

## Population differences in fever and sickness behaviors in a wild passerine: a role for cytokines

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### SUMMARY

Immune responses benefit hosts by clearing pathogens, but they also incur physiological costs and tissue damage. While wild animals differ in how they balance these costs and benefits, the physiological mechanisms underlying such differential investment in immunity remain unknown. Uncovering these mechanisms is crucial to determining how and where selection acts to shape immunological defense. Among free-living song sparrows (*Melospiza melodia*) in western North America, sickness-induced lethargy and fever are more pronounced in Southern California than in Washington and Alaska. We brought song sparrows from two populations (Southern California and Washington) into captivity to determine whether these differences persist in a common environment and what physiological signals facilitate such differences. As in free-living sparrows, captive California birds exhibited more pronounced fever and lethargy than Washington birds in response to lipopolysaccharide, a non-pathogenic antigen that mimics bacterial infection. After treatment, the two populations showed similar reductions in luteinizing hormone levels, food intake and body mass, although treated birds from California lost more breast muscle tissue than treated birds from Washington. Moreover, California birds displayed higher bioactivity of interleukin-6, a pro-inflammatory cytokine, and marginally higher levels of corticosterone, a steroid hormone involved in stress, metabolism and regulating inflammatory responses. Our results show that immunological differences between these populations cannot be explained by immediate environment alone and may reflect genetic, maternal or early-life effects. Additionally, they suggest that cytokines play a role in shaping immunological variation among wild vertebrates.

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### INTRODUCTION

Although the immune system provides critical defense against pathogens, this defense incurs measurable costs in terms of nutrients, time and damage to host tissues (Lochmiller and Deerenberg, 2000; Råberg et al., 2000; Martin et al., 2003; Klasing, 2004; Graham et al., 2005). Given these costs, hosts must balance investment in immune defenses against investment in crucial life history traits, such as reproduction (Stearns, 1992; Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Lee, 2006; Hasselquist, 2007; Martin et al., 2008b). While the precise balance between such investments will vary with ecological factors, including pathogen exposure, host population density and host residual reproductive value, the physiological mechanisms that modulate and optimize this differential allocation remain unknown (Yirmiya et al., 1995; Piersma, 1997; Hanssen, 2006; Møller et al., 2006; Owen-Ashley et al., 2008; Adelman and Martin, 2009). Revealing these mechanisms will help us understand how and where selection acts to shape diversity in immunological and life history investments (Zera and Harshman, 2001). Moreover, uncovering the mechanisms responsible for this diversity may help identify predictive biomarkers of host resistance (Poland et al., 2008;

Vollmer-Conna et al., 2008), clarify the impact of different parasitic organisms on host defenses (Jackson et al., 2009), and provide important tools for forecasting and mitigating the spread of wildlife diseases.

In this study, we focused on the acute phase immune response to determine what mechanisms underlie differential investment in immune defense among wild vertebrate populations. The acute phase response is widespread across animal taxa, protects against diverse types of pathogens, and includes fever, the production of antimicrobial peptides, and sickness behaviors such as lethargy and anorexia (Hart, 1988; Kluger, 1991; Exton, 1997). Despite its protective properties, the acute phase response carries substantial costs, making it highly likely to trade off with costly life history traits (Klasing, 2004). For instance, metabolic rate can increase during fever by 10–15% for each 1°C rise in core body temperature (Roe and Kinney, 1965). Additionally, sickness-induced lethargy can reduce the time available to forage, defend breeding territories and care for offspring (Aubert et al., 1997; Bonneaud et al., 2003; Owen-Ashley and Wingfield, 2006; Adelman et al., 2010). While the acute phase response has been shown to vary inversely with reproductive investment both within and among wild species (Lee et al., 2005; Owen-Ashley and Wingfield, 2006; Martin et al., 2008a;

Owen-Ashley et al., 2008; Adelman et al., 2010), the physiological underpinnings of these differences have not been elucidated.

In model organisms, however, many of the basic physiological mechanisms involved in the acute phase response are well understood. Cytokines, signaling molecules released by immune system cells, are critical in determining both the timing and magnitude of fever and sickness behaviors in the laboratory (Kluger, 1991; Elmquist et al., 1997; Dantzer, 2004). Because cytokines also interact directly with the neuroendocrine system (Besedovsky and del Rey, 2001; Dantzer, 2004), these molecules are likely to play a critical role in orchestrating the balance between reproductive investment and the acute phase response among wild vertebrates. Indeed, in mice and rats, cytokines that enhance the acute phase response, such as interleukin-1 $\beta$ , interleukin-6 and tumor necrosis factor- $\alpha$ , can decrease the secretion of reproductive hormones, such as luteinizing hormone and testosterone (Rivier and Vale, 1990; Turnbull and Rivier, 1997). Cytokines can suppress these reproductive signals directly by binding receptors that regulate activity in the hypothalamic–pituitary–gonadal axis (Rivier and Vale, 1990; Turnbull and Rivier, 1997). Alternatively, cytokines can downregulate reproductive hormones indirectly by increasing the secretion of glucocorticoids (Fleshner et al., 1995; Turnbull and Rivier, 1999), steroid hormones that can suppress both reproductive signals and immune responses (Goujon et al., 1995; Goujon et al., 1997; Sapolsky et al., 2000) [but see Dhabhar (Dhabhar, 2009) and Martin (Martin, 2009) for recent reviews of acute immunoenhancement by glucocorticoids].

Here we sought to uncover the physiological mechanisms that underlie differences in the acute phase response between two populations of a widespread North American vertebrate, the song sparrow (*Melospiza melodia*). In the wild, adult male song sparrows in southern California show more pronounced acute phase responses than conspecifics in Washington and Alaska (Adelman et al., 2010). Several previous studies have suggested that such population differences in immune defense may reflect factors that can co-vary with latitude, such as pathogen abundance, host density and investment in reproductive effort (Ardia, 2005; Martin et al., 2006; Møller et al., 2006; Owen-Ashley et al., 2008). However, it is important to note that all such studies focus on very few populations, meaning that drift or other ecological variables may also influence population differences (Garland and Adolph, 1994). So, while song sparrow populations differ in their acute phase responses in the wild, it remains unknown whether these differences result from immediate environmental differences or from other factors, such as maternal effects or genetic divergence.

