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RESEARCH ARTICLE

Photoperiod but not food restriction modulates innate immunity in an opportunistic breeder, *Loxia curvirostra*

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ABSTRACT

An organism's investment in immune function often varies seasonally but understanding of how fluctuations in environmental conditions directly modulate investment remains limited. This experiment investigated how changes in photoperiod and food availability affect investment in constitutive innate immunity and the acute phase response induced by lipopolysaccharide (LPS) injections in captive red crossbills (*Loxia curvirostra*). Crossbills are reproductively flexible songbirds that specialize on an unpredictably available food resource and display temporal variation in immunity in the wild. Birds were separated into four treatments and exposed to long or short day lengths for 6 weeks before continuing on an *ad libitum* diet or experiencing a 20% food reduction for 10 days. Birds were un-injected or injected with LPS both before and after diet change. Innate immunity was quantified throughout the experiment to assess effects of photoperiod, food availability and their interactions on hemolysis-hemagglutination, haptoglobin, bacterial killing ability and leukocyte counts. Overall, increasing day length significantly increased both bacterial killing ability and leukocyte counts. Surprisingly, food restriction had little effect on the immune parameters, potentially owing to the 'low-cost' environment of captivity and suggesting that investment in innate immunity is prioritized and maintained whenever possible. LPS injections induced stereotypical sickness behaviors and increased bacterial killing ability in short day birds and complement activity (hemolysis) both before and after food restriction. These results demonstrate robust seasonal modulation of immune investment and an ability to maintain innate immunity in the face of limited resources in these temporally flexible songbirds.

KEY WORDS: Ecoimmunology, Food restriction, Innate immunity, Lipopolysaccharide, Photoperiod, Red crossbill

INTRODUCTION

Natural selection dictates that multiparous organisms should balance energy allocation to both reproduction and survival-related processes to maximize fitness (King, 1974). Maintaining immune function is critical to survival in that the immune system detects pathogens and contains infection; however, its maintenance can be costly (e.g. Hasselquist et al., 2001; Martin et al., 2003). The immune system varies in the costs of maintenance and use, in that some components are more costly to maintain than others (Klasing, 2004). Broadly, the immune system can be categorized along an innate (nonspecific)-acquired (specific) axis and a constitutive (non-induced)-induced axis (Schmid-Hempel and Ebert, 2003). The constitutive or baseline responses require low energy investment, while induced responses such as an induced innate or acute-phase response often involving fever and inflammation are considered particularly demanding to initiate (Klasing, 2004). Owing to these potential energy costs, investment in immunity is often variable (Martin et al., 2008).

The state of an organism's energy budget is strongly influenced by the external environment. Throughout the annual cycle, organisms are exposed to seasonal variation in weather, resource availability and disease potential (King, 1974; Nelson et al., 2002). In predictable temperate environments, organisms use day length (photoperiod) as an initial predictive cue (Wingfield, 1983) to time investment in physiological processes to coincide with upcoming fluctuations in resources and changes in energy demand (Nelson et al., 1990). There have been many studies on seasonal variation in immunity in a variety of vertebrate taxa (reviewed in Adelman et al., 2013; Lee, 2006; Martin et al., 2008; Nelson and Demas, 1996; Nelson et al., 2002). Studies investigating the effects of photoperiod on changes in immune activity found that mammals decrease immune activity during the summer months (long days) and increase investment during the winter months (short days). This winter upregulation has been hypothesized to counteract environmentally induced immunosuppressive effects caused by challenges such as inclement weather and low food availability that occur during winter (Nelson and Demas, 1996; Sinclair and Lochmiller, 2000). In birds, both cellular and humoral immunity have been reported as higher during the winter or show no change when compared with summer (Adelman et al., 2013; Lee, 2006; Martin et al., 2008). In non-migratory great tits (*Parus major*), total immunoglobulin and heterophil levels were highest during the summer breeding months (Pap et al., 2010). Lymphocyte levels, however, were higher in the winter than in the summer. In skylarks (Hegemann et al., 2012), metrics of constitutive innate immunity (complement, natural antibody and haptoglobin levels) varied inter-annually but were consistently lower during fall migration than during the breeding season, suggesting that annual variation in environmental conditions contributed to variation in immunity. Overall, there appears to be considerable variation of seasonal effects on immunity in birds, which depends on the species and immune parameter sampled.

Prior studies on the effects of energy constraint on immune parameters have generally found that restrictions in food consumption resulted in decreases in lymphocyte-mediated responses in chickens (Hangalapura et al., 2005), yellow-legged gulls (*Larus cachinnans*) (Alonso-Alvarez and Tella, 2001) and deer mice (*Peromyscus maniculatus*), (Martin et al., 2007).

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However, there are several studies in which food restriction had little effect on constitutive innate and adaptive immunity, as demonstrated in Siberian hamsters (*Phodopus sungorus*) (Zysling et al., 2009) and red knots (*Calidris canutus*), although red knots did reduce the more demanding acute-phase response (Buehler et al., 2009).

Red crossbills (*Loxia curvirostra* Linnaeus 1758) are temperate zone songbirds that display exceptional temporal flexibility of breeding, despite highly seasonal changes in weather (Adkisson, 1996), primarily owing to their morphological specialization on conifer seeds, which are erratically available both spatially and temporally (Fowells, 1968; Koenig and Knops, 2000). In years and areas with large cone crops, these birds can breed approximately 10 months of the year, even when conditions are thermally challenging and day lengths are short (Benkman, 1987, 1990; Hahn, 1998). The timing and balance of energy investment in physiological processes is therefore strongly influenced by changes in food supply; however, it is not the only cue used to time their annual schedule – both photoperiod and social cues influence the timing and initiation of breeding (Hahn, 1995, 1998; Hahn et al., 1997, 1995). While previously considered to be completely flexible in terms of their annual cycle, crossbills are now considered to be 'seasonal opportunists', because while they exhibit a higher degree of behavioral flexibility when compared with seasonal migrants, they do exhibit seasonal cycles of reproduction, molt and migratory physiology that cannot be tied solely to changes in food supply in a proximate sense (Cornelius et al., 2012; Cornelius and Hahn, 2012; Hahn, 1995; Hahn et al., 2008).

The purpose of this experiment was to examine whether changes in innate immune function in red crossbills are mediated by a direct response to photoperiod and/or are affected by food restriction. Prior to the experiment, four years of field data on free-living crossbills demonstrated that, like many vertebrates, their investment in immunity varies by season and year sampled, with the highest immune activity occurring in the summer months and when conifer seeds are abundant and ambient temperatures are high (Schultz, 2015). These results revealed that food availability and temperature significantly predict these patterns. However, it is difficult to determine what effects either photoperiod or changes in food availability, alone, have on immune investment, without experimental manipulations.

To test whether photoperiodic changes and/or food restriction affect investment in innate immunity, captive crossbills were either exposed to long day lengths (LD) or short day lengths (SD), and were either fed *ad libitum* diets or restricted to 80% of their average daily food intake. This experiment tests the hypothesis that photoperiod and food availability have significant effects on immune activity, given that free-living crossbills exhibited the highest immune activity when cone crops were large and days were long (Schultz, 2015). Thus, we expected that the treatments would have additive effects with birds on LDs and provided *ad libitum* food having the highest immune activity and, conversely, birds on SDs and experiencing food restriction having the lowest immune activity. In addition to quantifying changes in constitutive innate immunity, we aimed to investigate both how these experimental conditions may affect investment in the systemic acute-phase response to inflammation by injecting a subset of birds with lipopolysaccharide (LPS) and how induction of the acute-phase response may affect other immune measures. Because the induction of the acute-phase response can increase investment in other immune parameters in other avian species (Buehler et al., 2009; Millet et al., 2007), we predicted that LPS-injected birds would have higher overall constitutive immune activity than control birds, with food restriction dampening the effect in injected birds because of energy limitations.

