Changes in Body Surface Temperature Play an Underappreciated Role in the Avian Immune Response

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ABSTRACT

Fever and hypothermia are well-characterized components of systemic inflammation. However, our knowledge of the mechanisms underlying such changes in body temperature is largely limited to rodent models and other mammalian species. In mammals, high dosages of an inflammatory agent (e.g., lipopolysaccharide [LPS]) typically leads to hypothermia (decrease in body temperature below normothermic levels), which is largely driven by a reduction in thermogenesis and not changes in peripheral vasomotion (i.e., changes in blood vessel tone). In birds, however, hypothermia occurs frequently, even at lower dosages, but the thermoeffector mechanisms associated with the response remain unknown. We immune challenged zebra finches (Taeniopygia guttata) with LPS, monitored changes in subcutaneous temperature and energy balance (i.e., body mass, food intake), and assessed surface temperatures of and heat loss across the eye region, bill, and legs. We hypothesized that if birds employ thermoregulatory mechanisms similar to those of similarly sized mammals, LPS-injected individuals would reduce subcutaneous body temperature and maintain constant surface temperatures compared with saline-injected individuals. Instead, LPS-injected individuals showed a slight elevation in body temperature, and this response coincided with a reduction in peripheral heat loss, particularly across the legs, as opposed to changes in energy balance. However, we note that our interpretations should be taken with caution owing to small sample sizes within each treatment. We suggest that peripheral vasomotion, allowing for heat retention, is an under-appreciated component of the sickness-induced thermoregulatory response of small birds.

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Introduction

In vertebrates, the response to infection is typically characterized by a suite of sickness symptoms, including reduced food intake, activity, and changes in body temperature ($T_b$; Owen-Ashley and Wingfield 2007). Changes in $T_b$ can take the form of fever (increase in $T_b$ above normothermia), hypothermia (decrease in $T_b$ below normothermia), or a combination of the two (e.g., a biphasic response consisting of initial hypothermia followed by fever; Romanovsky et al. 2005). During sickness, regulation of $T_b$ is coordinated through behavioral and autonomic responses that together result in increased or decreased heat conservation and heat production (Romanovsky et al. 2005). Behavioral responses may include seeking preferred temperatures and/or adopting postures to conserve or reduce heat loss, while autonomic responses may include adjustments in metabolic rate and peripheral vasomotion. The relative contribution of different thermoeffectors (e.g., shivering thermogenesis, changes in peripheral blood flow) on $T_b$ adjustment is known mostly from mammalian studies, which indicate that the dosage of endotoxin is a critical determinant of the resultant effector responses (reviewed in Romanovsky et al. 2005; Garami et al. 2018; Lopes et al. 2021).

In mammals, the febrile response is mediated by increases in both metabolic heat production and peripheral vasoconstriction, with the relative importance of each varying according to ambient temperature (Rudaya et al. 2005; Garami et al. 2018). In contrast, the hypothermic response is mostly mediated by the inhibition of thermogenesis, without changes in peripheral vasomotion (Roth and Blatteis 2014). In rats exposed to the endotoxin lipopolysaccharide (LPS), vasodilation in the tail remains relatively constant as core $T_b$ falls (Romanovsky et al. 1996; Steiner et al. 2005).

In birds, endotoxin exposure commonly elicits hypothermia instead of fever (Owen-Ashley et al. 2006; reviewed in Owen-Ashley and Wingfield 2007; Burness et al. 2010; Lopes et al. 2014; Sköld-Chiriac et al. 2015; Ben-Hamo et al. 2017). There is some evidence that mammals and birds share similar thermoregulatory pathways during the immune response (Dantiono et al. 2016), however, the role of peripheral vasomotion in birds and the
specific anatomical structures that may contribute to sickness-induced changes in $T_i$ have not yet been characterized. Such information is important for understanding how mechanisms driving the febrile response may have evolved across endotherms.

The avian bill is recognized as an important structure associated with regulation of peripheral heat exchange (Tattersall et al. 2016; Schraft et al. 2019). It is a highly innervated and uninsulated organ that rapidly responds to variation in environmental temperature via constriction or dilation of subcutaneous blood vessels (Tattersall et al. 2016). Like the bill, avian legs are largely uninsulated, and in many species, the legs are also an important source of heat exchange (Martineau and Larochelle 1988; Ward et al. 1999). However, because they conserve heat via counter-current heat exchange, the role of the legs in heat dissipation is likely minimized relative to the bill (Tattersall et al. 2016). Recently, the highly vascularized periorbital region (hereafter, “eye region”) has also been shown to play an important role in the regulation of heat exchange in response to a variety of stressors (Jerem et al. 2015, 2019; Robertson et al. 2020). However, because it comprises a relatively small proportion of total body surface and because local countercurrent heat exchangers are present in the area (Midtgård 1983; but see Powers et al. 2015), it too may play a relatively small role in total peripheral heat exchange. Nonetheless, despite our increasing understanding of the bill, legs, and periorbital region as modulators of heat exchange, to our knowledge, no studies have explored their individual contributions to heat exchange during the sickness-induced thermoregulatory response.