To identify (a) whether immediate environmental factors drive population differences in the acute phase response and (b) the potential physiological mechanisms underlying these differences, we brought adult male sparrows from both California and Washington into a common laboratory environment during the early breeding season. We then induced an acute phase response using lipopolysaccharide (LPS), a non-replicating component of Gram-negative bacterial cell walls and the same antigen used in our prior studies in the wild (Janeway et al., 2005; Adelman et al., 2010). If immediate environmental factors were not responsible for population differences in the acute phase response, we predicted that birds from California would show more pronounced fever, lethargy and anorexia than birds from Washington, as in the wild. For the first time in a wild passerine, we adapted a cell culture-based assay of interleukin-6 (IL-6) bioactivity (Van Oers et al., 1988) to test whether higher levels of pro-inflammatory cytokine signaling in California birds could underlie such population differences. In addition, we

predicted that downstream targets of pro-inflammatory cytokines would show stronger modulation in response to LPS treatment in California sparrows. In particular, we predicted that corticosterone concentrations would increase and that luteinizing hormone concentrations would decrease more sharply in California birds. Such differential modulation of cytokines and their neuroendocrine targets could explain, mechanistically, how populations invest differently in immune defense.

## MATERIALS AND METHODS

### Study species and field capture

Song sparrows (*Melospiza melodia* Wilson 1810) in western North America exhibit a latitudinal gradient in clutch size and breeding season length typical of most passerines (Johnston, 1954; Arcese et al., 2002). For this study, 57 adult males were captured at two latitudes: 29 of the subspecies *Melospiza melodia fallax* Baird 1854 near Niland, CA, USA (the Sonny Bono Salton Sea National Wildlife Refuge/Imperial Wildlife Management Area, 33°16'18"N, 115°34'49"W) and 28 of the subspecies *Melospiza melodia morphna* Oberholser 1899 near Eatonville, WA, USA (the Charles L. Pack Experimental Forest, 46°50'41"N, 122°17'32"W). Birds were captured using mist nets and conspecific song playback during the early breeding season in 2009 (6–12 March in CA and 11–16 April in WA). Sex and breeding condition were determined using the length and width of the cloacal protuberance, an androgen-dependent organ the size of which varies directly with testis volume and reproductive hormone levels in this species (Perfito et al., 2004). Populations differed in neither cloacal protuberance length (measured in the field,  $t_{53}=0.274$ ,  $P=0.79$ ) nor luteinizing hormone levels (see results for control birds below, supplementary material Table S3), suggesting that males from the two sites were in similar breeding condition during the experiments. Because breeding commences later in Washington than in southern California (Johnston, 1954; Arcese et al., 2002), we staggered our timing such that experiments on California birds were completed before fieldwork in Washington was begun. At each site, three nylon tents (3.05 m wide  $\times$  3.96 m long  $\times$  1.83 m high, Ozark Trails, Wal-Mart, Bentonville, AR, USA) served as temporary aviaries. Each tent was fitted with six hanging perches and housed a maximum of 10 birds, which were provided with *ad libitum* seed (Kaytee Supreme Finch; Kaytee Products, Inc., Chilton, WI, USA), water and fruit (blackberries, oranges and apples). In California, tents were placed within fenced enclosures and sheltered from rain and wind. In Washington, tents were placed inside buildings with exposure to natural light and temperature.

All work was conducted under Princeton University IACUC protocol 1745, California scientific collecting permit SC-009218, Washington scientific collecting permit 07-316, US Fish and Wildlife scientific collecting permit MB026193-0, and US Geologic Survey Bird Banding Laboratory permit 22965.

### Transport and housing

After no more than 1 week, birds were transferred to individual compartments (17.5 cm wide  $\times$  27.5 cm long  $\times$  19.5 cm high) within custom-built wooden travel carriers and provided with seed and fresh fruit sufficient for many days. Carriers were then driven to the nearest airport and shipped in a climate-controlled cabin to Newark, NJ, USA. One author (J.S.A.) rode on the same flight to ensure rapid transfer between ground and air transportation. Birds were then driven to Princeton University. Total travel time to Princeton University was approximately 14 h from each field site.

Upon arrival at Princeton University, birds were transferred to individual cages (25 cm wide  $\times$  55 cm long  $\times$  25 cm high) with two

perches, *ad libitum* water, grit and food [1:1 mix of Kaytee Supreme Finch seed and Mazuri Small Bird Maintenance pellets (Purina Mills, Gray Summit, MO, USA)], and housed in a room maintained at a constant temperature (23°C). The lighting regime in the room was adjusted weekly to reflect seasonal variation in day length at the birds' native latitudes. Lights were turned on at the average between sunrise and morning civil twilight and off at the average between sunset and evening civil twilight as calculated by standard charts (US Naval Observatory, 2008). Birds were allowed to acclimate to captive conditions for 2 weeks before the start of the experiments. During the experiments, only two to four birds were sampled each day, so total time in captivity before sampling ranged from 2 to 3.5 weeks. All birds survived transport, but one individual from Washington died while in captivity before the experiment began.

#### Immune challenge and experimental timeline

At the start of the experiment, all caged birds were randomly assigned to one of two chambers within the same room separated by a doorway covered in 75 mm thick plastic and black felt. Birds in one chamber could not see birds in the other, but could hear attenuated sounds from the other chamber. Each morning, four birds were selected arbitrarily, two from each chamber. One bird from each chamber was radio tagged and received an injection of LPS. The other bird from each chamber was handled and radio tagged (see below), but not injected. Both birds were measured for mass, fat and breast muscle thickness (see 'Morphological measurements' below). LPS (cat. no. L2880, serotype 055:B5, Sigma-Aldrich, St Louis, MO, USA) was first dissolved in sterile phosphate-buffered saline (cat. no. P3813, Sigma-Aldrich) and then mixed in a 1:1 ratio with Freund's incomplete adjuvant (cat. no. F5506, Sigma-Aldrich) to yield a final concentration of 2 mg ml<sup>-1</sup>. Injections were adjusted by body mass, with each bird receiving 2.1 µg LPS g<sup>-1</sup> body mass, consistent with previous work on this species (Owen-Ashley and Wingfield, 2006; Adelman et al., 2010). Adjuvant was included to match injections given in prior field studies on this species, in which adjuvant served to prolong fever and sickness behaviors (Owen-Ashley and Wingfield, 2006; Adelman et al., 2010). Adjuvant alone does not induce lethargy in this species, although it may cause some local immune response and a minimal decrease in food intake in similar passerines (Owen-Ashley, 2004; Owen-Ashley and Wingfield, 2006; Adelman et al., 2010).

#### Radio-telemetry

Following LPS injection and morphological measurements, birds were fitted with temperature-sensing radio transmitters (model no. LB-2NT, Holohil Systems, Ltd, Carp, ON, Canada) using previously published methods (Cochran and Wikelski, 2005). Briefly, a small patch of feathers was removed just lateral to the spine. A transmitter, weighing less than 3% of body mass, was then attached using surgical glue (Vetbond, 3M, St Paul, MN, USA).

