structural size (McDonald et al. 2005). We accounted for age and sex in our index of body condition because both body mass and culmen length increased with age, and growth curves differ between males and females (Collopy '83). As our index of body condition, we used the residuals of body mass (square-root transformed) from a mixed model that included $\log_{10}$- transformed culmen length, sex, a second-degree polynomial of age, and sex × polynomial of age as fixed effects. Individual identity nested within nest identity was included as a random effect to account for study design. This analysis was performed in R v 3.2.2 (R Development Core Team 2015) with package nlme (Kuhn et al. 2011) and the resulting equation is presented in the results section.

Statistical Analysis

We built linear mixed models to determine effects of the intrinsic factors measured and geographical region on bacteria-killing ability and hemolytic-complement activity. All analyses were performed in R v 3.2.2 (R Development Core Team 2015), with packages nlme (Kuhn et al. 2011) and multcomp (Hothorn et al. 2008). Models included region (California, Oregon, Idaho), age (in days), body condition, H:L ratio, sex, and Leucocytozoon parasites (presence or absence) as fixed effects. Haemoproteus and Plasmodium presence was excluded because of low prevalence (1 and 0 birds showed Haemoproteus and Plasmodium, respectively). Thus, Leucocytozoon presence was our only measure of parasite load. We included individual identity nested within nest identity as a random effect to account for study design; nest could have different intercepts but not slopes. We performed a log10-transformation on hemolytic-complement activity to meet the model assumption of normal residuals. Models were fit using restricted maximum likelihoods and no within-group correlation structure. We tested correlations among our predictor variables with variance influence factors, and found no evidence of such correlations. Significance of pairwise contrasts between factor levels of region was determined post-hoc with Tukey all-pair contrasts.

To help interpret our results about immune defense, we also tested for effects on body condition by building linear mixed models with H:L ratio and body condition as response variables. As in the models for the immune data, these models included region of origin, age, sex, and Leucocytozoon parasites as fixed effects. In addition, body condition was included as a fixed effect in the model for H:L ratio, and H:L ratio was included as a fixed effect in the model for body condition. We included individual identity nested within nest identity as a random effect to account for study design; nest could have different intercepts but not slopes. We performed a log10-transformation on H:L ratio data to meet the model assumption of normal residuals.

Results

We processed 158 plasma samples from 96 eagles. We had a single sample from 39 eagles, were missing some data from some individuals, and did not have enough aliquots of samples to rerun all samples that did not meet our inclusion requirements (see "Methods: Immunocompetence Assays"). In the end, our final sample for calculating body condition included 150 samples from 96 unique individuals. Our analysis of bacteria-killing ability included data from 142 plasma samples from 95 unique individuals, and our analysis of hemolytic-complement activity included data from 140 plasma samples from 93 unique individuals. Our analyses with body condition and H:L ratio as response variables included data from 139 observations of 91 unique individuals. Mean age of nestlings was 38 (± 9) days on the first entry and 50 (± 9) days on the second entry. Means (± sd) for each region are presented in Table 1. Note that Leucocytozoon infection rates differed among regions (Table 1).

Body mass (square root-transformed) of golden eagle nestlings was positively associated with culmen length (log10-transformed) (β = 64.2 ± 11.6, $F_{1,48}$ = 30.8, P < 0.001, Fig. 2A). Body mass of females was generally higher than that of males ($F_{1,33}$ =10.8, P = 0.02) and increased in a non-linear fashion with age (2° polynomial for age: $F_{2,48}$ = 10.8, P < 0.001). In addition, the interaction between age (2° polynomial for age) and sex was significant ($F_{1,48}$ = 3.43, P = 0.04), such that females grew faster than males and reached a larger size (Fig. 2B). The equation relating these predictor effects to body mass is as follows: $\sqrt{body\ mass} = -42.8 + 64.2\log(culmen\ length) - 2\text{male} + 20age - 17.9age^2 - 13.7males \times age + 4.8age^2 + error$. We calculated the residual of body mass (square root-transformed) to obtain a measure of structural size corrected mass for each individual and used this value as our measure of body condition.

Overall, there was a positive correlation between our two measures of immune defenses (r = 0.60, P < 0.001, Fig. 3), and some of the same effects predicted both measures of immune defenses. Bacteria-killing ability and hemolytic-complement activity differed among regions (Table 2, Fig. 4A&B). Specifically, birds from Oregon had higher bacteria-killing ability than those from either California (P = 0.003) or Idaho (P < 0.001); birds from Idaho and California did not differ (P = 0.43). Similarly, birds from Oregon had a higher hemolytic-complement activity than either those from California (P < 0.001) or Idaho (P < 0.001), but birds from Idaho and California did not differ (P = 0.93). In addition, H:L ratio was positively associated with the bacteria-killing ability ($\beta \pm$ S.E. $= 7.32 \pm 3.36$, Table 2, Fig. 4C) and hemolytic-complement activity ($\beta \pm$ S.E. $= 0.10 \pm 0.03$, Table2, Fig. 4D).

In contrast, *Leucocytozoon* infection and sex predicted hemolytic-complement activity, but not bacteria-killing ability (Table 2). Specifically, birds with a *Leucocytozoon* infection had higher hemolytic-complement activity than did those without infections (Fig. 5A), and females exhibited higher hemolytic-complement activity than males (Fig. 5B). Nestling age and body condition were not associated with either measure of immune defense (Table 2).