Using the zebra finch (Taeniopygia guttata, Gould 1837) as a model species, we assessed what thermoeffectors contribute to sickness-induced changes in $T_i$. To accomplish this we first had to determine whether individuals would mount a fever, or hypothermic response. Next, depending on the type of thermal response generated, we sought to understand how birds achieve this via the management of peripheral heat loss. Experimentally, sickness behaviors can be induced by exposure to an endotoxin, such as LPS. LPS is a component of the outer cell wall of a gram-negative bacteria, and injection of LPS results in $T_i$ adjustments, without the associated costs of pathogen infection. If individuals mount a fever in response to endotoxin exposure (here, LPS), we predicted an increase in subcutaneous $T_i$, coinciding with changes in peripheral vasomotion (i.e., decreased peripheral heat loss manifesting through lower surface temperatures) compared with controls. If, however, individuals become hypothermic in response to endotoxin exposure, we predicted a decrease in subcutaneous $T_i$ but no changes in peripheral vasomotion (i.e., peripheral heat loss remains constant, reflective of a constant surface temperature) compared with controls. It is important to note that our predictions relate to animals in a laboratory setting. In free-ranging individuals, the directionality of the thermal response may be related to an individual’s energy reserves. For instance, mounting a relatively “cheaper” fever by adjusting changes to peripheral blood flow while reducing the costs of raising the metabolic rate may be advantageous when individuals have low energy reserves. This line of reasoning is consistent with studies showing that the immune response is attenuated when animals have low energy availability (e.g., Carleton and Demas 2015, 2017). The results of our study could therefore be used to provide context to studies examining the immune response in wild animals.

**Methods**

**Animal Care and Housing**

Zebra finches were sex segregated and group housed in walk-in environmental chambers, provided with food and water ad lib., and maintained in a 14L:10D cycle. Environmental chambers were maintained at 26°C, a few degrees below the thermoneutrality of zebra finches (Calder 1964; Briga and Verhulst 2017). We chose this temperature because thermal responses to simulated infection have been demonstrated in finches at temperatures below thermoneutrality (Burness et al. 2010; Sköld-Chiriac et al. 2015). Two weeks before the onset of the experiment, we injected a temperature-sensitive passive integrated transponder (PIT) tag (BioThermo13, Biomark, Boise, ID) subcutaneously into the nape of 10 adult male and 10 adult female zebra finches. Before implantation, we sterilized the site of implantation with 70% ethanol, and after implantation, we sealed the perforation with tissue glue (VetBond; sensu Nicolaus et al. 2008). Final sample sizes, however, differed between analyses because of data loss associated with human and equipment error, as well as the death of two individuals (one LPS, one saline).

At 25.5 h before experimentation (0900 hours, day 0; fig. 1), we transferred each individual to an individual wooden cage to

![Figure 1. Study time line. Individuals were transferred from communal cages to individual experimental cages 25.5 h before injection (day 0). Monitoring of subcutaneous body temperature ($T_i$) began at 0930 hours on day 0 and ended at 0900 hours on day 2. On the day of injection (day 1), we collected blood samples at 0900 hours and subsequently recorded finches’ mass and weighed their food (~30 g). We then injected finches at 1030 hours with a saline or lipopoly saccharide solution. Monitoring of peripheral $T_i$ (via thermographic imaging) was used to calculate dry heat transfer and began at 0900 hours and ended at 1600 hours. A second blood sample was taken at 1500 hours (4.5 h after injection). On day 2, we again recorded individual body mass and food mass.](image-url)
(31 cm × 30 cm × 32 cm, L × W × H; 2.5-cm² polyethylene grid), located in an identical, adjacent, walk-in experimental chamber to reduce the effects of handling stress on Tb (Oka et al. 2001). We used a wooden cage because metal can interfere with temperature-sensitive PIT tags (see "Remote Monitoring of Body Temperature"). During experiments in the adjacent experimental chamber, we maintained a subset of individuals from the colony (~10 individuals, housed in flight cages) so that the experimental individuals could hear and see other members of the colony. All experiments followed the guidelines of the Canadian Council on Animal Care and were approved by Trent University's Animal Care Committee (AUP no. 25110).

**Injection and Blood Sampling Procedures**

On the day of experimentation (day 1; fig. 1) at 1030 hours, we injected finches either with 100 µL LPS kg⁻¹ (derived from *Escherichia coli*, L288, Sigma Aldrich, Oakville, Ontario) diluted in 50 µL of sterile phosphate buffered saline (PBS) or with 50 µL of PBS (control group) in the pectoral muscle. LPS-induced thermal responses have been demonstrated in zebra finches at 100 µg LPS kg⁻¹ (Sköld-Chiriac et al. 2015). Before injection, we sterilized the skin surrounding the injection site with 70% ethanol. To minimize order effects, we alternated between administering LPS and saline injections.

As part of a parallel study, we collected two blood samples (50–75 µL from the brachial vein) at 0900 and 1500 hours on the day of experimentation (day 1; fig. 1). After blood sampling, Tb fell below baseline levels for 15–30 min (table A1; fig. A1). Because of this temporary drop in Tb, data between 0900 and 0930 hours and between 1500 and 1530 hours were removed from our analysis. Exclusion of these data did not influence trends in our analyses at our chosen α level (tables A2, A3).

**Remote Monitoring of Body Temperature**

We recorded subcutaneous Tb of PIT tag–implanted individuals using an HPR Plus reader (Biomark, Boise, ID), which we connected to a loop antenna (17.5 cm) that encircled the center of the perch within the wooden experimental cage. PIT tag Tb has been shown to be correlated with core temperature (Nord et al. 2013) and to effectively capture the thermally induced immune response in birds (Sköld-Chiriac et al. 2015). We recorded the Tb of each individual for 47.5 h: from ~0930 hours on day 0 to 0900 hours on day 2 (fig. 1), but only data on day 1 were used in this study. Measurements were recorded every 5 min for the entire duration of the recording period.