An automated receiver (model no. 10-1000, Sparrow Systems, Champaign-Urbana, IL, USA) was then used to monitor inactivity (lethargy) and skin temperature (fever). Signal strength from each transmitter was used to determine periods of inactivity, defined as any length of time, 1 min or longer, during which consecutive measures of signal strength fluctuated by less than ±4 dB (Kjos and Cochran, 1970; Adelman et al., 2010). The 4 dB value has been calibrated in field studies and adequately represents passerine activity in the wild (Bisson et al., 2009; Lambert et al., 2009). The transmitters encoded skin temperature by varying the time interval between successive pulses. The relationship between temperature and inter-pulse interval was calibrated separately for each transmitter

by the manufacturer and a subset was retested in our laboratory to confirm accuracy. Data points were collected every 30 s, but were excluded if the signal strength from the transmitter came within 3 dB of the noise floor (approximately -123 dB). Additionally, if temperature varied by more than 1°C in 30 s and immediately returned to the prior level, this spike was excluded from the data set. Because blood sampling at 6 h (see below) may have disturbed birds in the neighboring chamber, telemetry data between 6 and 6.5 h post-treatment were excluded from the analysis.

#### Blood sampling

Because song sparrows are relatively small, blood sampling pre-treatment could have influenced both behavioral and physiological measures post-treatment. Therefore we did not take pre-treatment samples. Rather, each day, at 6 h post-treatment, blood samples were taken from the first two birds by puncturing the wing vein with a sterile 26 gauge needle and collecting the blood in heparinized capillary tubes. At 22 h post-treatment, blood samples were taken from the second two birds. For all but three birds, blood was taken for corticosterone measurement within 3 min of the researcher entering the room. For those three birds not sampled within 3 min, no corticosterone measurements were taken. Within 10 min of taking the corticosterone sample, additional samples were collected from the same birds in separate tubes for other blood measurements. The time taken for sample collection did not differ among populations, treatment groups or sampling time points (6 or 22 h) (corticosterone:  $F_{7,45}=0.96$ ,  $P>0.47$ ; other samples:  $F_{7,48}=0.49$ ,  $P>0.83$ ). Samples were stored in microcentrifuge tubes at 4°C until centrifugation, no more than 2 h following sampling. Plasma was then stored at -20°C.

#### Food intake

To measure changes in food intake, we first calculated the amount of food each bird ate 1 day prior to the experiment and used this as a baseline. Food dishes were weighed on a digital balance (to 1 mg) before they were placed in the cages immediately following lights on. At this time, trays at the bottom of the cages were cleaned and lined with fresh newspaper. Twenty-two hours later, food remaining in the dish and food spilled on the cage floor was weighed. Feces from the cage floors were removed by hand before weighing. This process was repeated on the day of the experiment. For each bird, the change in food intake between baseline and experimental days was expressed as a percentage of baseline.

#### Morphological measurements

Birds were weighed using a spring scale (30 g maximum, Pesola, Baar, Switzerland) to the nearest 0.1 g. The length of the left tarsus was measured using calipers (to 0.1 mm). Furcular fat was scored using previous methods (Wingfield and Farner, 1978), where a score of 0 indicates no fat and a score of 5 indicates fat spilling out of the furcular cavity. Breast muscle thickness was assessed by measuring the distance from a fixed point - 5 mm directly lateral from the center of the sternum - to the breast muscle (Schmidt-Wellenburg et al., 2008). Changes in fat scores and breast muscle thickness during the experiment were expressed as the difference between scores pre-treatment and 6 or 22 h post-treatment. To control for overall differences in mass between the populations, changes in mass during the experiment were expressed as a percentage of pre-treatment mass.

Because initial physiological reserves can affect the magnitude of the acute phase response in passerines (Owen-Ashley and Wingfield, 2007), we included a measure of overall initial body



condition in our analyses of fever, lethargy, hormones and IL-6. We calculated initial body condition by performing linear regressions of mass (measured immediately before the experiment) by tarsus length for each population separately. We then used each bird's residual from these regressions as our measure of initial body condition. We did not use mass/tarsus<sup>3</sup> to measure body condition because this correlates with tarsus length, which differs among song sparrow populations (Arcese et al., 2002; Adelman et al., 2010) and could therefore bias condition measurements.

#### IL-6-like bioactivity

IL-6-like bioactivity of plasma was determined by assaying a sample's ability to induce proliferation in mouse B-cell hybridoma cells, following previously published methods (Van Oers et al., 1988) with minor modification. Briefly, B9 hybridoma cells, which are highly dependent upon IL-6 for proliferation, were cultured in RPMI media (cat. no. 11875-085 Gibco, Invitrogen, Carlsbad, CA, USA) with 5% fetal bovine serum (cat. no. SH3007002 Hyclone, Thermo Scientific, Waltham, MA, USA), 100 IU ml<sup>-1</sup> penicillin/streptomycin (cat. no. SV30010, Hyclone), 50 µmol l<sup>-1</sup> 2-mercaptoethanol (cat. no. M6250, Sigma-Aldrich) and 50 pg ml<sup>-1</sup> recombinant human IL-6 (cat. no. I1395, Sigma-Aldrich). Before the assay, cells were washed twice and resuspended with RPMI containing no IL-6 and counted using Trypan Blue staining (cat. no. MT25900CI, Mediatech, Manassas, VA, USA) and a hemocytometer. The final suspension of cells contained 3 × 10<sup>6</sup> mol l<sup>-1</sup> polymyxin B (cat. no. P1004, Sigma-Aldrich) to inhibit the activity of any LPS that may have persisted in the plasma. Plasma samples were divided into two 5 µl replicates and added to wells of a 96-well culture dish. Each 5 µl replicate was added initially to 15 µl of RPMI (1:4 dilution) and then serially diluted to 1:256. Positive controls of recombinant human IL-6 were diluted in the same manner beginning at 1.25 pg µl<sup>-1</sup>. After sample dilution, 50 µl of a 1 × 10<sup>5</sup> cells ml<sup>-1</sup> suspension of B9 cells was added to each well to yield a final concentration of 5000 cells per well and a final polymyxin B concentration of 2.5 × 10<sup>-6</sup> mol l<sup>-1</sup>. At this concentration of polymyxin B, even 200 µg ml<sup>-1</sup> of LPS induced no B9 cell proliferation. After 72 h incubation at 37°C, each well was treated with 25 µl of 5 mg ml<sup>-1</sup> Thiazolyl Blue tetrazolium bromide (MTT, cat. no. M5655, Sigma-Aldrich), a yellow colored solution that is converted to blue by metabolically active cells, giving a measure of cell proliferation. Four hours later, the reaction was stopped and cells were lysed with 50 ml per well of an extraction buffer containing 20% SDS, 37.75% *N,N*-dimethyl formamide, 2% glacial acetic acid, 2.5% 1 mol l<sup>-1</sup> HCl and 37.75% de-ionized water. Cells were then incubated overnight at 37°C. IL-6-like bioactivity was measured as absorbance at 595 nm using a microplate reader (Tecan US, Inc., Durham, NC, USA).