MATERIALS AND METHODS

Study animals and pre-experimental conditions

We captured 38 red crossbills (*L. curvirostra*) of vocal types 2 and 4 (Groth, 1993) with mist nets near Naches, WA, USA (46°50′34.86″N, 120°59′31.70″W, elevation: 725 m) (n=19), and near Sisters, OR, USA (44°22′55.89″N, 121°35′56.48″W, elevation: 1081 m) (n=19) in September and October 2013. Red crossbill vocal 'types', of which there are 10, differ in body size, bill morphology, foraging performance on different conifer species (Benkman, 1993) and habitat (conifer) preference (Groth, 1993; Kelsey, 2008). Captured crossbills were coexisting in mixed ponderosa pine (*Pinus ponderosa*) and Douglas fir (*Pseudotsuga menziesii*) forests. Birds were transported to avian housing facilities at the University of California, Davis (Davis, CA, USA), shortly after capture, approximately 4 months prior to the start of the experiment. Both sexes (30 males, 8 females) were used and balanced across treatments. Birds were housed indoors at 21–23°C in large aviaries approximately 1.5 m wide, 2.43 m long and 2.1 m tall on a naturally declining photoperiod (light timers set to Davis, CA, latitude adjusted the room's lighting schedule daily to match seasonal decline in day length) until 21 December 2013. They were then moved to individual cages in acoustic isolation chambers (IAC 250 'Mini' Sound Shelters, 61 cm wide by 86 cm deep by 168 cm high inside dimensions; Industrial Acoustics Company, Bronx, NY, USA) and kept on a fixed photoperiod of 9 h 28 min of light, 14 h 32 min of dark corresponding to the winter solstice day length in Davis, CA (lights on: 07:20 h, lights off: 16:48 h). We housed each bird next to one neighbor of the same sex that they could see and hear, with each chamber housing a range of four to six individual birds. Additionally, we fed birds *ad libitum* amounts of Roudybush Small Maintenance Diet (pellet food) and 1.5 g of sunflower hearts daily. To measure changes in innate immunity in response to changes in photoperiod and food restriction, we used a repeated-measures design and birds were sampled prior to any changes in treatment (pre-sample), 3 weeks after photoperiod treatment, and prior to and after food restriction. All capture, handling and experimental protocols were approved by the University of California Davis Institutional Animal Care and Use Committee (protocol no. 17931).

Experimental design

Birds (total n=38) were initially separated into four treatment groups, which were staggered across the course of the experiment (Fig. 1). All treatments were balanced for sex, age at capture, vocal type and structural size (mass corrected by regression for tarsus length).

Photoperiod treatment

At day 1 of the experiment, birds were either advanced to long-day photoperiod (14 h 52 min light, 9 h 8 min dark; lights on: 05:41 h, lights off: 20:33 h, n=20) or remained on short-day photoperiod (9 h 28 min of light, 14 h 32 min of dark; lights on: 07:20 h, lights off: 16:48 h, n=18) for the duration of the experiment, 52 days total. Birds were divided among eight sound chambers (four chambers on LD, four on SD), with four to six birds per chamber.

Food treatment

At day 42 of the experiment, half of the birds in each photoperiod treatment (10 LD, 8 SD) experienced a 20% reduction in their daily average intake of pellets (calculated based on 5 days of daily intake

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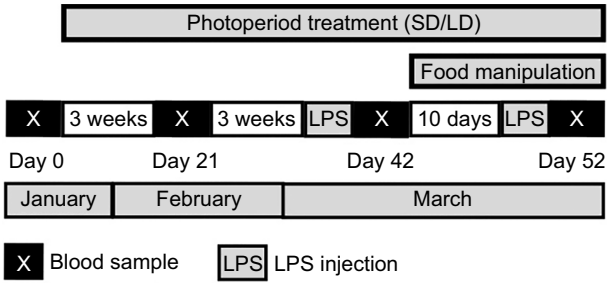

Fig. 1. Experimental setup. All birds (n=38) were kept on short day (SD) lengths for 33 days until a baseline blood sample was taken at day 0. Half of all birds were advanced to long day (LD) lengths for the remaining duration of the experiment. Lipopolysaccharide (LPS) injections occurred prior to and after the food manipulation (20% food reduction) for 10 days. After baseline sampling, blood samples were taken at day 21 (3 weeks post photoperiod change), day 42 (post first LPS injection) and day 52 (post second LPS injection and food restriction).

in grams; 1.5 g of sunflower hearts were still given to all birds) for a period of 10 days, after which birds were sampled and returned to an *ad libitum* diet. Within each chamber, birds on the same shelf experienced the same food treatment (both restricted or both *ad libitum*) to minimize across-treatment social effects that might influence stress physiology or behavior (Cornelius et al., 2010). At the start of the food restriction, all birds were weighed to the nearest 0.1 g, and subsequently weighed four times afterwards (days 44, 46, 49 and 51; when weighing food and birds, chambers were on average open for 14 min, mode: 5 min, range: 3–32 min) in order to calculate mass loss and to ensure that food-restricted birds specifically were not losing more than 20% of body mass as stipulated by IACUC policy.

Morphology measurements

We scored body mass, abdominal fat and reproductive measures 2 days prior to the first and second blood sampling (day 2, day 19) for all birds, and the day prior to the third and fourth blood sampling (day 41, and day 51; see Fig. 1). These measurements were staggered to match blood sampling, i.e. 22 birds were measured the first round and 16 birds were measured the second round (see blood sampling methods below). Further, because accurate masses and handling were required prior to LPS injections (see below), we took morphology measurements the afternoon before blood was collected between 15:00 and 16:30 h (mean time for chambers open was 12.71 min, range: 8–18 min). We measured body mass to the nearest 0.1 g using a Pesola spring scale and furcular and abdominal fat were scored on a scale from 0 (no fat) to 5 (bulging) (Helms and Drury, 1960; Nolan and Ketterson, 1983).

To estimate reproductive status, we measured cloacal protuberance length in males and incubation patch appearance in females. Three weeks post experimental end, these birds were euthanized and total testes volume (mm^3) and ovary mass (g) were measured and found to be poor predictors of cloacal protuberance lengths and incubation patch scores in these birds (Pearson correlation: total testes volume: r=−0.17, P=0.377, n=28, ovary mass: r=0.3, P=0.4675, n=8), and so were not used as measures of reproductive condition in this study.

Blood sampling

Prior to sampling, the area around the bird's alar (wing) vein was swabbed with an alcohol prep-pad and a sterile 26-gauge needle was used to puncture the vein to collect 300 µl of blood into pre-sterilized heparinized microhematocrit capillary tubes. All blood samples were taken between 08:30 and 10:30 h (LD birds lights on: 05:41 h, SD birds lights on: 07:20 h) within 13 min of opening the door of the isolation chamber, with all birds per chamber being bled at the same time. Owing to logistical constraints and to minimize handling and blood processing times, at each experimental time point we sampled birds across two days, with 22 birds bled on the first day and 16 birds bled on the second day. Samples were kept on ice for no more than 2 h prior to centrifuging, where plasma was separated from cellular components and immediately stored at −20°C until immune assays were performed.