Changes in peripheral vasoemotion are reflected as changes in surface temperature, which can be monitored noninvasively using infrared thermography (McCafferty et al. 2011). We therefore measured surface temperature across all body regions of exposed integument (i.e., legs, bill, and eye region) by infrared thermography from ~1 h before injection (0930 hours, day 1) to 5.5 h after injection (1600 hours; 6.5 h of filming total). We chose this time period for filming to capture the peak response to LPS, which occurs 3–5 h after injection (Burness et al. 2010; Lopes et al. 2014; Sköld-Chiriac et al. 2015). We captured thermographic images at 0.07 Hz (1 frame per 15 s; model SC660, FLIR Systems, Wilsonville, OR; lens focal length, 75 mm; field of view, 24°; image resolution, 640 × 480; accuracy, ± 1°C) from a distance of 1 m from the experimental cage. To estimate regional surface temperature of finches, we first converted infrared radiation values per pixel to temperature values per pixel (°C) according to our environmental conditions (ambient temperature and humidity), Planck’s law, and equations described elsewhere (Minkina and Dudzik 2009; Tattersall 2016) and as outlined in the R package Thermimage (Tattersall 2019). Emittance (ε) of the exposed integument was estimated as 0.95 (Best and Fowler 1981), and calibration constants for our camera were extracted from image metadata using ExifTool (https://exiftool.org/). Surface temperature of the bill, legs, and eye region of finches were then estimated from converted thermographic images in ImageJ (https://imagej.nih.gov/ij/) by manually drawing regions of interest (ROIs) around each region and extracting the maximum temperature within each ROI (Jerem et al. 2015; Robertson et al. 2020; fig. A2). Maximum temperature was selected as our index of surface temperature to minimize error associated with inaccurate regional perimeter selection (Jerem et al. 2015). Finally, images blurred from motion were excluded from analyses to minimize underestimation of surface temperature, as described by (Tattersall 2016). In total, surface temperature measurements from 1,381 thermographic images (n_LPS = 234, n_saline = 1,147) collected from 16 individuals (n_LPS = 7, n_saline = 9) were included in our analyses. Although the spatial orientation of an individual is known to influence estimates of its surface temperature by infrared thermography (Playà-Montmany and Tattersall 2021), we do not expect mean orientation to systematically differ across treatments or across time. Therefore, an influence of individual orientation on surface temperature estimates is unlikely to bias our reported findings. Nonetheless, we explored the possible influence of head angle on surface temperature (appendix; table A4). This subsequent analysis did not change our claims of statistical significance or influence our general conclusions.

To quantify the mean rate of dry heat transfer at each body region (q_Leg, q_Bill, q_Eye; mW) among finches, we used methods described by Nord and Nilsson (2019) and equations summarized in Robertson et al. (2020). Briefly, radiative and convective heat transfer rates (q_rad and q_conv, respectively) were calculated for the legs, bill, and eye region of finches from thermographic images, then summed per image (representing 15 s; q_Tot = q_rad + q_conv) and averaged across each half-hour interval. Given that there were many more thermal images from the saline group compared with the LPS group (n_LPS = 234, n_saline = 1,147), the ratio of individuals in each treatment differed across half-hour intervals, and our sample sizes, especially for the LPS group, were very small at each time point (table A5). This is because, in many thermographic images, we could not simultaneously detect the temperature of the legs, bill, and eye region, which was a consequence of several factors, including the birds facing away from the camera, one or more body regions being obscured by the food bowl, and images being unfocused (e.g., if the bird moved to the back of the cage and focus was at the front). Because of the small sample sizes
at each time point, we therefore acknowledge that our summative heat transfer results are preliminary in nature. Heat transfer by conduction was expected to be minimal across our anatomical ROIs (for legs, the sole of the foot was excluded), and as such, conductive heat transfer was excluded from all q calculations. Wind was absent during our experiment, and thus Reynold’s number was assumed to be 0 (McCafferty et al. 2011). Mean surface areas of the legs, bill, and eye region of our finches were estimated as 5.3 cm² (open cylinder; width = 0.5 cm), 0.7 cm² (open cone; length = 0.8 cm), and 0.7 cm² (planar oval; width = 1.0 cm), respectively, according to those reported for another species of comparable body size and morphometry (dark-eyed juncos, Junco hyemalis; legs and bill; Walsberg and King 1978) and according to measurements from our sample population (eye region).

Energy Balance

We indirectly measured changes in energy balance in response to sickness by quantifying the degree of movement, food consumption, and body mass loss during experimental treatment.

Activity Level. We quantified individual activity from thermographic images in R (ver. 4.0; R Core Team 2020) using an approach similar to that of Tattersall et al. (2020). Specifically, we estimated the occurrence of movement between temporally (i.e., 0.07 s apart) adjacent thermographic images by calculating the difference in temperature at each pixel ($n_{pixels/image} = 307,200$) and then categorizing pixels as “changed” or “unchanged” according to whether a temperature difference of ≥1.5°C was detected. Because ambient temperature was held constant during our experiment, regional changes in temperature exceeding 1.5°C were unlikely to occur in the absence of individual movement. Next, we calculated the percentage of pixels that were categorized as changed and then labeled thermographic images as “displaying movement” if this value exceeded 1% of the total number of pixels; otherwise, we assumed that no movement had occurred. Relative movement displayed by each individual throughout thermographic filming (0930–1600 hours) was then calculated as the percentage of thermographic images wherein movement was displayed. To ensure that movement estimates were not biased by experimental procedures (i.e., handling, LPS or saline injections), all images containing experimenters or lacking focal individuals were excluded from analysis ($n = 910$ of 27,928 thermographic images). Furthermore, one individual was excluded from analyses because of excessive movement as a result of a fallen perch. In total, movement data were included for 16 individuals ($n_{LPS} = 8$, $n_{saline} = 8$).