#### Corticosterone

Corticosterone was assayed by enzyme immunoassay using commercially available kits (cat. no. 900-097, Assay Designs, Ann Arbor, MI, USA) previously validated for use in similar passerines (Wada et al., 2007; Swett and Breuner, 2008). The assay was run as per the manufacturer's instructions with the following sample preparation steps. Plasma samples of 5 µl were diluted 1:1 with a 1:10 dilution of steroid displacement reagent and assay buffer 15 (provided in the kit) and incubated at room temperature for 5 min. Following this incubation, 240 µl of assay buffer 15 was added to each sample before continuing with the assay as per the manufacturer's instructions. The intra-assay variation was 11.1%

CV, the inter-plate variation was 4.75% CV, and the lower detection limit was 0.020 ng ml<sup>-1</sup>.

#### Luteinizing hormone

Plasma luteinizing hormone was measured by radioimmunoassay using a modification of a previously published method (Follett et al., 1975). Plasma samples were measured in duplicates of at least 10 µl in a single assay. Briefly, luteinizing hormone was measured by a postprecipitation, double-antibody radioimmunoassay (Follett et al., 1972; Follett et al., 1975; Sharp et al., 1987) using highly purified chicken luteinizing hormone for standard curves and for radio-iodination. Goat anti-rabbit γ-globulin precipitating serum was used as the secondary antibody. This assay has been used extensively for measurements of circulating concentrations of luteinizing hormone in a variety of avian species including *M. melodia* (e.g. Wingfield and Hahn, 1994). Further details of the luteinizing hormone assay are described elsewhere (Wingfield et al., 1991), and intra-assay variability was similar to that of previous studies. Intra-assay variation was 3.01% CV, and the lower detection limit was 0.039 ng ml<sup>-1</sup>.

#### Statistical analyses

Data were analyzed in R (version 2.7.1) (R Development Core Team, 2008). In all statistical models, interactions among main effects were removed, beginning with highest order interactions, if their *P*-value was above 0.10. When data violated the assumption of equal variance among groups (IL-6, corticosterone, luteinizing hormone), an identity variance function was added to the statistical models (Pinheiro and Bates, 2000).

Data on skin temperature and proportion of time spent active were analyzed using generalized additive mixed models (GAMM) with thin plate regression spline smoothing functions of the response variable over time (Zuur et al., 2009). We first constructed maximal models with main effects of initial body condition, population, LPS treatment and their interactions, and smoothing functions for all combinations of treatment group and population (CA control, CA LPS, WA control, WA LPS). The best combination of smoothing functions was chosen using Akaike's information criterion adjusted for small sample sizes (AICc) (Burnham and Anderson, 2002) as outlined in Table 1. To control for unequal variance, the proportion of time spent active was arc-sine square-root transformed. We analyzed the change in skin temperature from initial values (mean skin temperature from 30–60 min post-treatment), as this accurately reflects changes in core temperature in song sparrows (Adelman et al., 2010). Each model contained a random effect for individual bird. To control for autocorrelation, we used only one data point every 30 min for each individual and applied a rational quadratic correlation structure with a nugget effect, selected by AICc from among the following alternatives: exponential, linear, Gaussian and spherical (Pinheiro and Bates, 2000).

IL-6-like bioactivity, measured as absorbance at 595 nm, was also analyzed by GAMM. As using a single plasma dilution can bias cytokine bioassays (Mire-Sluis and Thorpe, 1998), each bird's data consisted of seven plasma dilutions in duplicate. To avoid pseudo-replication, the model contained a nested random effect for each replicate within each bird and an autoregressive (AR1) correlation structure was applied to control for correlation among dilutions. Separate smoothing functions of absorbance across dilutions were applied to LPS-treated 6 h birds, LPS-treated 22 h birds and control birds. Fixed effects included LPS treatment, population, time point of sampling (6 or 22 h), initial body condition and all of their interactions. To graph IL-6-like bioactivity, we averaged absorbance

Table 1. Generalized additive mixed models of (A) arcsin square-root transformed proportion of each 30 min spent active and (B) change in skin temperature, with different combinations of smoothing functions applied to the time since injection

Model no.	Separate functions of time since injection applied to:	AICc	$\Delta$ AICc
(A) Proportion of time spent active			
1*	CA control, CA LPS, all WA	-348.81	-
2	All CA, all WA	-344.05	4.76
3	CA control, WA control, all LPS	-341.25	7.55
4	CA control, CA LPS, WA control, WA LPS	-338.46	10.34
5	All control, all LPS	-335.61	13.20
6	All CA, WA control, WA LPS	-334.14	14.67
7	All control, CA LPS, WA LPS	-333.50	15.31
8	All birds	-330.11	18.70
(B) Change in skin temperature			
1*	All control, All LPS	1440.52	-
2	All birds	1442.51	1.99
3	All control, CA LPS, WA LPS	1461.50	20.97
4	CA control, CA LPS, all WA	1467.99	27.47
5	CA control, WA control, all LPS	1471.79	31.27
6	All CA, all WA	1476.91	36.38
7	CA control, CA LPS, WA control, WA LPS	1494.11	53.59
8	All CA, WA control, WA LPS	1504.20	63.67

The best-fit activity model (A1\*) contained smoothers for each treatment group in California (CA), but only one smoother in Washington (WA), suggesting that sickness behaviors progress differently between populations (Fig. 1A,C).

The best-fit skin temperature model (B1\*) contained one smoother for all control birds and one for all LPS-treated birds (Fig. 1B,D).

For all fixed effects, see supplementary material Table S1.

AICc, Akaike's information criterion adjusted for small sample sizes; LPS, lipopolysaccharide.

across dilutions for each bird and used these numbers to calculate group means and standard errors.

Changes in food intake, mass, fat score and breast muscle thickness were analyzed by general linear models with population, LPS treatment, time point of sampling and their interactions as fixed effects. Hormone data were also analyzed by general linear models, adding initial body condition and its interactions to the above fixed effects.

Seven birds were excluded from the analysis of skin temperature because of transmitter malfunctions that yielded constant temperature readings (while still yielding accurate activity readings). These transmitters were not reused. These birds were included in all other analyses. One control bird from California was excluded from the analysis of corticosterone because its blood samples had desiccated and readings were out of the range of the standard curve. One control bird from Washington was excluded from all analyses because of a wounded leg and elevated IL-6-like bioactivity, indicating infection. These exclusions explain the different sample sizes among treatment groups and time points.

## RESULTS

### Lethargy

The time course of lethargy in LPS birds differed between populations, as illustrated by the resumption of normal activity in Washington but not California birds at the later time points (Fig. 1A,C). The best-fit additive mixed model of activity confirmed this pattern as it contained three separate smoothing functions for activity over time: one for California control birds, one for California LPS birds and one for all Washington birds (Table 1A). However, the model revealed no interaction between the main effects of population and treatment ( $P>0.10$ , removed from model), suggesting that despite population differences in the time course of lethargy, LPS treatment decreased average locomotor similarly between populations (LPS treatment: estimate=-0.30,  $t_{51}=-9.8$ ,  $P<0.001$ ). Additionally, birds with a higher initial body condition showed a trend toward lower activity, independent of LPS treatment or

population of origin (initial body condition: estimate=-0.021,  $t_{51}=-1.74$ ,  $P=0.088$ ). All other fixed effects from this analysis can be found in supplementary material Table S1.