Measuring immune function

Complement and natural antibodies

Utilizing the protocol outlined in Matson et al. (2005), levels of plasma complement activity were determined by red blood cell lysis and non-specific natural antibodies were determined by red blood cell agglutination. Half scores were used to indicate partial lysis and agglutination. Samples were randomized across plates and scored blind to individual and sampling date by one observer (E.M.S.). A chicken plasma standard was run on all plates in duplicate. Ten microliters of plasma were used instead of the 25 µl described in the protocol to accommodate small blood sample volumes, and volumes of all reagents were adjusted accordingly. Samples were run in one batch in June 2014.

Bacterial killing ability

Following the basic procedure described in Millet et al. (2007) the capacity of fresh, whole blood to inhibit the growth of *Escherichia coli* (ATCC 8739) *ex vivo* was measured. Blood samples used for this assay (approximately 50 µl) were taken within 5 min of a chamber being opened to minimize the effects of elevated glucocorticoids (Matson et al., 2005, 2006; Millet et al., 2007) and processed through the assay within 1 h of collection. For each sample, the blood was diluted 1:6 in CO_2-independent medium (Invitrogen 18045-054) containing 4 mmol l^{-1} L-glutamine (Sigma G3126) to a volume of 100 µl, and 10 µl of microbe suspension was added from reconstituted lyophilized pellets (Microbiologics, St Cloud, MN, USA).

PIT54 (haptoglobin)

To quantify PIT54 (the avian acute-phase protein analog to mammalian haptoglobin) concentrations in the plasma, we used a commercially available colorimetric assay kit (TP801; Tri-Delta Diagnostics, NJ, USA), as was done in Millet et al. (2007). The protocol was modified by using 5 µl of plasma rather than the 7.5 µl the kit suggests; all reagents were adjusted accordingly, as was done in Zylberberg et al. (2012).

Circulating cellular immunity (leukocyte counts)

The quantity and type of leukocytes circulating in the blood as detected from prepared blood smears can indicate overall health and/or the present inflammatory status of the individual (Campbell, 1995). We collected a drop of blood from a baseline sample that was then smeared onto each glass slide, allowed to air-dry, fixed with 100% methanol and stained with Wright–Giemsa (Cambridge Diagnostic Camco Stain Pack). Smears were examined under 1000× magnification with oil immersion and were scored by one observer (E.M.S.). We detected leukocytes, including lymphocytes, heterophils, monocytes, eosinophils and basophils, based on the methods in Campbell (1995), across approximately 10,000 erythrocytes or 100 microscope 'fields'. The fields examined

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were counted evenly across the slide's surface to avoid oversampling. To account for changes in hematocrit (see below) during the study, we reported the number of leukocytes per volume (ml) of blood by multiplying hematocrit volume by total leukocyte number per approximate erythrocyte count.

Acute-phase response

The acute-phase response is induced when potentially dangerous microbes are encountered and recognized by macrophages and dendritic cells, which release pro-inflammatory cytokines causing local inflammation and later systemic inflammation (Klasing and Leshchinsky, 1999; Owen-Ashley and Wingfield, 2007). This response is also characterized by decreased locomotor and social activities, anorexia and fever (Janeway et al., 2004; Klasing and Leshchinsky, 1999). We induced the acute-phase response twice in the same individuals: first on day 41 (Fig. 1) of the experiment after birds had been exposed to the photoperiod treatment for approximately 6 weeks, and second on day 51 after approximately 7.5 weeks of the photoperiod treatment and 10 days of the food treatment. We injected birds (n=19) with a mass-dependent dose of 0.1 mg ml^{-1} (1 mg kg^{-1} body mass) LPS (L7261 Sigma, source strain *Salmonella typhimurium* ATCC 7823) in phosphate buffered saline (P3813, Sigma) intramuscularly (pectoralis major). This dosage was identical to that used to elicit the acute-phase response in house finches (*Haemorhous mexicanus*) (Toomey et al., 2010), zebra finches (*Taeniopygia guttata*) (Burness et al., 2010) and white-crowned sparrows (*Zonotrichia leucophrys gambelii*) (Owen-Ashley et al., 2006). We did not inject control birds (n=19) but they received the same handling procedure as injected birds – this is the most appropriate control treatment in this experiment because it minimizes the general inflammatory effects of breaking skin and other tissues through injection in the control birds (Koutsos and Klasing, 2001).

Following the general timing used in previous studies (Buehler et al., 2009; Matson et al., 2005; Millet et al., 2007), we collected blood samples to assess innate immunity between 08:30 and 10:30 h, 17 h after LPS injections given between 15:00 and 16:45 h the previous day. Additionally, we placed video cameras outside the chambers between 07:30 and 08:30 h both 8 h prior to injection and 16 h post injection to record sickness behaviors for 30 min through the window of the chamber. Because each bird on a shelf was either injected with LPS or not injected, we separated the pair with a cardboard divider 24 h before and during filming to minimize any social and behavioral effects of having a bird that appeared to be sick nearby (Zylberberg et al., 2013). Time budgets for feeding, drinking, self-care (preening) and activity (hops, short flights or movement around the cage) were scored as the number of seconds each activity was performed divided by the total number of seconds each bird was observed by one observer (H. Fakhri), who was blind to sampling day and treatment. Time not spent on these activities was categorized as resting behavior. To determine whether LPS injections affected these behaviors, we calculated the change in each behavior by subtracting the time devoted to a specific behavior after LPS injections from the time devoted to that behavior before LPS injections. Additionally, to determine whether photoperiod and/or food treatment affected the overall time budget of these behaviors, we examined treatment effects within each sampling time before and after each LPS injection.

Hematocrit

We measured hematocrit by centrifuging 300 µl of blood in capillary tubes for 10 min at 10,000 rpm in an IEC clinical centrifuge and measuring percent packed cell volume. Individual hematocrit scores were based on the averaged measurements from approximately five capillary tubes per individual.

Statistical analysis

Data were analyzed in R (version 3.1.1-3.3.0) (R Core Team, 2016) using linear mixed models (LMM) and generalized linear mixed models (GLMM) via lmer and glmer from the lme4 package (version 1.1-7-1.1-12). Overall, we tested the effects of photoperiod, food restriction and LPS injections on each of the five immune measures as well as on mass, hematocrit and changes in behavior post induction of the acute-phase response. To do this, we created a series of models that were compared using Akaike's information criterion for small sample sizes (AICc) in conjunction with calculated Akaike weights (w_i), which calculate the relative likelihood of each model compared given the data (Burnham and Anderson, 2002).

Specifically, for each response variable, we compared null models (intercept only) with those models containing treatment variables (photoperiod, LPS injection, food availability) as both main and all two-way and three-way interactive effects. Each model also included a random effect for bird ID to account for the repeated measures on each individual and a random effect for blood sampling date when it improved the fit of the model. The distribution of hemagglutination, hemolysis (complement) scores and circulating white blood cell count data best fit a log-normal error distribution, whereas all other measures were fit using a normal (Gaussian) error distribution. We calculated coefficient estimates, standard errors and 95% confidence intervals (CI) using the summary and confint function in the base and stats packages in order to determine the effect of each predictor and its precision. When CIs included zero, the parameter estimates were statistically non-significant and had minimal effect on the immune and behavior parameters. We considered models with a ΔAICc <2 from the lowest model score to be statistically supported or equivalent (Richards, 2005) and averaged the estimates from equivalent models using the model.avg function in MuMIn package. For all immune parameters, models were compared that included reproductive measures, sex, age at capture, vocal type, mass and fat scores the day prior to bleeding, chamber number and cage position. These models had low AICc w_i and never changed model outcomes.