Food Consumption and Mass Loss. To determine the effect of sickness on food consumption, we initially placed 30 g of seed into a tubular-shaped feeder that we fastened onto the side of the cage adjacent to the perch, and 24 h following injection, the food remaining in the container was reweighed. However, we found that it was difficult to measure the amount of food consumed by the birds because of seed scatter. We therefore switched methodologies partway through the experiment, placing the seed (30 g) into an open-top 946-mL plastic container on the floor of each individual’s cage immediately before injection. Of the 20 birds initially included in the analysis, the first eight fed from tubes ($n_{LPS} = 4$, $n_{saline} = 4$), and the remaining 12 ($n_{LPS} = 6$, $n_{saline} = 6$) fed from plastic containers. Because of the depth of the containers, we found that virtually no food was scattered throughout the cages; however, any food that was scattered was assumed to be random across treatments. To determine the effect of sickness on mass loss ($n_{LPS} = 10$, $n_{saline} = 10$), we measured individual body mass just before injection (day 1, 1030 hours) and 24 h following injection (day 2, 1030 hours; fig. 1), using a digital pan balance (±0.1 g).

Data Organization and Statistical Analyses

All data organization and statistical analyses were conducted with R, and we considered $P \leq 0.05$ as statistically significant.

Subcutaneous Temperature. To assess changes in subcutaneous $T_b$ after injection, we calculated the average $T_b$ ($T_b$ every 30 min for each individual from 0900 to 1600 hours on the experimental day (i.e., day 1; fig. 1). We removed all $T_b$ measurements that were 4 SDs either above (44.5°C; $n = 0$) or below (37.5°C; $n = 8$; −0.1% of observations) the mean, before calculating the half-hourly averages.

We used the lme4 package (Bates et al. 2015) to model $T_b$, with treatment, time (after injection), sex, and treatment × time as fixed effects and individual identity as a random intercept. Following Sköld-Chriac et al. (2015), we used a second-order polynomial ($time^2$ and treatment × time$^2$) to account for nonlinearity in the $T_b$ response. We used Wald 95% confidence intervals as a measure of error around our estimates.

Summative Heat Dissipation

To assess changes in heat dissipation over time, we used a generalized additive mixed model (mgcv package; Wood 2011) with treatment and sex as parametric predictors, time and treatment × time as fixed effects and individual identity as a random intercept. We weighted observations by the inverse variance of treatment to control for differences in residual variance with respect to heat dissipation.

Regional Body Temperatures. To assess the relative contribution of different body regions to summative heat dissipation during the sickness response, we examined whether saline-injected individuals and LPS-injected individuals differed from each other in eye, bill, or leg temperature at the peak of the immune response (3–5 h after injection). To be conservative, we therefore calculated the average temperature for each bird at each region between 2 and 4 h after injection. We excluded data past 4 h because of the potential influence of blood sampling (at 4.5 h after injection) on $T_b$. We subsequently compared the regional temperature means of each treatment using a two-sample t-test (for a total of three tests).
Energy Balance. To determine how endotoxin exposure influenced movement and mass loss, we compared relative movement (%) and change in body mass (g; preinjection mass – postinjection mass) of individuals between treatments using unpaired two-sample t-tests with unequal variance. To determine how endotoxin exposure influenced food consumption, we used a linear model with seed consumption as the dependent variable and treatment and initial body mass as fixed factors. We did not include sex to avoid overparameterizing our model. We removed two (LPS) individuals from the food consumption analysis because these individuals were >4 Pearson residuals above 0 (amount of food consumed = 15.7 and 8.4 g; grand mean = 3.5 g), and the recorded values were a consequence of observer measurement error and/or food scattering associated with collecting data from the tube feeders. To ensure that the food consumption method (i.e., feeder type) did not differentially influence our results, we also ran the linear model including feeder type as a fixed factor. Feeder type did not significantly affect seed consumption or otherwise alter the trends in our results, and thus we excluded this factor from our final model.

Results

Subcutaneous Temperature

Subcutaneous $T_b$ differed between treatments as a function of time (i.e., treatment × time; table 1). In saline-injected birds, subcutaneous $T_b$ decreased by ~0.4°C from 1.5 h before injection to ~4 h after injection, before rising again for the duration of the measurement period (hour 4.5 to hour 5.5; fig. 2A). A similar decrease in subcutaneous $T_b$ was not observed in LPS-treated birds, with subcutaneous $T_b$ in these individuals remaining consistently elevated relative to saline-injected birds (5.5 h; table 1; fig. 2A). Males and females did not significantly differ in subcutaneous $T_b$ (i.e., sex; table 1).

Summative Heat Dissipation

The rate of heat dissipation across the bill, eye region, and legs was nonlinear over time (i.e., time; table 2) and differed significantly with respect to treatment (i.e., treatment × time; table 2; fig. 2B). Saline-injected birds maintained a relatively consistent heat dissipation rate of 75 mW across the duration of the measurement period (e.g., 5.5 h), while LPS-injected birds restricted heat loss over time (fig. 2B). Minimum heat loss in LPS-injected birds occurred at ~2.5 h after injection (45 mW) and returned to preinjection levels (1.5 h before injection; 60 mW) by 5.5 h after injection (fig. 2B). Male and females did not significantly differ in heat dissipation output (i.e., sex; table 2).

Regional Body Temperatures

Between 2 and 4 h after injection (fig. 3A), when the response to LPS is expected to be greatest, LPS-injected birds and saline-injected birds did not differ from each other in average bill ($t = 0.06, 95\% CI: \pm 2.51$ to $2.67, P = 0.949$), eye ($t = 0.79, 95\% CI: \pm 0.59$ to $1.27, P = 0.442$), or leg temperature ($t = -2.00, 95\% CI: \pm 7.50$ to $0.32, P = 0.068$). However, LPS-injected birds, but not saline-injected birds, experienced a decrease in leg temperature between 2 and 3.5 h after injection (fig. 3B). This decrease coincided with the increase in subcutaneous $T_b$ (fig. 2A), but a similar pattern was not seen for eye and bill temperature (fig. 3C, 3D). Regardless, a lack of statistical difference at the individual tissues/structures suggests that summative heat dissipation is more important than each individual body region.