### Fever

All birds exhibited nocturnal decreases in skin temperature (Fig. 1B,D), typical of small passerines. Beginning roughly 2 h after injection, LPS-treated birds increased their temperature, which is reflected in the different smoothing functions for control and LPS-treated birds present in the best-fit additive mixed model (Table 1B). As the model with only one smoothing function for all birds had a  $\Delta$ AICc of 1.99, it is worth noting that fixed effect parameter estimates and their significance within these two models were nearly identical. We therefore chose to report the results from the model with two smoothers, as it best describes the data. The significant interaction between population and treatment in this model (estimate=0.54,  $t_{42}=2.52$ ,  $P=0.016$ ) suggests that LPS treatment increased temperature by roughly 0.5°C more in California than in Washington. Control groups from the different populations did not differ, but birds in California with a better initial body condition tended to have higher temperatures (initial body condition: estimate=0.15,  $t_{42}=1.70$ ,  $P=0.097$ ). All other fixed effects from this analysis can be found in supplementary material Table S1.

### Anorexia

Birds treated with LPS also showed pronounced anorexia, consuming less food and losing more mass than controls (Fig. 2A,B; supplementary material Table S2; food intake: LPS treatment: estimate=-46.79,  $t_{49}=-8.87$ ,  $P<0.001$ ; mass change: LPS treatment: estimate=-7.23,  $t_{48}=-6.30$ ,  $P<0.001$ ). The non-significant interactions between population and treatment suggest that food consumption and mass loss after LPS treatment did not differ between populations ( $P>0.10$ , removed from models). Decreases in food consumption were more pronounced at 6 h after treatment for both LPS-treated and control birds in each population (time of

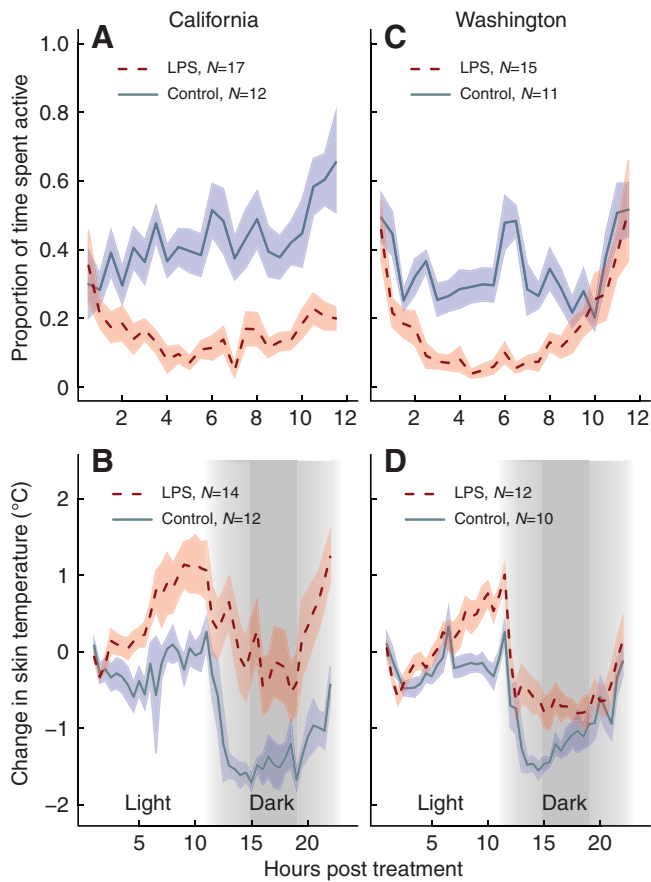


Fig. 1. Song sparrows from California show more sustained lethargy (A) and more pronounced fever (B) after injection with lipopolysaccharide (LPS; red dashed lines) than do sparrows from Washington (C,D). Note that x-axes differ: lethargy was measured only until 12 h post-treatment, before lights went out, whereas fever was measured throughout the night. Lines depict group means, shaded regions show  $\pm 1$  s.e.m.

sampling: estimate=33.31,  $t_{48}=6.22$ ,  $P<0.001$ ). Overall, mass loss was more pronounced at 22 h, although differences between control and LPS-treated birds were smaller at 22 h than at 6 h (LPS treatment  $\times$  time: estimate=3.62,  $t_{47}=2.65$ ,  $P=0.011$ ).

#### Changes in body composition

Population differences in fat scores were driven by an increase in fat among the three control birds from Washington sampled at 6 h post-treatment (Fig. 2C). Because these birds all had an increase in fat score of exactly 1.0, results from the generalized linear model of fat scores (supplementary material Table S2) suggest a strong effect of population origin (estimate=-1.00,  $t_{47}=-3.49$ ,  $P<0.001$ ) and a marginally significant interaction between population origin and LPS treatment (estimate=0.71,  $t_{47}=1.94$ ,  $P=0.059$ ). However, repeating the analysis excluding all control birds (apart from the 6 h Washington birds, no other control group showed changes significantly different from zero) revealed no significant population or temporal differences among fat scores for LPS-treated individuals (all  $P>0.10$ ).

Similarly, results from the initial analysis of breast muscle volume were likely driven by the Washington control birds sampled at 6 h post-treatment (Fig. 2D, supplementary material Table S2). While the initial model suggests that LPS treated birds lost more breast

muscle than controls (estimate=-0.30,  $t_{48}=-2.32$ ,  $P=0.025$ ) and that California birds lost more than Washington birds (estimate=-0.41,  $t_{48}=-3.12$ ,  $P=0.003$ ), high values for 6 h Washington controls made the interaction between population and treatment non-significant ( $P>0.10$ , removed from model). Repeating the analysis using only LPS-treated birds suggested that treated birds from California lost more breast muscle than did birds from Washington (estimate=-0.50,  $t_{26}=-2.56$ ,  $P=0.017$ ).

#### IL-6 and hormones

LPS-treated birds displayed higher levels of IL-6-like bioactivity than did control birds (Fig. 3A, supplementary material Table S3, estimate=0.55,  $t_{45}=7.98$ ,  $P<0.001$ ). This effect was more pronounced at 6 h after treatment than at 22 h after treatment (LPS treatment  $\times$  time: estimate=-0.023,  $t_{45}=-7.87$ ,  $P<0.001$ ). The significant interaction between population and treatment (estimate=0.21,  $t_{45}=2.38$ ,  $P=0.021$ ) shows that LPS treatment induced more IL-6-like bioactivity in birds from California than in birds from Washington. The significant three-way interaction among population, treatment and time suggests that although treated birds from the two populations still differed in IL-6 levels at 22 h, this difference was less pronounced than at 6 h (estimate=-0.008,  $t_{45}=-2.15$ ,  $P=0.037$ ).