Our analyses using linear models of H:L ratio and body condition indicate that these two indices exhibited different patterns regarding their significant effects. H:L ratio decreased with age (Table 2) and was higher in individuals in which *Leucocytozoon* infections were absent (Table 2). Region, body condition, and sex were not significant predictors of H:L ratios (Table 2). In contrast, body condition decreased with increasing H:L ratio (Fig. 3D), but not related to region, sex, age, or *Leucocytozoon* presence (Table 2)

Discussion

As expected, both region and parasite load impacted immune function in golden eagle nestlings. Nestlings in Oregon had higher constitutive immune function than nestlings from California or Idaho. There was no latitudinal gradient with respect to immune function. This is perhaps related to the scale of our study. In most studies where a relationship

has been found, the gradients were along larger distances than those in our study. For example, differences in immune defenses were observed in song sparrows (*Melospiza melodia*) from Alaska and those from the west coast of the continental U.S., but not within the continental U.S. (Adelman et al. 2010a).

Differences in vector prevalence or differences in the diversity of parasites among the regions may provide one possible explanation for our result (Horrocks et al. 2011). Indeed, a higher proportion of eagles from Oregon had evidence of *Leucocytozoon* infections (Table 1), and although inclusion of this measure of parasite load in our analysis removed some variation in immunity caused by parasites, our measure of parasite load was incomplete. For example, we did not quantify avian pox, conjunctivitis, avian flu, or West Nile virus—all contagous diseases to which raptors are susceptible (Wrobel et al. 2016; Wilcoxen et al. 2016). Perhaps Oregon birds had higher overall microparasite loads and thus invested more energy in immune function to combat these parasites than did the California and Idaho birds. This possibility is consistent with our result that *Leucocytozoon* infection also increased investment in hemolytic-complement activity. Alternatively, soregions. Environmental factors including toxicant exposure, food availability, reproductive costs,me other environmental factor may be driving differences among population density, and habitat diversity can impact immune function (Franson ‘86; Wiehn et al. ‘99; Ardia 2005; Ortego and Espada 2007). A longer-term and larger cross-region study is needed to determine the mechanism driving the relationships between immune function, parasite load, and environmental factors.

Leucocytozoon presence affected immune function as measured by the hemolytic-complement activity assay, but not as measured by bacteria-killing ability. The proteins and cells involved in complement activity are always circulating, but the circulating concentration increases when an animal is challenged by a parasitic infection (Millet et al. 2007), as suggested by our results from the hemolytic-complement activity assay. *Leucocytozoon* presence, however, did not affect bacteria-killing ability. Although both assays quantify complement activity, the differences in the methodologies mean that results from each assay must be interpreted differently. The complement system detects foreign cells by recognizing conserved antigens, and it then controls and eliminates parasites by inducing inflammation, lysis, or opsonization via three proteolytic cascade pathways—the classical, lectin, or alternative pathway (Dunkelberger and Song 2010). Each pathway is initiated by a different process: the classical pathway is initiated by natural antibodies, the lectin pathway is initiated by other opsonizing molecules, and the alternative pathway is initiated when C3, a complement protein, spontaneously cleaves and binds to the surface of a pathogen (Dunkelberger and Song 2010). Although we cannot directly distinguish among these pathways in the bacteria killing assay, we can draw conclusions about the pathways by integrating results from both complement assays used in this study.

The bacterial killing assay relies solely on the plasma sample for all components of the immune defenses and thus provides an integrated measure of all three complement pathways. That is, that assay relies on natural antibodies, other opsonizing molecules, and C3 present in the plasma sample to opsonize bacterial cells and initiate the complement pathway. Thus, even if enough complement proteins are present to cause high levels of *E.coli* lysing, an individual would show a low killing ability if there were insufficient opsonizing molecules to initiate the complement cascade. In contrast, opsonizing molecules required to initiate the complement cascade are not a limiting step in the hemolytic-complement assay because we added enough antibodies to mark all of the foreign cells (i.e., SRBC) as part of the procedure. Therefore, our results indicate that although the propensity to opsonize *E. coli* did not change in response to *Leucocytozoon* presence, the concentrations of complement proteins did increase. We concluded that *Leucocytozoon* presence increased concentrations of complement in golden eagles, but not circulating antibodies for all microbes.

Similarly, hemolytic-complement activity, but not bacterial killing ability was higher in females than males. This suggests that concentrations of constitutive complement proteins, but not proteins that initiate the complement cascade (e.g., natural antibodies for *E. coli*), are elevated in female nestlings relative to males. These results corroborate results from other bird studies that found lower immune defenses in males. For example, male nestling great tits had reduced cellular immunity relative to females and when experimentally infested with parasites, males were more susceptible than females (Tschirren et al. 2003). Similarly, relative to counterparts in unmanipulated nests, male nestlings from experimentally enlarged broods exhibited a greater reduction in their cell-mediated immunity than females (Dubiec et al. 2006). Presumably, differences in immune defenses might lead to differences in susceptibility to parasites; however, we could not directly test this hypothesis.