Sickness Behaviors

Across the duration of the experiment (i.e., 0930–1600 hours, day 1), LPS-injected individuals spent significantly less time moving than saline-injected individuals (fig. 4A; $t = -2.58, 95\% CI: \pm 28.36$ to $2.13, df = 10, P = 0.027$; percentage of time spent moving $\pm \text{SEM} = 24.53\% \pm 4.76\%$ and 9.28% ± 3.62% for saline and LPS treatments, respectively). On average ($\pm \text{SEM}$), LPS-injected individuals did not differ in 24-h mass loss compared with saline-injected individuals (fig. 4B; saline: 1.58% ± 0.88%; LPS: 1.79% ± 0.67%; 95% CI: $-2.13$ to $2.55, t = 0.19, df = 15, P = 0.681$). However, LPS-injected individuals had

| Predictor                  | Estimate | 95% CI     | $t$   | $P$   |
|----------------------------|----------|------------|-------|-------|
| Intercept [LPS]            | 41.20    | 40.88 to 41.50 | 257.01 | <.001 |
| Treatment [saline]         | -.20     | -.57 to .17  | -1.05 | .309  |
| Time                       | 1.02     | -.39 to 2.42 | 1.41  | .160  |
| Time$^2$                   | 1.55     | .14 to 2.97  | 2.15  | .033  |
| Sex [male]                 | .26      | -.11 to .63  | 1.34  | .198  |
| Treatment × time           | -3.18    | -5.25 to -1.11 | -2.99 | .003  |
| Treatment × time$^2$       | 1.01     | -1.06 to 3.07 | .95   | .342  |

Note. Data from 0900 to 0930 hours and from 1500 to 1530 hours have been removed from the analysis (see text). Brackets indicate reference category. Bold values indicate statistical significance at $P \leq 0.05$. LPS = lipopolysaccharide; CI = confidence interval.
Figure 2. Subcutaneous temperature (A) and summative dry heat transfer (B) of zebra finches in response to injection of lipopolysaccharide (LPS; 100 μg LPS kg⁻¹) or saline solution. Points represent the raw data averaged (±SEM) at each half hour, according to treatment. Time 0 is 1030 hours, the time of injection. Gray bars indicate the periods of time removed from the analysis (i.e., 0900–0930 and 1500–1530 hours). Solid and dashed lines indicate the saline and LPS treatments, respectively. In B, numbers within boxes indicate the number of individuals within each treatment at each time point (top row shows LPS, bottom row shows saline). Points without error bars represent estimates from a single individual because data were not available for multiple birds.

Table 2: Factors affecting the heat dissipation output of zebra finches injected with a saline or endotoxin (100 μg LPS kg⁻¹) solution

| Predictor           | Estimate | 95% CI       | t    | P      |
|---------------------|----------|---------------|------|--------|
| Intercept [saline]  | 61.28    | 41.94 to 80.61| 6.21 | <.001  |
| Treatment [LPS]     | -15.94   | -33.92 to 2.03| -1.74| .09    |
| Sex [female]        | 2.42     | -23.06 to 27.90| .19  | .85    |

| Predictor               | EDF; ref. EDF | SE  | F    | P   |
|-------------------------|---------------|-----|------|-----|
| Time                    | 2.36; 2.62    | 4.90| 3.86 | .021|
| Treatment × time        | 2.43; 2.75    | 10.84| 2.90 | .030|
| Bird identity           | 11.75; 13.00  | 12.72| 15.77| <.001|

Note. Data from 0900 to 0930 hours and from 1500 to 1530 hours have been removed from the analysis. Brackets indicate reference categories. Standard errors of smoothed terms represent averages across predictor knots. Confidence intervals (CIs; linear terms) and standard errors have been corrected for smoothness of smooth terms. Bold values indicate statistical significance at P ≤ 0.05. EDF = estimated degrees of freedom; ref. = reference; LPS = lipopolysaccharide.
lower 24-h food consumption compared with saline-injected individuals (fig. 4C; saline: 4.1 ± 0.27 g; LPS: 2.9 ± 0.30 g; linear model, $\beta = -1.17$, 95% CI: $-2.04$ to $-0.29$, $t = -2.60$, df = 15, $P = 0.012$). We did not find an effect of body mass on seed consumption ($\beta = -0.04$, 95% CI: $-0.30$ to 0.22, $t = -0.336$, df = 15, $P = 0.742$).

**Discussion**

In our study, zebra finches responded to an LPS endotoxin by maintaining a relatively elevated subcutaneous $T_s$ compared with saline-injected birds. Previous studies have shown that when small birds, and in particular zebra finches, are injected with LPS during the daytime (as replicated here), $T_s$ tends toward hypothermia (summarized by Sköld-Chiriac et al. 2015). It is surprising that we saw the opposite trend, because both the endotoxin (LPS serotype) and dosage (100 $\mu$g LPS kg$^{-1}$) have been previously shown to elicit hypothermia in zebra finches in other studies (Burness et al. 2010; Lopes et al. 2014; Sköld-Chiriac et al. 2015), and the ambient temperature used here (26°C) is also within the bounds of other studies. Furthermore, daytime hypothermia has been recorded with both PIT tags (Sköld-Chiriac et al. 2015) and cloacal probes (Burness et al. 2010; Lopes et al. 2014), suggesting that factors other than the acute effect of handling,
or measurement error, are important in determining the thermal response. Additionally, we housed our birds in social contexts similar to those in other studies (i.e., individuals were isolated in single cages but could see and hear other colony members; Burness et al. 2010; Sköld-Chiriac et al. 2015), meaning that it is unlikely that the effect of social context could explain our results in isolation. Such contrasting results could however be explained by the combined effects of stress exposure (i.e., blood sampling and handling) and LPS on \( T_b \). Blood loss itself is likely to lead to short-term decreased subcutaneous \( T_b \) (here, 15 min; see fig. A1) as heat is carried away from the peripheral tissues. Handling can either increase (stress-induced hyperthermia; Kluger et al. 1987; Cabanac and Guillemette 2001; Nord and Folkow 2019) or decrease (stress-induced hypothermia; Møller 2010; Maggini et al. 2018; Muise et al. 2018; Andreasson et al. 2020) \( T_b \), and it is unclear what factors invoke which type of thermal response. In our study, the stress-induced thermal response may therefore have been conflated with individual differences in the LPS-induced thermal response, leading to relatively higher \( T_b \) in LPS-injected birds. However, we acknowledge that subcutaneous \( T_b \) is not a direct measure of core \( T_b \) (e.g., cloacal \( T_b \)), and we are unsure whether the \( T_b \) response that we detected would have been relatively greater if we had measured the core \( T_b \) of individuals.