Circulating concentrations of corticosterone were also higher in LPS-treated birds (Fig. 3B, supplementary material Table S3, estimate=8.77,  $t_{44}=2.51$ ,  $P=0.016$ ). The marginally significant interaction between population and treatment (estimate=9.27,  $t_{44}=1.95$ ,  $P=0.058$ ) suggests that this effect was slightly more pronounced in California birds than in Washington birds. There was no effect of time since treatment on this difference (population  $\times$  treatment  $\times$  time,  $P>0.10$ , removed from model). In Washington, but not California, birds in better initial body condition tended to have higher corticosterone concentrations (population  $\times$  initial body condition: estimate=-4.22,  $t_{44}=-2.57$ ,  $P=0.014$ ).

Plasma concentrations of luteinizing hormone were reduced in LPS-treated birds (Fig. 3C, supplementary material Table S3, estimate=-1.74,  $t_{44}=-3.58$ ,  $P=0.001$ ). The non-significant interaction between population and treatment ( $P>0.10$ , removed from model) shows that this pattern did not differ between populations. Overall, luteinizing hormone levels were lower at 22 h after treatment than at 6 h after treatment, a trend that was driven by control rather than LPS-treated birds (Fig. 3C, time of sampling: estimate=-0.070,  $t_{44}=-2.86$ ,  $P=0.006$ , treatment  $\times$  time: estimate=0.077,  $t_{44}=2.98$ ,  $P=0.005$ ).

#### DISCUSSION

When housed in a common laboratory environment, male song sparrows from California exhibited more pronounced levels of fever and lethargy in response to simulated infection (LPS) than did conspecifics from Washington (see Table 2 for a summary of all variables measured). These results are consistent with differences in fever and lethargy between these populations in the wild (Adelman et al., 2010). This outcome suggests that population differences in the wild do not result exclusively from immediate environmental conditions, but could represent genetic or early-life divergence in immune defense. Furthermore, differences in pro-inflammatory cytokine signaling may underlie this divergence in the acute phase immune response, as IL-6-like bioactivity was higher in birds from California than in birds from Washington at both 6 and 22 h post-treatment. In contrast to fever and lethargy, anorexia did not differ between populations, suggesting that different

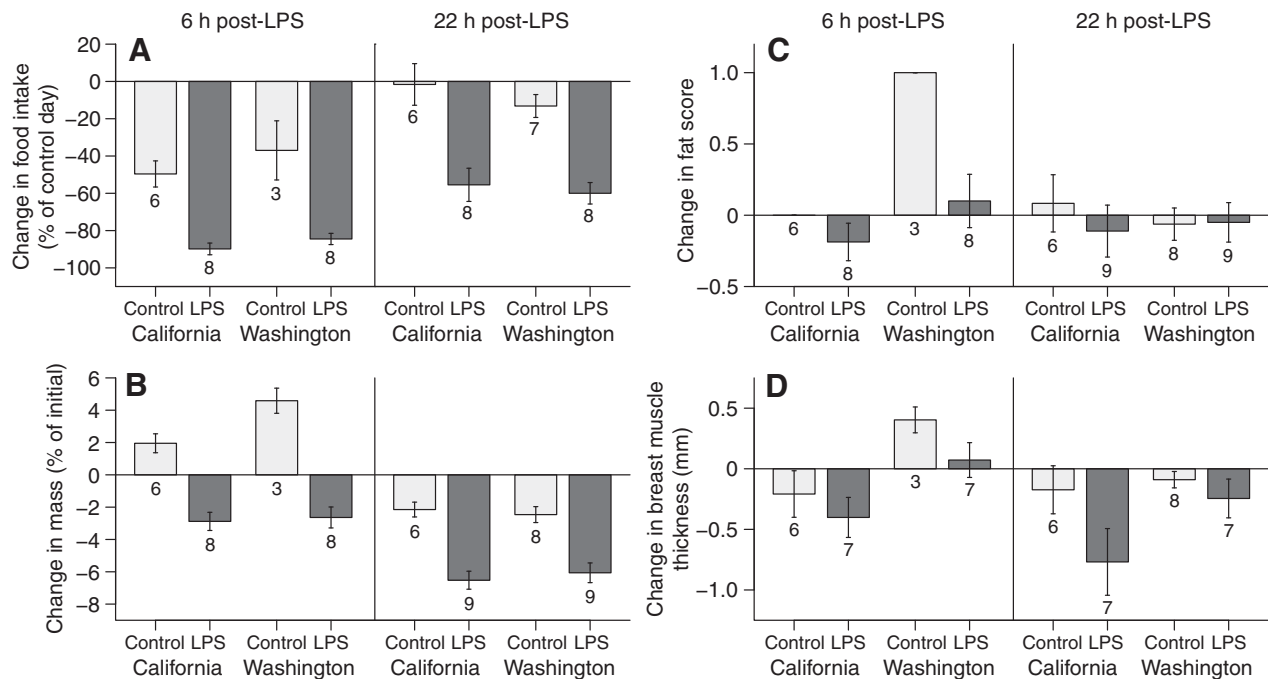


Fig. 2. Decreases in food intake and mass loss were similar in LPS-treated birds from California and Washington (A,B). Fat scores did not show strong patterns with LPS treatment, but breast muscle thickness tended to decline more sharply in LPS-treated birds from California (C,D). Sample sizes are indicated below the bars, error bars show  $\pm 1$  s.e.m.

behavioral components of the acute phase response may be regulated independently among song sparrow populations (Martin et al., 2008a; Adelman and Martin, 2009).

This study's factorial design was intended specifically to probe for such inter-population differences. This design, however, limited the sample sizes and statistical power needed to assess correlations among different physiological and immunological measurements at the inter-individual level within treatment groups. Future studies designed to test such questions will help refine the precise relationships among signaling molecules and population differences in the acute phase response. Nevertheless, this study represents an important first step in determining how and where selection could act to alter investment in immune function among wild vertebrate populations.

### Population differences in the acute phase response

#### Lethargy and fever

In response to LPS treatment, male song sparrows from California showed prolonged lethargy, as measured by decreased locomotor activity, when compared with birds from Washington (Fig. 1A,C, Table 1). Despite these population differences in the duration of lethargy, the magnitude of the decrease in activity was similar: the lowest average level of activity for treated birds from each population was 1–3 min (5–10%) out of a 30-min sampling period (Fig. 1A,C, supplementary material Table S1). However, assessing only the magnitude of such decreases would risk missing differences from 9 to 12 h after injection, when treated birds from California remained inactive but treated birds from Washington returned to control levels of activity (Fig. 1A,C). These differences in the duration of lethargy could have important consequences for territorial defense and reproductive success in the wild (Bonneaud et al., 2003; Owen-Ashley and Wingfield, 2006), as recovering normal levels of activity 3 h earlier can represent 20–25% of the daylight hours during the early breeding

season. This result suggests that birds from California invest more time in their acute phase responses. The populations also showed marked differences in fever, with LPS-treated California birds mounting fevers that were, on average, 0.5°C above those of LPS-treated Washington birds throughout the night (Fig. 1B,D, Table 1, supplementary material Table S1). As fever can increase metabolic rate by 10–15% for every 1°C rise in core body temperature (Roe and Kinney, 1965), this result suggests that California birds invest not only more time but also more energy in the acute phase response.