We found a positive relationship between H:L ratio, our measure of general health and chronic stress, and both immune measures, suggesting that healthier or less stressed individuals had lower constitutive, complement defenses—the opposite of our prediction. If H:L ratio is interpreted as a measure of general health, then our results support a growing body of literature that suggests individuals in lower overall condition invest more in constitutive immunity than those in better condition (Arsnoe et al. 2011; Downs et al. 2015). Specifically, maintenance of constitutive immunity is generally less costly than mounting cellular or humoral induced, adaptive responses during an infection (Demas et al. '97; Derting and Compton 2003; Armitage et al. 2003), and individuals in poorer health may opt for a strategy of higher daily costs of high constitutive immunity with the potential advantage of clearing an infection prior to the need to mount high-cost induced responses (Downs et al. 2015). If this interpretation is correct, it would contrast previous literature that interpreted low H:L ratios as an indicator of compromised immune defenses (Hanauska-Brown et al. 2003). Alternatively, these results could have arisen because H:L ratios also increase with parasite infections (Davis 2005; Lobato et al. 2005; Wilcoxen et al. 2015). That is, our results could be interpreted as an indicator of parasite load and could suggest that individuals with higher H:L ratios have higher parasite loads, and thus, have invested more in circulating immunity. Contrary to this prediction, H:L ratio was lower in eagles with hematological evidence of *Leucocytozoon* infections than in eagles without evidence of infection. However, we only have this one indicator of parasite load and, thus, would need to collect more information about overall parasite load to distinguish between these hypotheses.

Body condition often trades off with investment in immune defenses (Dawson and Bortolotti '97; Dawson and Bortolotti 2000; Whiteman and Parker 2004), but we found that body condition was not associated with immune function in this observational study. Nestlings use resources for rapid structural growth (Penteriani et al. 2005), so our results could indicate that individuals invest in a baseline level of immune defense and then allocate remaining resources to growth. This idea is corroborated by a study in captive zebra finches (*Taeniopygia guttata*) that found that food-restricted zebra finch nestlings had reduced body mass and structural growth relative to non-restricted controls, although they did not differ in antibody responses to a novel antigen, keyhole limpet hemocyanin (Killpack et al. 2014). Similarly, food restriction did not alter concentrations of total circulating immunoglobulin Y and enhanced lysis activity in zebra finches—although it did reduce structural growth, mass, and circulating haptoglobin after a challenge with lipopolysaccharide (Killpack et al. 2015). Alternatively, energy entering the nest may be allocated between generations and within generations resulting in energetic trade-offs between generations. That is, parents might suffer costs to improve fledgling success of nestlings, as seen in great tits (*Parus major*) (Ots and Horak '96; Norte et al. 2009). Thus, to fully understand the lack of relationship between body condition and immune defense seen in our study, a full energy profile of both nestlings and adults is required.

Similarly, nestling age was not associated with immune function in this observational study. Although the immune system of the nestlings was developing from hatching until the age at the time of sampling (Tieleman et al. 2010; Killpack et al. 2013), our sampling range (18-68 days old) only represented a small portion of the golden eagles' entire lives. The age range samples may have been too narrow to capture significant differences in development of innate immune defenses. The litertature about the relationship between age and innate immune function is inconclusive. European stonechats (*Saxicola rubicola*) showed an increase in constitutive bacterial killing ability from ages one to seven years (Tieleman et al. 2010). Similarly, induced adaptive and constitutive innate immune defenses both increased over the nestling period in house sparrows (*Passer domesticus*), yet immune defenses did not fully mature until after fledging (Killpack et al. 2013). Likewise, zebra finches did not achieve a mature, induced secondary antibody response level until after fledging (Killpack and Karasov 2012). Future studies on both constitutive and induced responses are needed to tease apart these contradictions.

Our study contributes to the understanding of the evolutionary and environmental pressures on immune function in birds. To date, most immune studies in birds have been conducted on songbirds; however, passerines are very different from raptors in ways that may affect evolutionary and ecological pressures on immunity. For example, nestling raptors remain in the nest much longer than passerines, therefore increasing their exposure risk to parasites and vectors of parasites. Raptors, including golden eagles, eat meat and allow excess food to remain in their nest, which could provide a breeding ground for parasites (Bent '61; Collopy '83). In addition, raptors often reuse nests across years, further increasing risk of parasitic infections. Our study showed that immune defenses differed among regions of golden eagles and helps illuminate how parasites may impact immunity early in the life history of this long-lived species. These early energetic costs could have carry-over effects that reduce survival and ultimately affect population

dynamics (Downs and Stewart 2014). By integrating information about toxin and parasite exposure and developing a more comprehensive understanding of the immune defenses in birds, we will better understand how the environment impacts allocation choices with respect to immunity.

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Literature Cited

Adelman JS, GE Bentley, JC Wingfield, LB Martin, M Hau. 2010a. Population differences in fever and sickness behaviors in a wild passerine: a role for cytokines. J Exp Biol, 213: 4099-4109.

Adelman JS, S Cordoba-Cordoba, K Spoelstra, M Wikelski, M Hau. 2010b. Radiotelemetry reveals variation in fever and sickness behaviours with latitude in a free-living passerine. Funct Ecol, 24: 813-823.

Ahmed SA, WJ Penhale, N. Talal. 1985. Sex hormones, immune responses, and autoimmune diseases: mechanisms of sex hormone action. Am J Pathol, 121: 531-551.

Appleby BM, SM Redpath. 1997. Indicators of male quality in the hoots of tawny owls (Strix aluco). J Raptor Res, 31: 65-70.

Ardia DR. 2005. Tree swallows trade off immune function and reproductive effort differently across their range. Ecology 86: 2040-2046.

Ardia DR, HK Parmentier, LA Vogel. 2011. The role of constraints and limitation in driving individual variation in immune response. Funct Ecol 25: 61-73.

Armitage SAO, JJW Thompson, J Rolff, MT Siva-Jothy. 2003. Examining costs of induced and constitutive immune investment in *Tenebrio molitor*. J Evol Biol 16: 1038-1044.

Arsnoe DM, HS Ip, JC Owen. 2011. Influence of body condition on influenza A virus infection in mallard ducks: experimental infection data. PLoS One 6: e22633.