Some microbial killing scores were not included in the analysis (n=3, day 0; n=22, day 42) owing to contamination of media. Because of small blood volume, complement and natural antibodies, leukocyte counts, hematocrit and PIT54 concentration were not measured for one individual at day 21.

The list of generalized linear models ranked by AICc score, as well as model parameter estimates from best-supported GLMMs are included in Tables S1 and S2, respectively.

RESULTS

Effects of photoperiod treatment

Morphology and hematocrit

Photoperiod treatment was included in the best-fit model predicting mass (ΔAICc=3.6). Overall, birds held on SDs weighed on average 2.01 g more (5.64% of total body mass) than those birds advanced to LDs (estimate: 2.58, CI: 1.04–4.12). Best-fit models predicting hematocrit also contained photoperiod treatment (ΔAICc=6.4). Birds held on SDs had on average 2.45 and 2.05 lower % hematocrit than those advanced to LDs at 3 and 6 weeks, respectively (estimate: −1.69, CI: −3.42 to −0.62; Fig. 2A).

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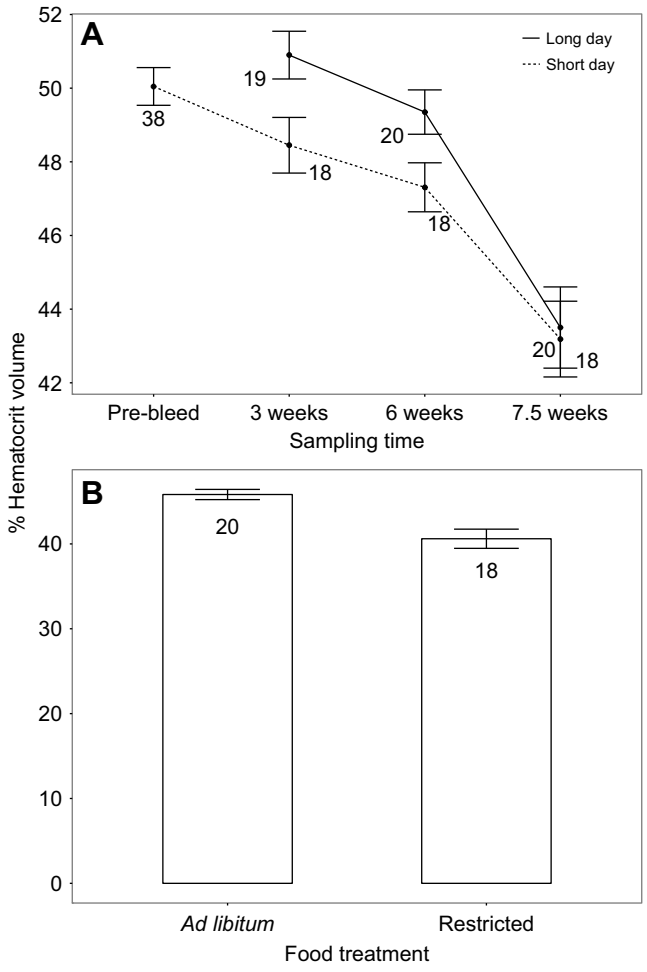

Fig. 2. Effects of photoperiod treatment and 20% food restriction on hematocrit in red crossbills. (A) Photoperiod treatment; (B) food treatment. Hematocrit was higher in crossbills advanced to long days at day 21 (3 weeks) and day 42 (6 weeks); on sampling day 52, food-restricted birds had lower hematocrit than those birds on *ad libitum* diets. Error bars are ±s.e.m. Sample sizes are given in each panel.

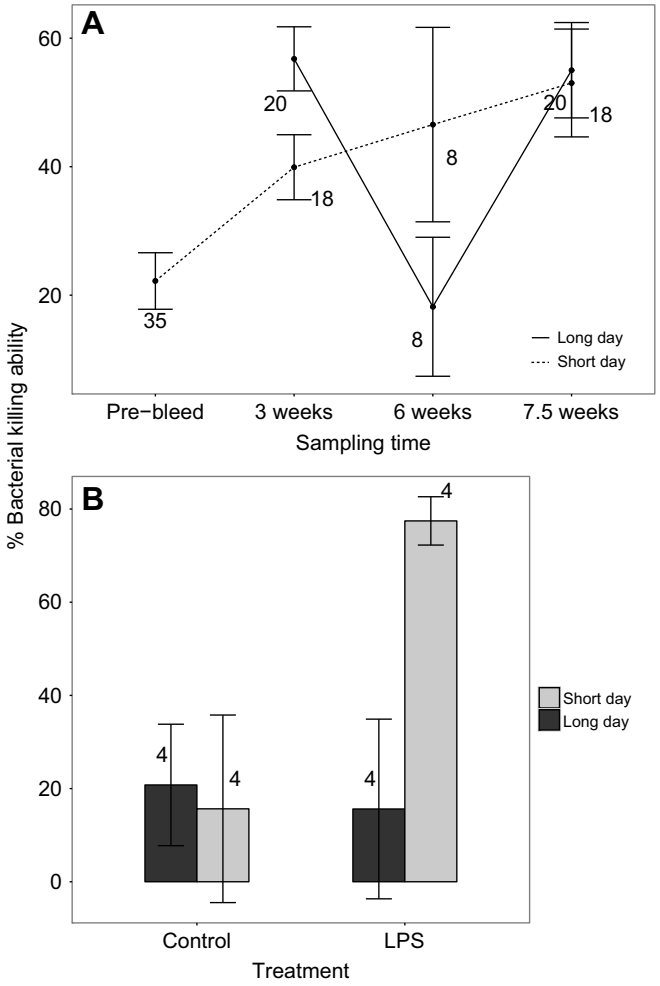

Fig. 3. Effects of photoperiod treatment and photoperiod×LPS treatment on bacterial (*Escherichia coli*) killing ability in red crossbills. (A) Photoperiod treatment; (B) photoperiod×LPS treatment. Bacterial killing ability was higher in crossbills advanced to long days on day 21 (3 weeks) but when birds were injected with LPS on day 42 (6 weeks) killing ability was only higher in short day birds. Error bars are ±s.e.m. Sample sizes are given in each panel. Owing to media contamination, 22 data points were excluded on day 42.

Innate immunity

The best-fit model predicting *E. coli* killing ability (ΔAICc=3.7) and leukocyte counts (ΔAICc=5.0) contained photoperiod treatment. Birds held on SDs had on average 16.80% lower *E. coli* killing ability than those advanced to LDs after 3 weeks post photoperiod change (estimate: −0.13, CI: −0.26 to −0.01; Fig. 3A), and lower average % leukocytes per blood volume by 17.38 and 7.04 after 3 and 6 weeks post photoperiod change, respectively (estimate: −0.63, CI: −0.88 to −0.39; Fig. 4A). Additionally, photoperiod treatment and LPS injection had an interactive effect on *E. coli* killing ability. SD birds injected with LPS on day 42 had on average 60.10% higher killing ability when compared with controls, whereas LPS injections had no effect for those birds on LDs (estimate: 1.12, CI: 0.51–1.75; Fig. 3B). Hemolysis, hemagglutination and haptoglobin scores were not significantly affected by photoperiod (ΔAICc was <2 between null models or null models outperformed models containing photoperiod treatment).

Sickness behavior

While photoperiod treatment was included in the best-fit models as both a main and interactive effect predicting changes in activity (ΔAICc=22.0), resting (ΔAICc=23.4) preening (ΔAICc=7.5) and feeding (ΔAICc=17.2), none of these estimates were significant. However, in general, birds on SDs were less active and preened less, and spent more time resting and feeding. Additionally, photoperiod did not affect the total time spent active, resting, preening or feeding at any of the experimental time points (ΔAICc was <2 between null models or null models outperformed models containing photoperiod treatment).