#### Anorexia

While all birds, even controls, showed lower levels of food intake (compared with baseline) at 6 h post-treatment than at 22 h (Fig. 2A), this is most likely due to the calculation of baseline food intake: each animal's baseline was calculated as the total mass (g) of food eaten during an entire non-treatment day (*ca.* 13 h of light). Based upon this calculation, it is reasonable that controls from both populations had eaten only 40–50% of baseline levels by 6 h on the treatment day (Fig. 2A).

LPS-treated birds displayed clear anorexia and mass loss, but neither of these responses differed between populations (Fig. 2A,B). This similarity between the populations in food intake and mass loss suggests that anorexia in song sparrows may be regulated independently of other sickness behaviors, such as lethargy. Such differential regulation is known to occur in at least one other passerine: house finches (*Carpodacus mexicanus*) infected with *Mycoplasma gallisepticum* show little or no anorexia despite pronounced lethargy (Kollias et al., 2004; Bouwman and Hawley, 2010). Differences in the relative costs and benefits of individual sickness behaviors may drive their differential regulation among populations, species, or ecological settings (Adelman and Martin, 2009). For instance, because song sparrows in Washington have a shorter potential breeding season than those in California, with fewer



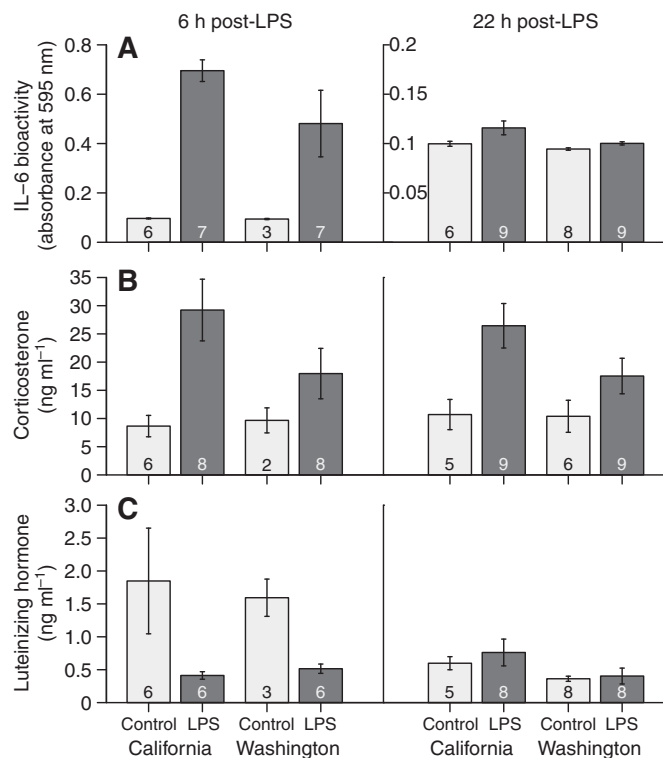


Fig. 3. Immune and endocrine signals in plasma differ between LPS-treated song sparrows from California and Washington. Interleukin-6 (IL-6)-like bioactivity (A) was higher in LPS-treated birds from California than in treated birds from Washington at both 6 and 22 h after injection (note that in order to display the small, but significant, differences at 22 h, the  $y$ -axes have been scaled differently). Corticosterone (B) was also marginally higher ( $P=0.058$ ) in LPS-treated birds from California than in treated birds from Washington. Luteinizing hormone levels (C) showed no difference between populations, but were lower in LPS-treated birds than in control birds at 6 h after injection. Sample sizes are indicated inside the bars, error bars show  $\pm 1$  s.e.m.

opportunities to make up reproductive losses, time may be a more critical resource in Washington, making sickness-induced lethargy more constrained in this population. By contrast, the benefits of anorexia, including reduced bacterial access to iron and slower bacterial replication (Grieger and Kluger, 1978; Hart, 1988; Exton, 1997), may outweigh its costs in both populations. Such differential cost-benefit ratios could exert selective pressure to maintain anorexia despite differences and lethargy.

Alternatively, the lack of population differences in anorexia may reflect coarser temporal sampling. Because data for food intake and mass loss were collected at only two time points post-treatment, rather than every 30 min, the power to detect differences in recovery rates in these parameters was lower than for fever and lethargy.

While overall changes in mass and fat scores among LPS-treated birds were similar between populations (Fig. 2B,C), LPS-treated birds from California tended to lose more breast muscle than LPS-treated birds from Washington (Fig. 2D). In birds, breast muscle represents a significant repository of protein (Jenni and Jenni-Eiermann, 1998; Bauchinger and Biebach, 2001) and is important for thermoregulation and thermogenesis (Marjoniemi and Hohtola, 1999). The patterns of breast muscle loss in Fig. 2D are consistent with California birds making more use of this resource to fuel their higher fevers. Such differences could also help explain results from

Table 2. Summary of results for all measurements of the acute phase immune response for California and Washington song sparrows

Measurement	Response to LPS	Magnitude of response
Body temperature	+	CA>WA
Locomotor activity	-	CA>WA
Food intake	-	CA=WA
Body mass	-	CA=WA
Breast muscle	-	CA>WA
Interleukin-6	+	CA>WA
Corticosterone	+	CA>WA
Luteinizing hormone	-	CA=WA

+, increase; -, decrease.

the wild in which California birds mounting higher nocturnal fevers made fewer territorial flights 24 h after injection (Adelman et al., 2010). If birds utilize flight muscle to fuel fever, animals mounting the highest fevers may initiate fewer costly flights to conserve this fuel. Future research into the avian acute phase response should examine breast muscle more closely as it may represent a limiting resource to be allocated between immune defenses (fever) and reproductive investment (territorial defense).