Bent AC. 1961. Life Histories of North American Birds of Prey. Dover Publications. Caldwell RM, JF Schafer, LE Compton, FL Patterson. 1958. Tolerance to cereal leaf rusts. Science, 128: 714-715.

Campbell TW 1995. Avian Hematology and Cytology. Iowa State University Press.

Chandra, RK. 1975. Serum complement and immunoconglutinin in malnutrition. Archives of disease in childhood 50: 225-229.

Christe P, AP Moller, F de Lope. 1998. Immunocompetence and nestling survival in the house martin: the tasty chick hypothesis. Oikos, 83: 175-179.

Cohen AA, LB Martin, JC Wingfield, SR McWilliams and JA Dunne. 2012. Physiological regulatory networks: ecological roles and evolutionary constraints. Trends Ecol Evol, 27: 428-435.

Collopy MW. 1983. A comparison of direct observations and collections of prey remains in determining the diet of Golden Eagles. J Wildl Manage, 47: 360-368.

Davis AK, D Maney, J Maerz. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct Ecol, 22: 760-772.

Davis AK. 2005. Effect of handling time and repeated sampling on avian white blood cell counts. J Field Ornithol, 76: 334-338.

Dawson RD, GR Bortolotti. 2000. Effects of hematozoan parasites on condition and return rates of American kestrels. Auk, 117: 373-380.

Dawson RD, GR Bortolotti. 1997. Ecology of parasitism of nestling American kestrels by *Carnus hemapterus* (Diptera: Carnidae). Can J Zool, 75: 2021-2026.

Demas G.E. and R.J. Nelson. 2012. Ecoimmunology. Oxford University Press, New York, NY, USA.

Demas G.E., V. Chefer, M.I. Talan and R.J. Nelson. 1997. Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. Am J Physiol Regul Integr, 273: R1631-R1637.

Demas GE, DA Zysling, BR Beechler, MP Muehlenbein, SS French. 2011. Beyond phytohaemagglutinin: assessing vertebrate immune function across ecological contexts. J Anim Ecol, 80: 710-730.

Derting TL, S Compton. 2003. Immune response, not immune maintenance, is energetically costly in wild white-footed mice (*Peromyscus leucopus*). Physiol Biochem Zool, 76: 744-752.

Downs CJ, KM Stewart. 2014. A primer in ecoimmunology and immunology for wildlife research and management. Calif Fish Game 100: 369-393.

Downs CJ, KM Stewart, BL Dick. 2015. Investment in constitutive immune function: effects of density-dependent processes. PLoS One 10: e0125586.

Downs CJ, JS Adelman, GE Demas. 2014. Mechanisms and methods in ecoimmunology: integrating within-organism and between-organism processes. Integr Comp Biol, 54: 340-352.

Driscoll D. 2010. Protocol for Golden Eagle occupancy, reproduction, and prey population assessment. American Eagle Research Institute, Apache Jct. , AZ. p. 55.

Dubiec A, M Cichon, K Deptuch. 2006. Sex-specific development of cell-mediated immunity under experimentally altered rearing conditions in blue tit nestlings. Proc Biol Sci, 273: 1759-1764.

Dunkelberger JR, W Song. 2010. Complement and its role in innate and adaptive immune responses. Cell Res. 20: 34-50.

Ellis VA, MR Kunkel, RE Ricklefs. 2014. The ecology of host immune responses to chronic avian haemosporidian infection. Oecologia, 176: 729-737.

Franson J.C. 1986. Immunosuppressive effects of lead.

French SS, DF DeNardo, TJ Greives, CR Strand, GE Demas. 2010. Human disturbance alters endocrine and immune responses in the Galapagos marine iguana (*Amblyrhynchus cristatus*). Horm Behav, 58: 792-799.

French SS, LA Neuman-Lee. 2012. Improved ex vivo method for microbiocidal activity across vertebrate species. Biol Open, 1: 482-487.

Fridolfsson A, H Ellegren. 1999. A simple and universal method for molecular sexing of nonratite birds. J Avian Biol, 30: 116-121.

Hanauska-Brown LA, AM Dufty Jr, GJ Roloff. 2003. Blood chemistry, cytology, and body condition in adult Northern Goshawks (*Accipiter gentilis*). J Raptor Res, 37: 299-306.

Hawley DM, SM Altizer. 2011. Disease ecology meets ecological immunology: understanding the links between organismal immunity and infection dynamics in natural populations. Funct Ecol, 25: 48-60.

Hoechlin D. 1976. Development of golden eaglets in southern California. Western Birds 7: 137-152.

Horrocks NPC, KD Matson, BI Tieleman. 2011. Pathogen pressure puts immune defense into perspective. Integr Comp Biol 51: 563-576.

Horrocks NPC, A Hegemann, KD Matson, K Hine, S Jaquier, M Shobrak, JB Williams, JM Tinbergen, BI Tieleman. 2012. Immune indexes of larks from desert and temperate regions show weak associations with life history but stronger links to environmental variation in microbial abundance. Physiol Biochem Zoo 85: 504-515.

Hothorn T, F Bretz, P Westfall. 2008. Simultaneous inference in general parametric models. Biom J 50: 346-363.

Houston AI, JM McNamara, Z Barta, KC Klasing. 2007. The effect of energy reserves and food availability on optimal immune defence. Proc Biol Sci. 274: 2835-2842.

Isaacs, F.B. 2012. Golden eagles (*Aquila chrysaetos*) nesting in Oregon, 2011 and 2012. Draft Annual Report, 20 December 2012. Oregon Eagle Foundation, Inc., Klamath Falls, Oregon, USA.