Our results provide novel, albeit preliminary, evidence that birds use peripheral vasomotion to adjust \( T_b \) during an immune challenge. Although dry heat transfer at the leg did not statistically differ between treatments during peak infection, it was lower for LPS-injected birds compared with saline-injected

Figure 4. Sickness behaviors in zebra finches injected with a saline or endotoxin solution (100 \( \mu \)g lipopolysaccharide [LPS] kg\(^{-1}\)). Box limits indicate the interquartile range, black lines indicate the median, and whiskers indicate \( \pm 1.58 \) times the interquartile range. Points represent the raw data. Asterisks indicate a statistically significant difference between treatments at \( P < 0.05 \). A, Percentage of time individuals spent moving after injection (\( n_{\text{saline}} = 8, n_{\text{LPS}} = 7 \)). B, Amount of body mass lost over 24 h (\( n_{\text{saline}} = 10, n_{\text{LPS}} = 10 \)). C, Amount of food consumed over 24 h (two outlier birds were removed from the analysis; \( n_{\text{saline}} = 10, n_{\text{LPS}} = 8 \)).
birds for most of the recording period, with the greatest drop at around the 3-h mark (fig. 3B). Additionally, in LPS-injected birds, the minimum rate of dry heat transfer across the leg coincided with the relative elevation in subcutaneous \( T_b \) at \( \sim 4 \) h after injection (compare fig. 3B with fig. 2A). Taken together, these results suggest that the increase in subcutaneous \( T_b \) in LPS-injected birds was probably driven, at least in part, by a reduction in heat loss via vasoconstriction at the leg, although all three regions together contributed to the thermal response. The legs have been shown to play an important role in thermoregulation during flight (Baudinette et al. 1976; Martineau and Larochelle 1988) and at thermoneutral and higher temperatures (Johansen and Bech 1983; Tattersall et al. 2009, 2018). Here, we show that they also play an important role in regulating the \( T_b \) during the immunological response.

The bill has been demonstrated to be an effective thermoregulatory organ across species (Tattersall et al. 2016) and in different contexts (e.g., flight [Schaft et al. 2019], energy conservation [Winder et al. 2020]). Here, we found that the role of the bill in the thermoregulatory immune response was minor, given that saline-injected birds and LPS-injected birds did not differ in average bill temperature at \( 2-4 \) h after injection and had largely overlapping bill temperatures for the duration of the immune challenge (fig. 3D).

We also found that eye temperature did not differ between treatments at peak infection, although LPS-injected individuals did trend toward higher values during this period (\( 2-4 \) h after injection; fig. 3C). Heat transfer at the eye may reflect changes in core temperature in birds (Ikikata and Watanabe 2015), and the slightly higher eye temperature in LPS-injected birds found here is consistent with this observation. Therefore, the eye may be an indicator, rather than a driver, of changes in \( T_b \) during the immune response.

Our results also provide evidence that the febrile response in our LPS-injected birds may differ from the mechanism used by mammals. In mammals, the thermoeffectector response during fever is dependent on the ambient temperature (Rudaya et al. 2005). For instance, at subneutral temperatures, increases in metabolic heat production tend to drive up \( T_b \); at supraneutral temperatures, peripheral vasoconstriction tends to predominate the response; and at thermoneutral temperatures, peripheral vasoconstriction and thermogenesis can occur simultaneously (reviewed in Garami et al. 2018). In our study, zebra finches were held slightly below thermoneutrality (29.5°C–40°C; Calder 1964). This means that if finches were to have used a mechanism similar to that of mammals, then we would have expected the relative rise in subcutaneous \( T_b \) among LPS-injected individuals to stem primarily from heightened metabolic heat production relative to saline-injected individuals rather than from increased peripheral vasoconstriction. While we cannot directly address changes in metabolic rate, our results provide little evidence for a relative increase in metabolically derived heat production among LPS-injected birds (i.e., shivering and nonshivering thermogenesis). First, LPS-injected birds spent significantly less time moving compared with saline-injected birds and were presumably generating less heat as a result. Second, despite a reduction in 24-h food consumption by \( \sim 25\% \) among LPS-injected birds, mass loss did not differ between treatments. Third, we found that LPS-injected birds restricted heat transfer compared with saline-injected birds over several hours after injection (at least at the legs). Finally, while subcutaneous \( T_b \) remained relatively constant among LPS-injected individuals, rates of peripheral heat loss decreased, suggesting that expenditure toward heat production was likely lower after injection than before injection. At our observed rates of peripheral heat loss, we estimate that expenditure toward heat production probably fell by as much as 7% following LPS treatment (according to metabolic rate estimates and conversion coefficients by Renning et al. [2005] and Gessaman and Nagy [1988], respectively).