#### Differences in immune and endocrine signaling IL-6

In this study, IL-6-like bioactivity was higher in LPS-treated California sparrows than in LPS-treated Washington sparrows (Fig. 3A). In vertebrates, LPS stimulates an acute phase response by binding to toll-like receptor 4 (predominantly), which triggers a cascade of gene expression that ultimately causes the release of pro-inflammatory cytokines, including tumor necrosis factor- $\alpha$  (in mammals), interleukin-1 $\beta$  and IL-6 (Janeway et al., 2005). These cytokines interact with both the central and peripheral nervous system to induce fever and sickness behaviors, including lethargy and anorexia (Dantzer, 2004). In addition, pro-inflammatory cytokines act centrally to stimulate the hypothalamic-pituitary-adrenal axis, resulting in increased release of corticosterone (Fleshner et al., 1995; Turnbull and Rivier, 1999). Given these critical roles, pro-inflammatory cytokine pathways are likely to be involved in population and species differences in acute phase responses. Our results suggest that mechanisms at or above the level of pro-inflammatory cytokine release underlie the divergent acute phase responses between these populations.

#### Corticosterone

Among control birds, circulating concentrations of corticosterone were comparable to other laboratory studies in this species (Owen-Ashley et al., 2004) and generally below baseline levels from studies in the wild (Wingfield, 1984; Wingfield, 1994; Newman et al., 2008). Corticosterone levels are typically elevated by pro-inflammatory cytokines associated with the acute phase response (Fleshner et al., 1995; Turnbull and Rivier, 1999; Besedovsky and del Rey, 2001). Indeed, in this study, birds from California released more corticosterone during the acute phase response than Washington birds (Fig. 3B, Table S3). As corticosterone can act as a brake on the acute phase response (Goujon et al., 1995; Goujon et al., 1997; Dantzer, 2004), this higher induction in California may result from the higher levels of cytokines and fever, and could prevent acute phase responses from progressing to pathological levels (Graham et al., 2005). Another non-exclusive explanation is that corticosterone stimulates metabolism and catabolism to fuel the



energetic needs of the higher fevers in this population (Landys et al., 2006). As corticosterone remained elevated at 6 and 22 h in both populations, both roles are feasible, potentially with enhancement of metabolism more pronounced at the earlier time point and downregulation of the acute phase responses occurring later.

#### Luteinizing hormone

At 6 h after treatment, LPS-injected birds from both populations showed pronounced reductions in circulating luteinizing hormone concentrations. This result is consistent with prior work in other wild passerines and laboratory animals (Rivier and Vale, 1990; Turnbull and Rivier, 1997; Besedovsky and del Rey, 2001; Owen-Ashley et al., 2006). In song sparrows, suppression of luteinizing hormone may be driven by direct, central actions of cytokines, rather than corticosterone, as exogenous corticosterone does not reduce luteinizing hormone levels in this species in the wild (Wingfield and Silverin, 1986).

The lack of population differences in luteinizing hormone was not consistent with our predictions. Based upon their weaker territorial responses in the wild (Adelman et al., 2010), we expected that California sparrows mounting an acute phase response would suppress reproductive hormones more than Washington sparrows. As with our data on anorexia, the lack of population differences in luteinizing hormone may be due to the coarse temporal scale of sampling. Additionally, the universally low levels of luteinizing hormone at 22 h likely reflect the timing of these samples: just after lights on, when avian luteinizing hormone secretion is near its circadian minimum (Chowdhury et al., 2010). Alternatively, the lack of social stimuli, or conditions in the lab generally, may have muted secretion of luteinizing hormone. In the wild, these patterns may be more nuanced because behavioral interactions, with both females and other males, have been shown to modulate levels of luteinizing hormone and testosterone (Moore, 1982; Wingfield et al., 1990). Future experiments could test whether luteinizing hormone suppression differs between populations when birds are allowed to interact following LPS treatment.

#### Comparison to results in the wild

In captivity, song sparrows from California and Washington showed qualitatively similar differences in fever and lethargy to those in the wild, but several distinctions were apparent. First, birds mounted fevers earlier in this study (*ca.* 2 h after injection, Fig. 1B,D) than in the wild [*ca.* 6 h after injection (Adelman et al., 2010)]. These differences may result from methodological constraints because cutaneously mounted radio transmitters record skin temperature most accurately when birds are not exposed to direct sunlight (Adelman et al., 2010). Therefore, the noise associated with movement between sun and shade could mask fever during daylight hours in the wild. In addition, *ad libitum* access to food or the stress of captivity and subsequent effects on body condition may have altered the progression of fever in captivity when compared with the wild. While overall mass was not significantly higher on the morning of injection than at initial capture in the wild (paired *t*-test,  $P=0.38$ ), fat scores were significantly higher in captivity (paired Wilcoxon test,  $P<0.001$ ), suggesting that changes in physiological reserves could contribute to febrile differences between captive and wild birds.

Population differences in lethargy were somewhat less dramatic in the present study than in the wild. The limited space and opportunity for mobility in captivity likely contributed to this difference. Control birds in captivity spent an average of 40–60% of their time active (Fig. 1A,C), in sharp contrast to the 80–85% of time spent active in the wild (Adelman et al., 2010). Therefore, in

captivity, a decrease to 0% of time spent active necessarily represents a smaller relative change than in the wild. Alternatively, such differences could reflect the influence of stress due to the laboratory, as captivity can alter immune parameters in other small passerines (Kuhlman and Martin, 2010). Additionally, because the doses of LPS used in this study and in the field study were adjusted per unit mass, and because some birds were heavier in captivity than they would have been in the field, some captive birds may have received a higher absolute dose. However, as mass was not significantly different between the field and laboratory, LPS dosage alone is unlikely to account for differences in lethargy between the field and laboratory. Moreover, these differences were apparent in birds from both California and Washington, suggesting that captivity did not differentially influence immune responses between populations.

#### Evolved *versus* plastic differences in the acute phase response

Given that population differences in the acute phase response persist in a common environment, it is unlikely that the population differences observed in the wild were directly caused by immediate environmental factors like temperature or humidity. However, maternal effects and other early-life experiences, including pathogen exposure, could contribute to these differences (Saino et al., 2002; Jackson et al., 2009; Morrison et al., 2009). For instance, maternal transfer of hormones or other molecules to eggs can influence physiological, behavioral and immunological phenotypes in offspring (Saino et al., 2002; Groothuis et al., 2005; Grindstaff et al., 2006; Gasparini et al., 2009; Hasselquist and Nilsson, 2009). In addition, in several mammalian species, early life exposure to stressors and pathogens can program immune responses in later life (Galic et al., 2009; Bilbo et al., 2010).

In contrast to these examples of plasticity *in ovo* and during early life, differential cytokine and febrile responses have been artificially selected between strains of chickens (Leshchinsky and Klasing, 2001), suggesting a potential genetic component to variation in the acute phase response. Coupled with the results of the present study, this finding suggests that selection on cytokine production, or immunological recognition processes upstream, may play an important role in shaping population differences in the acute phase responses of wild animals. With continued sequencing of cytokine genes and other molecules involved in the passerine acute phase response, we will be able to better pinpoint the precise mechanisms underlying the observed population differences. Through such advances, we can determine whether selection has acted on these mechanisms and clarify how differential investment in immunity can evolve among wild populations.

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