Kabat, EA, MM Mayer. 1961. Experimental immunochemistry. 2nd ed. Charles C. Thomas, Springfield, IL.

Killpack TL, WH Karasov. 2012. Ontogeny of adaptive antibody response to a model antigen in captive altricial zebra finches. PloS One, 7: e47294.

Killpack TL, E Carrel, WH Karasov. 2015. Impacts of Short-Term Food Restriction on Immune Development in Altricial House Sparrow Nestlings. Physiol Biochem Zool, 88: 195-207.

Killpack TL, DN Tie, WH Karasov. 2014. Compensatory growth in nestling Zebra Finches impacts body composition but not adaptive immune function. Auk, 131: 396-406.

Killpack TL, Y Oguchi, WH Karasov. 2013. Ontogenetic patterns of constitutive immune parameters in altricial house sparrows. J Avian Biol, 44: 513-520.

Kuhn M, S Weston, J Wing, J Forester. 2011. Contrast: a collection of contrast method. See http://CRAN.R-project.org/package=contrast.

Labocha MK, JP Hayes. 2012. Morphometric indices of body condition in birds: a review. J Ornithol, 153: 1-22.

Lee KA 2006. Linking immune defenses and life history at the levels of the individual and the species. Integr Comp Biol, 46: 1000-1015.

Lifjeld JT, PO Dunn, LA Whittingham. 2002. Short-term fluctuations in cellular immunity of tree swallows feeding nestlings. Oecologia, 130: 185-190.

Lobato E, J Moreno, S Merino, J Sanz, E Arriero. 2005. Haematological variables are good predictors of recruitment in nestling pied flycatchers (*Ficeduld hypoleuca*). Ecoscience, 12: 27-34.

Lochmiller RL, C Deerenberg. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos, 88: 87-98.

Martin LB. 2009. Stress and immunity in wild vertebrates: Timing is everything. Gen Comp Endocrinol, 163: 70-76.

Martin LB, M Pless, J Svoboda, M Wikelski. 2004. Immune activity in temperate and tropical house sparrows: a common-garden experiment. Ecology, 85: 2323-2331.

Martin LB, ZM Weil, RJ Nelson. 2008. Seasonal changes in vertebrate immune activity: Mediation by physiological trade-offs. Phil. Trans. R. Soc. B, 363: 321-339.

Martin LB, AL Liebl, JH Trotter, CL Richards, K McCoy, MW McCoy. 2011. Integrator networks: illuminating the black box linking genotype and phenotype. Integr Comp Biol, 51: 514-527.

Maxwell M. 1993. Avian blood leucocyte responses to stress. Worlds Poult Sci J, 49: 34-43.

Mayer MM. 1948. Complement and complement fixation. In Kabat EB, Mayer MM, editors. Experimental Immunochemistry. Charles C. Thomas, Springfield, Ill. 905

McDonald PG, PD Olsen, A Cockburn. 2005. Selection on body size in a raptor with pronounced reversed sexual size dimorphism: are bigger females better? Behav Ecol 16: 48-56.

McEwen B, JC Wingfield. 2003. The concept of allostasis in biology and biomedicine. Horm Behav, 43: 2-15.

Millet S, J Bennett, KA Lee, M Hau, KC Klasing. 2007. Quantifying and comparing constitutive immunity across avian species. Dev Comp Immunol, 31: 188-201.

Muller C, S Jenni-Eiermann, L Jenni. 2011. Heterophils/Lymphocytes-ratio and circulating corticosterone do not indicate the same stress imposed on Eurasian kestrel nestlings. Funct Ecol, 25: 566-576.

Newton I. 1979. Population Ecology of Raptors. Berkhamsted: T. & A. D. Poyser. Hertfordshire, England.

Norte AC, PM Araujo, HL Sampaio, JP Sousa, JA Ramos. 2009. Haematozoa infections in a great tit *Parus major* population in Central Portugal: relationships with breeding effort and health. Ibis, 151: 677-688.

Ortego J, F Espada. 2007. Ecological factors influencing disease risk in eagle owls *Bubo bubo*. Ibis, 149: 386-395.

Ots, I, P Horak. 1996. Great Tits *Parus major* trade healthfor reproduction. Proc R Soc Lond B, 263: 1443–1447.

Penteriani V, MM Delgado, C Maggio, A Aradis, F Sergio. 2005. Development of chicks and predispersal behaviour of young in the Eagle Owl *Bubo bubo*. Ibis, 147: 155-168.

R Development Core Team 2015. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Raberg L, AL Graham, AF Read. 2009. Decomposing health: tolerance and resistance to parasites in animals. Phil Trans R Soc B, 364: 37-49.

Remple JD. 2004. Intracellular hematozoa of raptors: a review and update. J Avian Med Surg, 18: 75-88.

Saks L, I Ots, P Horak. 2003. Carotenoid-based plumage coloration of male greenfinches reflects health and immunocompetence. Oecologia, 134: 301-307.

Sapolsky RM, LM Romero, AU Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev, 21: 55-89.

Schmid-Hempel P, D Ebert. 2003. On the evolutionary ecology of specific immune defence. Trends Ecol Evol, 18: 27-32.

Schmitt C, D Garant, M Belisle, F Pelletier. 2017. Agricultural intensification is linked to constitutive innate immune function in a wild bird population. Physiol Biochem Zool, 90: 000-000.

Schuurs A, HAM Verheul. 1990. Effects of gender and sex steroids on the immune response. J Steroid Biochem, 35: 157-172.

Simms EL. 2000. Defining tolerance as a norm of reaction. Evol Ecol 14: 563-570.