To our knowledge, our study is the first to provide evidence that the endotoxin-induced thermoregulatory response in birds can be mediated by peripheral heat loss (i.e., vasoconstriction). During sickness, birds may favor a reduction in heat loss over an increase in heat production (as a means to conserve energy), owing to their relatively elevated metabolic rate compared with similarly sized mammals. Reducing the amount of energy required to mount an immune response may also apply at an individual level, with individuals of relatively poor body condition favoring a reduction in peripheral heat loss over an increase in metabolic rate. However, it remains unclear whether there is a relationship between the thermal mechanics of the immune response (e.g., fever vs. hypothermia) and the ability to clear pathogens from the body. Comparisons of populations that exist under different pathogen pressures or under different levels of food availability may provide interesting avenues for future research. To improve understanding of when and why animals utilize different thermal responses, future studies could also compare the metabolic rate and antibody response of individuals displaying hypothermia or fever, while examining changes in surface temperature. Additionally, our results are suggestive of mechanistic differences between mammals and birds in the thermal component of the immune response, which could derive from variations in the avian and mammalian cellular components used to detect pathogens (e.g., types of toll-like receptors; Gray et al. 2013) or from differences in cellular signaling pathways between taxa (Brownlie and Allan 2011), among other reasons. Future research comparing the cellular and molecular pathways involved in both mammals and birds during the sickness-induced thermoregulatory response will be necessary in bridging this gap.

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APPENDIX

Effects of Blood Sampling on Subcutaneous Temperature

To control for the effects of blood sampling on subcutaneous body temperature ($T_b$), we first had to determine whether, and for how long, $T_b$ deviated from baseline following blood sampling. To accomplish this, we first averaged the $T_b$ of each bird ($n = 20$) across six equally spaced 15-min time intervals (0830–0844, 0845–0859, 0900–0914, 0915–0929, 0930–0944, 0945–1000 hours) and subsequently ran a linear mixed effects model with average $T_b$ as the dependent variable and time interval as a fixed effect. We also included a random intercept for individual identity to control for repeated observations from the same individual.

$T_b$ significantly decreased in the first 15-min window following blood sampling (table A1), returning to baseline 30 min after blood sampling (fig. A1). The drop in $T_b$ (estimated marginal mean ± 1 SEM) from the first (0830–0844 hours; before blood sample) to the third (0900–0914 hours; after blood sample) time interval was $0.58 ± 0.25$°C. Note that we assessed changes in subcutaneous $T_b$ only, because we did not have thermographic data for birds before blood sampling.

Table A1: Effect of blood sampling on the subcutaneous body temperature ($T_b$) of finches ($n = 20$) across time

| Time interval | Estimate  | 95% CI     | t     | P       |
|---------------|-----------|------------|-------|---------|
| T1: 0830–0844 (intercept) | 41.71     | 41.30 to 42.13 | 194.15 | <.001   |
| T2: 0845–0859 | −.04      | −.52 to .44  | −.17  | .864    |
| T3: 0900–0914 | −.58      | −1.05 to −.11 | −2.38 | .020    |
| T4: 0915–0929 | −.35      | −.80 to .11  | −1.46 | .148    |
| T5: 0930–0944 | −.38      | −.83 to .07  | −1.61 | .110    |
| T6: 0945–1000 | −.43      | −.88 to .02  | −1.83 | .071    |

Note. Blood sampling occurred at approximately 0900 hours. From T1 (before blood sample) to T3 (15 min after blood sample), $T_b$ decreased by $−0.58$°C but had returned to preinjection levels by 30 min after blood sampling (i.e., T4). Bold values indicate statistical significance at $P ≤ 0.05$. CI = confidence interval.

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Figure A1. Changes in body temperature ($T_b$) in response to blood sampling before performing experimental injections. Blood sampling occurred at ~0900 hours (i.e., at the onset of T3). On average ($\pm$ 1 SEM), $T_b$ dropped by 0.58$\pm$0.27°C in the first 15-min period (T1 vs. T3) following blood sampling ($P = 0.020$). The difference in $T_b$ at 30 min after blood sampling (T1 vs. T4; 0.35$\pm$0.24°C) was no longer statistically different from $T_b$ before blood sampling ($P = 0.148$). Black points represent the estimated marginal means $\pm$ 95% confidence intervals, and white and gray points represent the raw data (i.e., each point represents an individual bird; sample size for each time period is given). White points indicate before blood sampling, and gray points indicate after blood sampling. To reduce the scale and improve clarity of the figure, seven data points (one $<39^\circ$C and six $>42.5^\circ$C) have been removed from the figure.

Subcutaneous $T_b$ and Heat Transfer Models
Tables A2 and A3 show the model results of the subcutaneous $T_b$ and heat transfer analyses, respectively, when the data from 0900 to 0930 and 1500 to 1530 hours were included in the analysis. In both models, the interaction between treatment and time is significant at $P \leq 0.05$, which is consistent with the results when the data are excluded (refer to tables 1 and 2).

Effects of Head Orientation on Surface Temperatures
To determine the influence of head orientation on eye region and bill surface temperatures of LPS-injected birds and saline-injected birds, we followed previously established methods in Tabh et al. (2021) for calculating head angle. However, because of the small body size of finches and relatively low resolution of our images, we were unable to reliably identify and mark more than four spatial landmarks in our imaged birds with precision. With such a limited number of identifiable landmarks and with such limited precision in our estimates of landmark location, algorithms used by Tabh et al. (2021) to estimate head orientation performed poorly. Given that we could not follow methods described by Tabh et al. (2021), we took a different approach to correct for possible differences in head orientation between treatment groups. Here, we assumed that LPS-injected birds had
a complete bias in head preference away from the camera. We therefore added 0.5°C to all eye region and bill temperature measurements of LPS-injected birds to make our estimates more liberal. We then reran our statistical analyses with the new estimated surface temperature measurements and examined whether our results changed. Results from this new analysis show that even if LPS-injected birds solely faced away from our thermographic camera while saline-injected birds did not, we still detect a reduction in heat loss among LPS-injected birds relative to saline-injected birds (i.e., our conclusions do not change; table A4). Note that a preference of individuals facing toward the camera (i.e., subtracting 0.5°C from all measurements) would not influence the results because surface temperatures do not significantly change between a yaw of −90°C (i.e., facing the camera) and a yaw of 0°C (i.e., perpendicular to the camera; Tabb et al. 2021).