Effects of food and LPS treatment

Morphology and hematocrit

Food and LPS treatment terms were contained within best-fit models predicting mass (ΔAICc=3.6) and hematocrit (ΔAICc=6.4); however, only food restriction significantly impacted mass and hematocrit. Food restriction had negative effects on both mass and hematocrit (mass: estimate: −2.33, CI: −4.24 to −0.43; hematocrit: estimate: −5.04, CI: −7.28 to −2.80, Fig. 2B), reducing the average mass by 2.54 g (7.34% of total body mass) and hematocrit by 5.21%. LPS injections were included in best-fit models and had predicted positive effects on mass (estimate: 0.64, CI: −2.59 to 5.53); however, the estimate was non-significant. Additionally, an interaction between food treatment and photoperiod was included in best-fit

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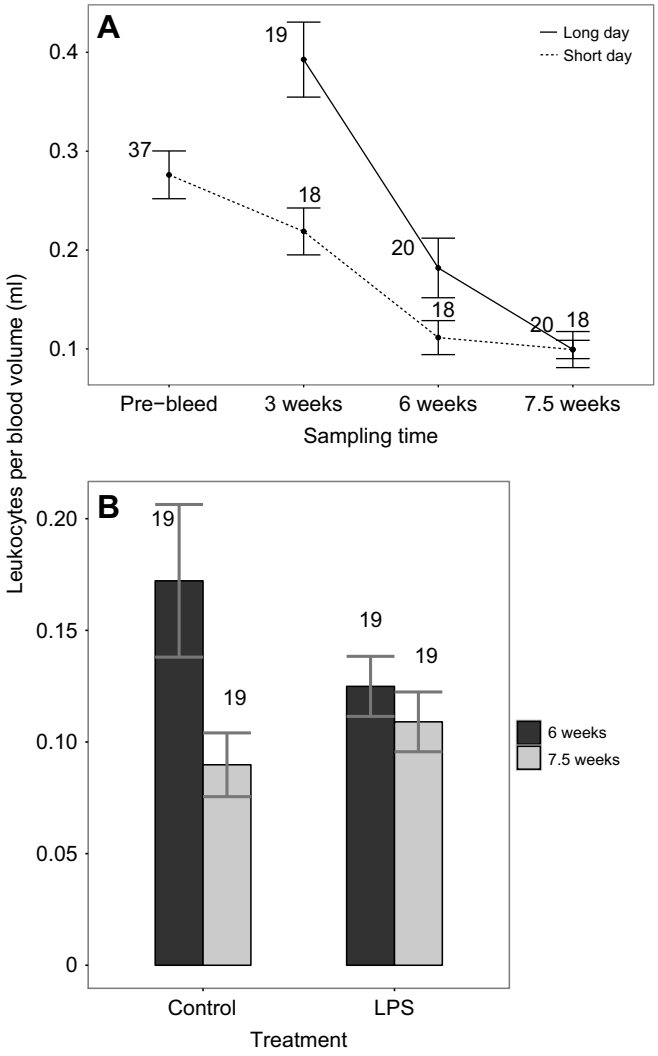

Fig. 4. Effects of photoperiod treatment and LPS treatment on leukocyte counts per blood volume (ml) in red crossbills. (A) Photoperiod treatment; (B) LPS treatment. Leukocyte counts were higher in crossbills advanced to long days at day 21 (3 weeks) and day 42 (6 weeks; A) and lower in birds injected with LPS at day 42 (6 weeks; B) (significant interaction between sampling time and LPS treatment, GLMM: LPS×sampling date estimate: 0.85, CI: 0.45–1.25). Error bars are ±s.e.m. Sample sizes are given in each panel.

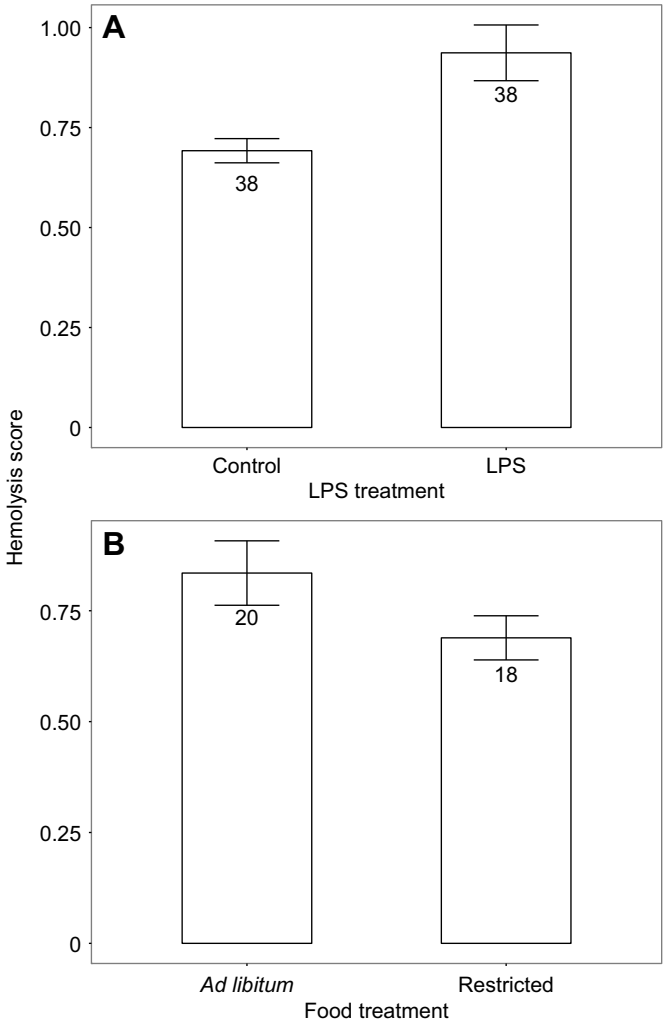

Fig. 5. Effects of LPS treatment and food restriction on hemolysis scores in red crossbills. (A) LPS treatment; (B) food treatment. Hemolysis scores were higher in crossbills injected with LPS but lower in food-restricted birds. Bars represent group means taken from sampling on days 42 and 52 (no significant interaction between LPS and sampling date on hemolysis) (A) and sampling day 52 (B), respectively. Error bars are ±s.e.m. Sample sizes are given in each panel.

models predicting hematocrit, but estimates were non-significant (estimate: 0.46, CI: −1.24 to 4.17). In contrast, LPS injections had predicted negative effects on hematocrit (estimate: −0.68, CI: −5.55 to 2.97), with an interaction parameter between food restriction and LPS injection (estimate: −1.11, CI: −16.05 to 2.44), although the estimates were non-significant.