Sinclair JA, RL Lochmiller. 2000. The winter immunoenhancement hypothesis: associations among immunity, density, and survival in prairie vole (*Microtus ochrogaster*) populations. Can J Zool, 78: 254-264.

Snyder NF, JW Wiley. 1976. Sexual size dimorphism in hawks and owls of North America. Ornithol Monogr, 20: 1-96.

Stearns SC. 1992. The Evolution of Life Histories. Oxford University Press, New York, NY.

Tieleman BI, E Croese, B Helm, MA Versteegh. 2010. Repeatability and individual correlates of microbicidal capacity of bird blood. Comp Biochem Phys, A 156: 537-540.

Tieleman, BI, JB Williams, RE Ricklefs, KC Klasing. 2005. Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. Proc Biol Sci, *272*:1715-1720.

Tschirren B, PS Fitze, H Richner. 2003. Sexual dimorphism in susceptibility to parasites and cell-mediated immunity in great tit nestlings. J Anim Ecol, 72: 839-845.

Whiteman NK, PG Parker. 2004. Body condition and parasite load predict territory ownership in the Galapagos hawk. Condor, 106: 915-921.

Wiehn J, E Korpimaki, I Pen. 1999. Haematozoan infections in the Eurasian kestrel: effects of fluctuating food supply and experimental manipulation of parental effort. Oikos, 84: 87-98.

Wilcoxen TE, E Wrobel, J Seitz, J Nuzzo. 2016. Prevalence of antibodies against Avipoxvirus, Aspergillus, and West Nile Virus in birds of prey from Central Illinois. Integr Comp Biol, 56: E238-E238.

Wilcoxen TE, DJ Horn, BM Hogan, CN Hubble, SJ Huber, J Flamm, M Knott, L Lundstrom, F Salik, SJ Wassenhove. 2015. Effects of bird-feeding activities on the health of wild birds. Conserv Physiol, 3: cov058.

Wrobel ER, TE Wilcoxen, J. Nuzzo, J Seitz. 2016. Seroprevalence of avian pox and *mycoplasma gallisepticum* in raptors in central Illinois. J Raptor Res 50: 289-294.

Zajac AM, GA Conboy. 2012. Veterinary Clinical Parasitology. John Wiley & Sons, Oxford

Figure Legends

Figure 1. Location of eagle nests sampled for this study. Each filled black circle represents one golden eagle nest. Nests from the same region are within the same large unfilled circle.

Figure 2: Model-estimated relationship (a) between body mass (square root-transformed) of golden eagle nestlings and culmen length (log10-transformed) and (b) between body mass and age by sex. Dashed lines represent standard errors.

Figure 3. Correlation between bacteria-killing ability and hemolytic-complement activity, two measures of constitutive innate immune defense.

Figure 4. Model-estimated mean ± SE of (a) percent of bacteria killed and (b) hemolyticcomplement activity for samples from different regions, and the relationship between (c) percent of bacteria killed and (d) hemolytic-complement activity with heterphile:lymphocyte (H:L) ratio. Statistical models for hemolytic-complement activity were performed on $\log_{10}$-transformed percent SRBC lysed, but model-derived, back-transformed data are presented in the figures. Different letters indicate a difference at $\alpha = 0.05$.

Figure 5. Model-estimated mean ± SE hemolytic-complement activity by (a) *Leucocytozoon* infection and (b) sex. Error bars represent model estimated standard errors and different letters indicate a significant different at $\alpha = 0.05$. Statistical models for hemolytic-complement activity were performed on $\log_{10}$-transformed percent SRBC lysed, but model derived, back-transformed data are presented in the figures.

Table 1. Sample sizes, % individuals with *Lecutocytozoon* infections, and mean (±s.d.) age, body condition, and heterophil:lymphocyte ratio (H:L ratio) by region and entrance period.

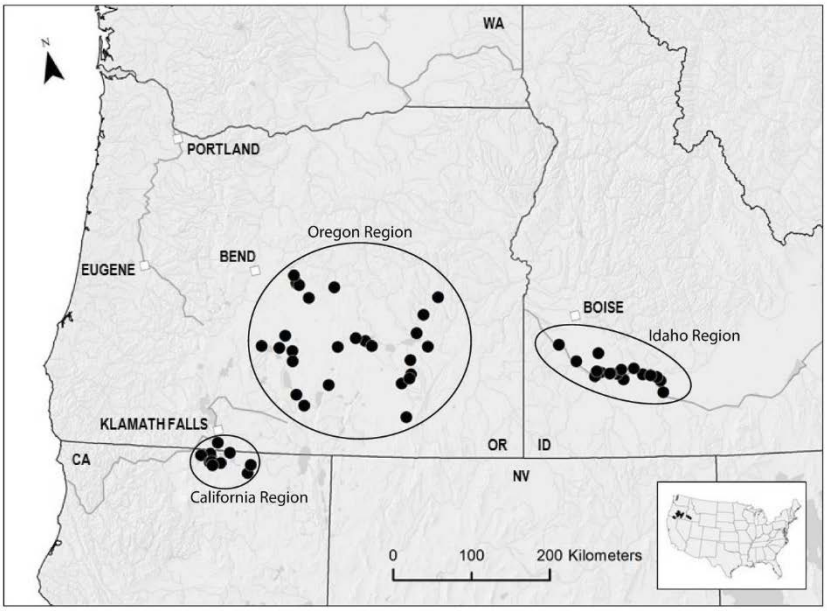
	n[1]		Age (days)		Body condition		H:L ratio		Leucocytozoon infections (%)	
Nest entry	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Region										
California	18	13	37.3 ±8.1	52.3 ± 3.5	-0.13 ± 1.26	0.61 ± 1.96	2.03 ± 0.75	1.50 ± 0.42	5.9	15.4
Idaho	28	22	36.4 ± 6.7	52.5 ± 4.1	-0.17 ± 1.92	0.05 ± 2.21	1.89 ± 0.56	1.58 ± 0.57	0	4.8
Oregon	44	20	38.6 ± 10.4)	45.8 ± 12.2	0.55 ± 2.05	-1.33 ± 2.75	1.67 ± 1.03	1.21 ± 0.68	11.4	43.8