It is also worth noting that most of the heat transfer in LPS-injected birds and saline-injected birds came from the legs, which are not subject to temperature biases as a function of orientation (i.e., surface temperature of the leg does not vary according to orientation because heat transfer occurs both at the front and at the back of the leg). Given the relatively small contribution of heat transfer from the eyes and bill, we therefore think it is unlikely that head orientation would have influenced our results in any meaningful way.

Table A2: Factors affecting the subcutaneous temperature response in zebra finches injected with a saline or endotoxin (100 µg LPS kg⁻¹) solution

| Predictor            | Estimate | 95% CI      | t      | P     |
|----------------------|----------|-------------|--------|-------|
| Intercept [LPS]      | 41.15    | 40.85 to 41.46 | 254.45 | <.001 |
| Treatment [saline]   | −.19     | −.57 to .18  | −.99   | .337  |
| Time                 | .75      | −.78 to 2.29 | .96    | .340  |
| Time²                | 1.01     | −.53 to 2.56 | 1.28   | .202  |
| Sex [male]           | .28      | −.09 to .66  | 1.45   | .166  |
| Treatment × time     | −3.55    | −5.81 to −1.30 | −3.08 | .002  |
| Treatment × time²    | .97      | −1.27 to 3.22 | .85    | .398  |

Note. Brackets indicate reference category. Bold values indicate statistical significance at P ≤ 0.05. LPS = lipopolysaccharide; CI = confidence interval.

Table A3: Factors affecting the heat dissipation output in zebra finches injected with a saline or endotoxin (100 µg LPS kg⁻¹) solution

| Predictor            | Parametric predictors | 95% CI      | t      | P     |
|----------------------|-----------------------|-------------|--------|-------|
| Intercept [saline]   | Estimate              | 41.05 to 80.20 | 6.07   | <.001 |
| Treatment [LPS]      | −13.84                | −32.10 to 4.43 | −1.49  | .140  |
| Sex [female]         | 5.33                  | −20.45 to 31.11 | .41    | .686  |

Smoothed predictors

| Predictor            | EDF; ref. EDF | SE | F   | P     |
|----------------------|---------------|----|-----|-------|
| Time                 | 2.17; 2.54    | 4.81 | 1.44 | .162  |
| Treatment × time     | 2.48; 2.79    | 9.77 | 4.80 | .007  |
| Bird identity        | 12.09; 13.00  | 12.53 | 18.84 | <.001 |

Note. Brackets indicate reference categories. Standard errors of smoothed terms represent averages across predictor knots. Confidence intervals (CIs; linear terms) and standard errors have been corrected for smoothness of smooth terms. Bold values indicate statistical significance at P ≤ 0.05. EDF = estimated degrees of freedom; ref. = reference; LPS = lipopolysaccharide.
Table A4: Factors affecting the heat dissipation output in zebra finches while controlling for the effects of head orientation on surface temperatures

| Predictor          | Estimate | 95% CI      | t    | P    |
|--------------------|----------|-------------|------|------|
| Intercept [saline] | 61.63    | 42.29 to 80.96 | 6.25 | <.001|
| Treatment [LPS]    | −15.45   | −33.43 to 2.52 | −1.69| .096 |
| Sex [female]       | 2.42     | −23.06 to 27.90 | .19  | .853 |

Smoothed predictors

| Predictor          | EDF; ref. EDF | SE  | F    | P    |
|--------------------|---------------|-----|------|------|
| Time               | 2.17; 2.54    | 4.90| 1.44 | .162 |
| Treatment × time   | 2.48; 2.79    | 10.84| 4.80 | .007 |
| Bird identity      | 12.09; 13.00  | 12.72| 18.84| <.001|

Note. Data from 0900 to 0930 hours and from 1500 to 1530 hours are excluded from the analysis. Brackets indicate reference categories. Standard errors of smoothed terms represent averages across predictor knots. Confidence intervals (CIs; linear terms) and standard errors have been corrected for smoothness of smooth terms. Bold values indicate statistical significance at \( P \leq 0.05 \). Controlling for head orientation did not change our claims of statistical significance or influence our general conclusions. EDF = estimated degrees of freedom; ref. = reference; LPS = lipopolysaccharide.

Table A5: Number of individuals in each treatment (lipopolysaccharide [LPS] or saline) and the ratio of LPS-injected birds to saline-injected birds at each half-hour interval for the summative heat dissipation analysis

| Time after injection | LPS | Saline | Ratio |
|----------------------|-----|--------|-------|
| −1.5                 | 1   | 8      | .13   |
| −1                   | 1   | 7      | .14   |
| −.5                  | 1   | 7      | .14   |
| 0                    | 3   | 8      | .38   |
| .5                   | 4   | 7      | .57   |
| 1                    | 2   | 7      | .29   |
| 1.5                  | 3   | 6      | .50   |
| 2                    | 3   | 6      | .50   |
| 2.5                  | 3   | 5      | .60   |
| 3                    | 3   | 7      | .43   |
| 3.5                  | 1   | 7      | .14   |
| 4                    | 1   | 5      | .20   |
| 4.5                  | 3   | 8      | .38   |
| 5                    | 2   | 7      | .29   |
| 5.5                  | 0   | 4      | .00   |
Figure A2. Thermal photograph of a zebra finch (here, female and saline treated) used for extracting surface temperature measurements. The regions of interests are circled in white and denote the eye region, bill, and legs (including the feet). The inset is the original image, shown here for clarity. The red circle surrounding the perch is the radio frequency identification antenna, used to extract the individual identification number, date, time, and subcutaneous temperature measurements.

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