Innate immunity

The best-fit model for hemolysis (complement) scores contained LPS treatment (ΔAICc=2.0) and had a positive effect (estimate: 0.49, CI: 0.10–0.89; Fig. 5A); the average hemolysis scores of LPS-njected birds were 0.25 units higher than controls at both experimental time points (days 42 and 52). The best-fit model predicting *E. coli* killing ability contained LPS injection as a main effect as well as an interactive effect of photoperiod and LPS (ΔAICc=3.7). LPS injection alone and its interaction with photoperiod were both positive (LPS: estimate: 0.53, CI: 0.03–0.98; LPS×photoperiod: estimate: 1.12, CI: 0.51–1.75; Fig. 3B), with an average 37.64% higher killing ability in birds injected with LPS at both time points, and a 60.10% higher average among birds injected with LPS the first time (day 42) and held on SDs (estimate: 1.12, CI: 0.51–1.75; Fig. 3B). This result suggests that the LPS treatment increased *E. coli* killing during SDs and had no effect during LDs. The best-fit model predicting leukocyte counts contained LPS treatment as a main effect (ΔAICc=5.0), with the parameter estimates revealing negative effects (estimate: −1.20, CI: −2.09 to −0.31). The interaction between sampling date and LPS treatment was significant, however (estimate: 0.85, CI: 0.45–1.25), indicating that LPS injections decreased average crossbill % leukocyte concentration by 4.73 at after the first injection (experimental day 42) but not after the second (day 52) (Fig. 4B).

Food restriction, which was contained within the best-fit model predicting hemolysis scores (ΔAICc=2.2) and leukocyte counts (ΔAICc=5.0), reduced average hemolysis scores by 0.15 units (estimate: −0.10, CI: −0.39 to 0.04; Fig. 5B) and average leukocyte concentrations by 1.04% (estimate: −0.25, CI: −0.84 to 0.35); however, in both cases the estimate was not significant. For

hemagglutination scores (natural antibodies) and haptoglobin concentration, models containing either LPS or food treatment were statistically equivalent to null models (ΔAICc<2).

Sickness behavior

LPS injections and food restriction, as both main and interactive effects, were included in best-fit models predicting changes in activity (ΔAICc=11.5), resting (ΔAICc=11.5), preening (ΔAICc=2.5) and feeding behavior (ΔAICc=7.8); however, estimates were not significant. In general, LPS injections and food restriction increased resting behavior and caused birds to spend less time preening and more time feeding compared with control birds. Additionally, activity tended to decrease in response to LPS injections but increased in response to food restriction. Food restriction had a positive main effect on time spent active on day 52 of the experiment (model output not shown, AICc w_i=0.83, estimate: 14.60, CI: 0.69–28.50), suggesting that food restriction alone was sufficient to elevate activity rate in crossbills.

DISCUSSION

The goal of this study was to examine the effects of changes in photoperiod and food restriction on five measures of innate immunity in a reproductively flexible songbird, the red crossbill. Overall, crossbills that were advanced to LDs exhibited lower mass, but higher hematocrit, *E. coli* killing ability and leukocyte counts. However, LDs markedly blunted their ability to increase bacterial killing ability following LPS challenge compared with SDs. Changes in photoperiod had no significant effect on haptoglobin concentration, levels of natural antibodies and complement, or sickness behavior as measured by time spent active, resting, preening, feeding and drinking. Limiting crossbills to 80% of their *ad libitum* daily intake of pellet food significantly reduced mass and hematocrit but had little detectable effect on the immune parameters measured, suggesting that these immune parameters had higher priority than maintaining other tissues. Food restriction had no significant effects on sickness behavior; however, it did significantly enhance overall activity levels. Crossbills injected with LPS had higher levels of complement and *E. coli* killing ability (but only those on SDs), and lower leukocyte counts. The hepatic secretion of many protective proteins increases during the acute-phase response induced by LPS and they are likely responsible for the increased *E. coli* killing ability.

Seasonality of crossbill immunity

Because crossbills modulated some parameters of immune activity in response to changes in photoperiod in the absence of other environmental cues (no changes in food availability or social conditions), it can be inferred that changes in day length are acting as a strong predictive cue in modulating a crossbill's investment in components of innate immunity. Additionally, in this study, changes in energy allocation resulting from reproductive investment that accompany increases in day length are likely minimal, although not non-existent – the photoperiod treatment had no significant effects on any of the reproductive measures taken (the model containing reproductive measures was statistically equivalent with the null model; data not shown), nor did any of the birds exhibit incubation patches or large cloacal protuberance lengths greater than 5 mm, indicative of high reproductive potential (Cornelius et al., 2012), during the study. Measurement of male testes volume 22 days post experimental end revealed that LD males exhibited larger testes than those of SD males (LD mean right testis volume: 22.57 mm^3; SD mean right testis volume: 8.58 mm^3), suggesting that increasing day lengths did affect gonadal growth in males. However, these gonad sizes were not indicative of reproductively competent males (Hahn, 1995, 1998; Tordoff and Dawson, 1965).

In corroboration with previous studies that have found crossbills to maintain seasonal cycles of migratory physiology, gonadal development and plumage molt despite their persistent reputation as opportunistic breeders with complete reproductive flexibility (Cornelius et al., 2012; Cornelius and Hahn, 2012; Hahn et al., 2008), it appears that crossbills also maintain seasonal patterns of immune activity in the absence of other local predictive cues such as food availability. Hematocrit, which reflects oxygen transport capacity in the blood and often increases in anticipation or as the result of higher energy demand (e.g. migration, winter thermoregulatory challenges) (Maina, 2000; Schmidt-Nielsen, 1997), increased in response to increases in day length. Previous studies on captive red crossbills that were kept on natural day lengths and *ad libitum* food found the highest hematocrit levels in the late spring and early summer (Hahn, 1995). For crossbills, the abrupt change in photoperiod from short to very long day lengths likely acted as an anticipatory cue to prepare for the typical nomadic migration or movement that occurs in spring to locate areas with good cone crops (Adkisson, 1996; Cornelius and Hahn, 2012).

LPS effects on innate immunity

The injection of LPS increased *E. coli* killing ability at both experimental time points but a stronger effect was seen in SD birds that were also injected (Fig. 3B). Increases in *E. coli* killing ability in response to LPS injections has also been documented in red knots (Buehler et al., 2009) and chickens (Millet et al., 2007). Hemolysis scores, or what is correlated to complement-mediated lysis and opsonization (Matson et al., 2005), also increased in response to LPS injections at both experimental time points (day 42 and 52). This innate immuno-enhancement in response to LPS injections is expected: injected LPS isolated from Gram-negative bacteria increases pro-inflammatory cytokine and acute-phase protein production, which in turn increases phagocytic and thus bacterial killing ability in the blood (Bliss et al., 2005; Leshchinsky and Klasing, 2001). In contrast, LPS injections decreased crossbill leukocyte counts at experimental day 42 but not day 52. This difference between injections of LPS into naive versus previously exposed birds is likely due to differing responses, with the naive birds having a primarily inflammatory response and the previously exposed birds having high levels of anti-LPS immunoglobulins that would blunt the inflammatory response. This inflammatory response results in movement of leukocytes out of the blood and into affected tissues (Dhabhar and McEwen, 1997).

Neither hemagglutination (natural antibodies) nor haptoglobin (PIT54) significantly increased in individuals injected with LPS. Given that natural antibodies have relatively low developmental, maintenance and use costs (Lee, 2006), are not sensitive as other immune parameters to changes in infection status (Matson et al., 2005), and fluctuate less across the annual cycle in red knots (Buehler et al., 2008) and in free-living red crossbills (Schultz, 2015), it is not surprising that natural antibodies did not respond to LPS injections in the present study. Additionally, despite the fact that increases in haptoglobin activity have been shown in other bird species (Buehler et al., 2009; Hegemann et al., 2013), haptoglobin is only one acute-phase protein that can increase in response to LPS-induced inflammation. Other positive acute-phase proteins such as C-reactive protein (CRP) can activate complement (reviewed in Gruys et al., 2005; Owen-Ashley and Wingfield, 2007), which is involved in mediating hemolysis and *E. coli* (ATCC 8739) killing ability (Matson et al., 2005; Millet et al., 2007). We did not measure

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CRP in the present study, but given that both *E. coli* killing ability and hemolysis increased in response to LPS injections, we conclude that the acute-phase response was induced.