[1] Number of golden eagles samples entered during each entrance period. We present the means and s.d. for the data set used to calculate body condition, our largest data set. We did not have all data for all individuals so actual sample sizes used in our statistical models are smaller. See results section for samples sizes for each analysis.

Table 2. Analysis of variance for the fixed effects from the linear models for bacteria-killing ability, hemolytic-complement activity, heterophil:lymphocyte ratio (H:L ratio), and body condition. All models included individual-identity nested within nest-identity as random effects. **Bold** indicates that the effect is significant at α=0.05.

Response variable	Fixed effect	df	F-value	p-value
Bacteria-killing ability	Intercept	1, 55	2.06	0.157
	Region	**1, 55**	**14.17**	**<0.0001**
	Body condition	1, 42	0.38	0.543
	Sex	1, 32	1.90	0.178
	Age	1, 42	1.30	0.261
	H:L ratio	**1, 42**	**4.74**	**0.035**
	Leucocytozoon presence	1, 42	0.05	0.825
Hemolytic-complement	**Intercept**	**1, 53**	**62.39**	**<0.0001**
activity	**Region**	**2, 53**	**15.90**	**<0.0001**
(log10-transformed)	Body condition	1, 42	1.15	0.289
	Sex	**1, 32**	**4.46**	**0.043**
	Age	1, 42	0.92	0.343
	H:L ratio	**1, 42**	**14.55**	**0.0004**
	***Leucocytozoon* presence**	**1, 42**	**12.54**	**0.001**
H:L ratio	**Intercept**	**1, 55**	**38.46**	**<0.0001**
(log10-transformed)	Region	2, 55	2.44	0.097
	Body condition	1, 45	2.65	0.111
	Sex	1, 32	0.56	0.460
	Age	**1, 45**	**11.16**	**0.002**
	***Leucocytozoon* presence**	**1, 45**	**10.64**	**0.002**
Body condition	Intercept	1, 55	1.86	0.178

Region	2, 55	0.36	0.703
H:L ratio	**1, 45**	**7.47**	**0.009**
Sex	1, 32	0.07	0.793
Age	1, 45	0.00	0.962
Leucocytozoon presence	1, 45	3.56	0.066



Location of eagle nests sampled for this study. Each filled black circle represents one golden eagle nest. Nests from the same region are within the same large unfilled circle.

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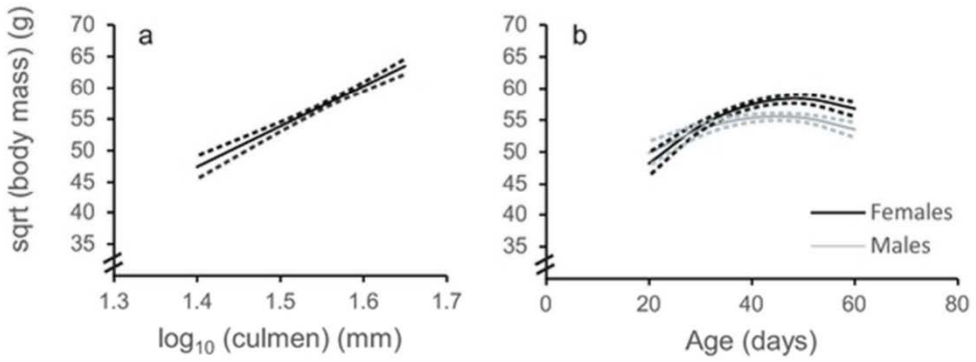

Model-estimated relationship (a) between body mass (square root-transformed) of golden eagle nestlings and culmen length (log10-transformed) and (b) between body mass and age by sex. Dashed lines represent standard errors.

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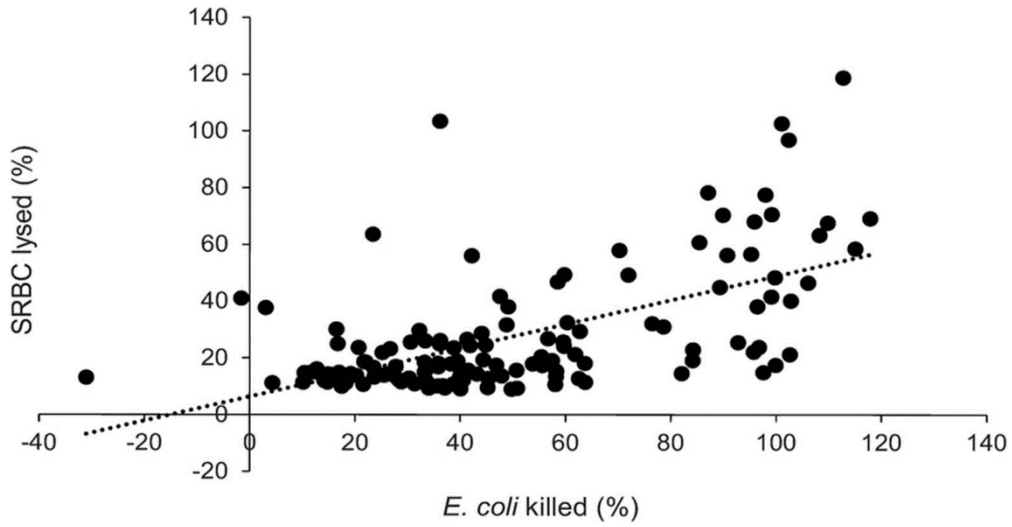

Correlation between bacteria-killing ability and hemolytic-complement activity, two measures of constitutive innate immune defense.