LPS-injected birds did not show significant differences in activity levels, time spent preening, drinking, eating or resting. However, injected birds tended to be less active, preened less and spent more time feeding, which are considered to be stereotypical sickness behaviors in animals generally (Hart, 1988) and in other bird species specifically (Bonneaud et al., 2003; Burness et al., 2010; Owen-Ashley et al., 2006). We measured sickness behavior over a relatively short time frame (30 min) following LPS injection because of logistical reasons, and this likely diminished our ability to observe moderate changes in behavior. However, given that all of the measures of sickness behavior tended to change in the expected direction, it is likely that a focused study would show that crossbills have sickness behavior that is similar to that of other species.

Minimal effect of food restriction on innate immunity

Restricting crossbills to 80% of their *ad libitum* daily intake of pellet food had little effect on the immune parameters measured. However, it did significantly reduce mass and hematocrit. Based on the mass and hematocrit loss seen in birds that were food restricted, the food restriction clearly negatively impacted energy balance. Other studies have shown minimal effects of food restriction on constitutive immunity, such as was demonstrated for red knots (Buehler et al., 2009;Vezina et al., 2009) and for Siberian hamsters (Zysling et al., 2009). Despite resource restriction, red knots maintained constitutive levels of immunity and may have conserved energy so as not to affect their investment in immunity, perhaps owing to the cost of not maintaining immune defense in energetically limited situations (Buehler et al., 2009). The crossbills in the present study were kept in small cages in thermally neutral conditions (20–23°C); never at any point were the birds presented with a thermoregulatory or energetic challenge that a food-restricted bird in the wild would experience, e.g. low temperatures, or flight away from a predator or in search of food. The activity data in this study indicate that food-restricted birds increased their activity in response to food restriction. A similar result was found in white-crowned sparrows (*Zonotrichia leucophrys gambelii*), which also dramatically increased their activity or 'escape behavior' during a 40 h fast (Astheimer et al., 1992). In the wild, crossbills may experience bouts of low food availability that may induce irruptive migratory behavior (Svardson, 1957) and food restriction increases activity in captive crossbills (Cornelius et al., 2010). Thus, it seems unlikely that these food-restricted birds were behaviorally modulating (limiting their energy cost) in such a way as to conserve energy that was funneled into maintaining immune function at the expense of other tissues.

Conclusions

Overall, this study has demonstrated that changes from short to long day lengths are sufficient to significantly increase multiple measures of constitutive innate immune function (leukocyte counts and bacterial killing ability), even in a temporally flexible songbird. This suggests that changes in day length are a salient cue when timing energy investments for these birds. LPS injections were found to significantly increase complement levels (hemolysis) and bacterial killing ability but in short day birds only, demonstrating a modulation in immune activity in response to an induction of the acute-phase response that may be blunted by increases in day length. Lastly, food restriction, while having significant negative effects on mass and haematocrit, had little effect on any of the immune parameters measured, suggesting that in a thermo-neutral, 'low-stakes' environment, investment in innate immune function is prioritized and maintained.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

E.M.S. performed all immune assays, fieldwork necessary to bring crossbills into captivity, statistical analyses, and wrote/edited the manuscript. T.P.H. provided partial funding, contributed to fieldwork and participated in the editing of the manuscript. K.C.K. provided resources and laboratory space for immune assays and participated in the editing of the manuscript.

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Supplementary information

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Table S1: List of generalized linear models ranked in order of lowest to highest AICc score. Variable codes are lps dose (mass-dependent LPS dose received at time of injection), food (food treatment: ad-libitum or restricted), photoperiod (short day or long day).

Structure	ΔAICc	ω_i
Mass Models[1], Model Link=Identity		
~ food + photoperiod + lps dose	0.0	0.3908
~ food + photoperiod + food*photoperiod	0.4	0.3246
~ food + photoperiod	1.6	0.1790
~ lps dose + photoperiod + lps dose*photoperiod	3.6	0.0640
~ food + lps dose + food*lps dose	5.8	0.0214
~ photoperiod + lps dose	7.5	0.0091
~ food + lps dose	8.6	0.0053
~ photoperiod	9.5	0.0034
~ food	10.3	0.0023
~ lps dose	15.7	<0.001
~ 1 (null model)	17.7	<0.001
~ food*photoperiod* lps dose	18.2	<0.001
Hematocrit Models[2], Model Link=Identity		
~ food + photoperiod + lps dose	0.0	0.357
~ food + photoperiod + food*photoperiod	0.3	0.309
~ food + lps dose + food*lps dose	1.6	0.159
~ food + photoperiod	1.7	0.154
~ food + lps dose	6.4	0.014
~ food	8.2	0.006
~ lps dose + photoperiod + lps dose*photoperiod	24.9	<0.001
~ photoperiod + lps dose	27.9	<0.001
~ photoperiod	29.4	<0.001
~ lps dose	33.0	<0.001
~ 1 (null model)	34.5	<0.001
Hemagglutination Models[1], Model Link=Log		
~ 1 (null model)	0.0	0.341
~ lps dose	1.6	0.150
~ food	2.1	0.120

~ photoperiod	2.1	0.120
~ food + photoperiod + food*photoperiod	3.4	0.062
~ photoperiod + lps dose	3.7	0.053
~ food + lps dose	3.8	0.052
~ food + photoperiod	4.2	0.042
~ lps dose + photoperiod + lps dose*photoperiod	5.3	0.024
~ food + photoperiod + lps dose	5.9	0.018
~ food + lps dose + food*lps dose	5.9	0.018
~lps dose*food*photoperiod	10.6	0.0017
Hemolysis Models[1], Model Link=Log		
~ food + lps dose	0.0	0.308
~ lps dose	0.7	0.2281
~ food + lps dose + food*lps dose	2.0	0.1189
~ food + photoperiod + lps dose	2.2	0.1092
~ photoperiod + lps dose	2.8	0.0783
~ 1 (null model)	3.9	0.0447
~ food	4.6	0.0318
~ lps dose + photoperiod + lps dose*photoperiod	4.8	0.0297
~ photoperiod	5.7	0.0186
~ food + photoperiod	6.2	0.0145
~ food + photoperiod + food*photoperiod	8.2	0.0054
~lps dose*food*photoperiod	8.9	0.0037
Bacterial Killing Ability Models[2], Link=Identity		
~ photoperiod + lps dose + photoperiod*lps dose	0.0	0.8482
~ lps dose	3.7	0.1318
~ food + lps dose	9.1	0.0088
~ photoperiod + lps dose	9.5	0.0074
~ food + lps dose + food*lps dose	11.1	0.0032
~lps dose*food*photoperiod	12.1	0.0020
~ food + lps dose + pp	14.9	<0.001
~ 1 (null model)	24.6	<0.001
~ food	29.8	<0.001
~ photoperiod	29.9	<0.001
~ food + photoperiod	35.1	<0.001

~ food + photoperiod + food*photoperiod	39.5	<0.001
Haptoglobin/PIT54 Models[2], Link=Identity		
~ 1 (null model)	0.0	0.6385
~ lps dose	2.1	0.2248
~ photoperiod	5.2	0.0470
~ food	5.4	0.0437
~ photoperiod + lps dose	7.3	0.0169
~ food + lps dose	7.5	0.0153
~ food + lps dose + food*lps dose	9.7	0.0051
~ photoperiod + lps dose + lps dose*photoperiod	10.3	0.0038
~ food + photoperiod	10.6	0.0031
~ food + lps dose + photoperiod	12.7	0.0011
~ food + photoperiod + food*photoperiod	13.5	<0.001
~lps dose*food*photoperiod	21.4	<0.001
White Blood Cell Models[2], Link=Log		
~ photoperiod + lps dose	0.0	0.465
~food + lps dose + photoperiod	1.5	0.221
~photoperiod + lps dose + photoperiod*lps dose	1.7	0.197
~lps dose*food*photoperiod	5.0	0.038
~ photoperiod	5.0	0.037
~ food + photoperiod + food*photoperiod	5.6	0.028
~ food + photoperiod	7.0	0.014
~ lps dose	23.3	<0.001
~ food + lps dose	24.7	<0.001
~ food + lps dose + food*lps dose	25.7	<0.001
~ 1 (null model)	26.6	<0.001
~ food	28.6	<0.001
Sickness Behavior:[1] Activity Models, link=Identity		
~ lps dose*food*photoperiod	0.0	1
~ lps dose + photoperiod + lps dose*photoperiod	22.0	<0.001
~ lps dose + food + lps dose*food	23.3	<0.001
~ lps dose	33.5	<0.001
~ 1 (null model)	40.2	<0.001

Sickness Behavior[1]: Resting Models, link=Identity		
~ lps dose*food*photoperiod	0.0	1
~ lps dose + food + lps dose*food	23.4	<0.001
~ lps dose + photoperiod + lps dose*photoperiod	23.9	<0.001
~ lps dose	34.9	<0.001
~ 1(null model)	41.3	<0.001
Sickness Behavior[1]: Preen Models, link=Identity		
~ lps dose*photoperiod*food	0.0	0.9184
~ lps dose+ photoperiod + lps dose*photoperiod	7.5	0.0218
~ lps dose + food + lps dose*food	7.6	0.0208
~ lps dose	10.1	0.0062
~ 1 (null model)	11.7	0.0028
Sickness Behavior[1]: Feed Models, link=Identity		
~ lps dose*food*photoperiod	0.0	1
~ lps dose + food + lps dose*food	17.2	<0.001
~ lps dose +photoperiod + lps dose*photoperiod	18.8	<0.001
~ lps dose	25.0	<0.001
~ 1 (null model)	28.1	<0.001
Sickness Behavior[1]: Drink Models, link=Identity		
~ 1 (null)	0.0	0.8037
~ lps dose	3.0	0.1821
~ lps dose + food + lps dose*food	9.0	0.0089
~ lps dose + photoperiod + lps dose*photoperiod	10.0	0.0053
~ lps dose*photoperiod*food	20.5	<0.001

[1]All models include a random effect for bird ID

[2] All models include a random effect for bird ID and blood sampling date

Table S2: Model parameter estimates from best-supported GLMMs

Mass Model, link= Identity				
Parameter	Estimate	S.E.	2.5%	97.5%
Intercept	35.59	1.26	33.09	38.08
Food Restriction	-2.33	0.96	-4.24	-0.43
LPS Dose	0.64	1.54	-2.59	5.53

Photoperiod Short Day	2.58	0.78	1.04	4.12
Food Restriction *Photoperiod SD	-0.48	1.01	-3.91	1.26
Hematocrit Model, link=Identity				
Parameter	Estimate	S.E.	2.5%	97.5%
Intercept	50.53	1.32	47.94	53.13
Food Restriction	-5.04	1.14	-7.28	-2.80
LPS Dose	-0.68	1.69	-5.55	2.97
Photoperiod Short Day	-1.69	0.99	-3.42	-0.62
Food Restriction *Photoperiod	0.46	1.03	-1.24	4.17
Food Restriction *LPS Dose	-1.11	3.14	-16.05	2.44
Hemolysis Model, link=Log				
Parameter	Estimate	S.E.	2.5%	97.5%
Intercept	-0.28	0.06	-0.40	-0.17
Food Restriction	-0.10	0.12	-0.39	0.04
LPS Dose	0.49	0.20	0.10	0.89
E. coli Model, link= Identity				
Parameter	Estimate	S.E.	2.5%	97.5%
Intercept	1.39	0.08	1.22	1.56
Photoperiod Short Day	-0.13	0.07	-0.26	-0.01
LPS Dose	0.53	0.24	0.03	0.98
Photoperiod*LPS Dose	1.12	0.32	0.51	1.75
White Blood Cell Model, link= Identity				
Parameter	Estimate	S.E.	2.5%	97.5%
Intercept	-1.95	0.35	-2.64	-1.26
Photoperiod Short Day	-0.63	0.12	-0.88	-0.39
LPS Dose	-1.20	0.45	-2.09	-0.31

Food Restriction	-0.25	0.30	-0.84	0.35
LPS Dose * Photoperiod	0.56	0.79	-0.99	2.11
Changes in Activity Model, link=Identity				
Parameter	Estimate	S.E.	2.5%	97.5%
Intercept	0.22	6.49	-11.92	12.38
LPS Dose	-39.89	24.82	-86.25	6.52
Food Restriction	5.85	11.65	-16.12	28.16
Photoperiod Short Day	-6.91	9.39	-24.45	10.63
LPS Dose * Food Restriction	-2.86	51.84	-99.82	98.40
LPS Dose *Photoperiod	49.49	37.13	-19.91	118.86
Food Restriction*Photoperiod	-1.31	17.33	-29.64	32.00
LPS dose*Photoperiod*Food Restriction	-23.00	78.18	-174.02	123.61
Changes in Resting Model, link=Identity				
Parameter	Estimate	S.E.	2.5%	97.5%
Intercept	0.35	6.66	-12.09	12.80
Photoperiod Short Day	5.90	9.62	-12.05	23.85
LPS Dose	33.44	25.45	-14.02	80.90
Food Restriction	-6.68	12.78	-31.23	17.29
Photoperiod*LPS Dose	-43.42	38.07	-111.50	25.33
Photoperiod*Food Restriction	-6.52	18.98	-42.22	29.25
LPS Dose*Food Restriction	3.11	56.74	-105.87	107.68
Photoperiod*Food	30.00	85.56	-	191.80

Restriction*LPS Dose			130.19	
Changes in Preening Model, link=Identity				
Parameter	Estimate	S.E.	2.5%	97.5%
Intercept	0.96	1.06	-1.03	2.95
Food Restriction	-0.73	1.71	-3.97	2.54
LPS Dose	-1.78	4.07	-9.42	5.84
Photoperiod Short Day	-0.25	1.54	-3.15	2.64
Food Restriction*LPS Dose	6.84	7.64	-7.93	21.19
Food Restriction*Photoperiod	1.20	2.55	-3.83	5.98
LPS Dose*Photoperiod	3.04	6.09	-8.37	14.49
Food Restriction*LPS Dose*Photoperiod	-10.33	11.52	-31.94	12.12
Changes in Feeding Model, link=Identity				
Parameter	Estimate	S.E.	2.5%	97.5%
Intercept	-1.42	1.93	-5.04	2.20
Photoperiod Short Day	1.46	2.78	-3.77	6.69
Food Restriction	1.34	3.87	-5.93	8.61
LPS Dose	8.84	7.36	-4.97	22.66
Photoperiod*Food Restriction	6.16	5.75	-4.63	16.95
Photoperiod*LPS Dose	-9.88	11.00	-30.54	10.77
Food Restriction*LPS Dose	-11.61	17.17	-43.85	20.62
Photoperiod*LPS Dose*Food Restriction	7.60	25.89	-41.00	56.21