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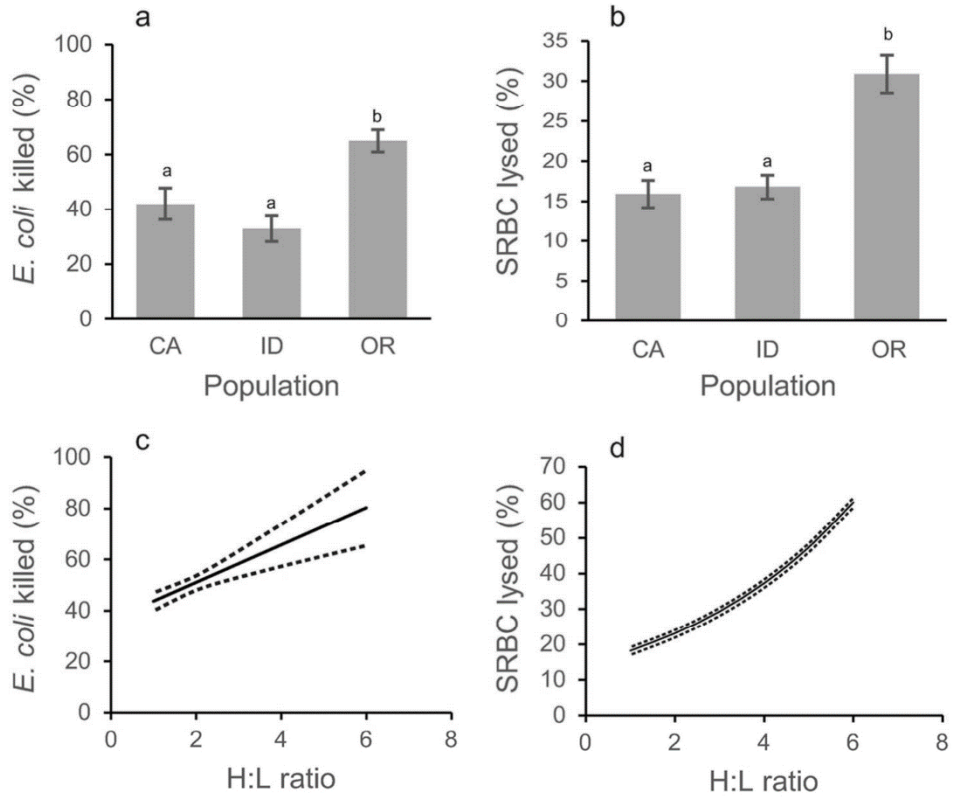

Model-estimated mean ± SE of (a) percent of bacteria killed and (b) hemolytic-complement activity for samples from different regions, and the relationship between (c) percent of bacteria killed and (d) hemolytic-complement activity with heterphile:lymphocyte (H:L) ratio. Statistical models for hemolytic-complement activity were performed on $\log_{10}$-transformed percent SRBC lysed, but model-derived, back-transformed data are presented in the figures. Different letters indicate a difference at $\alpha = 0.05$.

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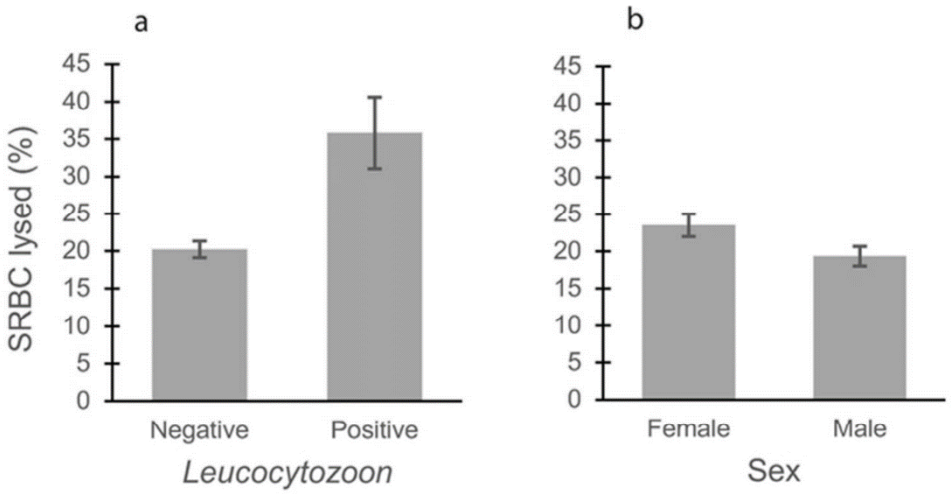

Model-estimated mean ± SE hemolytic-complement activity by (a) *Leucocytozoon* infection and (b) sex. Error bars represent model estimated standard errors and different letters indicate a significant different at α = 0.05. Statistical models for hemolytic-complement activity were performed on log10-transformed percent SRBC lysed, but model derived, back-transformed data are presented in the figures.

59x30mm (300 x 300 DPI)