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Is there an energetic-based trade-off between thermoregulation and the acute phase response in zebra finches?

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SUMMARY

There has been recent interest in understanding trade-offs between immune function and other fitness-related traits. At proximate levels, such trade-offs are presumed to result from the differential allocation of limited energy resources. Whether the costs of immunity are sufficient to necessitate such energy reallocation remains unclear. We tested the metabolic and behavioural response of male zebra finches (*Taeniopygia guttata*) to the combined effects of thermoregulation and generation of an acute phase response (APR). The APR is the first line of defence against pathogens, and is considered energetically costly. We predicted that at cold temperatures zebra finches would exhibit an attenuated APR when compared with individuals at thermoneutrality. We challenged individuals with bacterial lipopolysaccharide (LPS), an immunogenic compound that stimulates an APR. Following LPS injection, we measured changes in food intake, body mass, activity, and resting and total energy expenditure. When challenged with LPS under *ad libitum* food, individuals at both temperatures decreased food intake and activity, resulting in similar mass loss. In contrast to predicted energetic trade-offs, cold-exposed individuals injected with LPS increased their nocturnal resting energy expenditure more than did individuals held at thermoneutrality, yet paradoxically lost less mass overnight. Although responding to LPS was energetically costly, resulting in a 10% increase in resting expenditure and 16% increase in total expenditure, there were few obvious energetic trade-offs. Our data support recent suggestions that the energetic cost of an immune response may not be the primary mechanism driving trade-offs between immune system function and other fitness-related traits.

Key words: immunity, anorexia, life history, lipopolysaccharide, sickness, trade-off, thermoregulation, energy expenditure.

INTRODUCTION

Over the past decade, there has been increasing interest in trade-offs between immune system function and other fitness-related traits. Experimental induction of immune activity has been shown to negatively affect various fitness components, including breeding effort (Bonneaud et al., 2003; Ilmonen et al., 2000), nestling growth rates (Soler et al., 2003) and adult survival (Hõrak et al., 1999; Soler et al., 2003; Hanssen et al., 2004). At proximate levels, the differential allocation of limited internal energy resources is thought to drive such trade-offs. In support of this, increased quality and quantity of food can result in improved immune responses (Brzek and Konarzewski, 2007). However, whether the costs of mounting an immune response are sufficient to drive energy allocation decisions remains unclear (Eraud et al., 2005).

Relatively few studies have measured the energetic costs of mounting an immune response, and when they have, the results have been equivocal. For example, Martin et al. (Martin et al., 2003) showed that in house sparrows (*Passer domesticus*), generating a cutaneous immune response to phytohaemagglutinin (PHA) injection resulted in a 29% elevation in resting oxygen consumption ($\dot{V}_{O_2}$), an amount calculated to represent about one-half of the cost of producing an egg. As such, they argued that such an energetic increase would be sufficient to influence an important life-history trait. By contrast, great tits (*Parus major*) injected with higher doses of PHA had stronger immune responses; however, an individual's metabolic rate (MR) was not dose-dependent, and was relatively low (Nilsson et al., 2007).

The energetic cost of humoral responsiveness also remains equivocal. While some studies demonstrate significant elevations of MR in response to novel antigens [e.g. mice *Mus musculus* (Demas et al., 1997); great tits *Parus major* (Ots et al., 2001)], others find no increase in metabolism [e.g. greenfinch *Carduelis chloris* (Hõrak et al., 2003)]. Furthermore, while an 8–13% elevation of basal metabolic rate in response to an immune system challenge is considered low in blue tits [*Parus caeruleus* (Svensson et al., 1998)], a similar elevation is interpreted as a serious energetic challenge in great tits (Ots et al., 2001). Even when such increases in MR are reported, the magnitude of the increased energetic expenditure may be minimal when compared with other tasks, such as thermoregulation (Eraud et al., 2005).

Most research exploring the energetic basis for immunity in an evolutionary context has focused on the humoral and/or cell-mediated branches of the adaptive immune system. One aspect of infection increasingly believed to be an important mediator of trade-offs between immunity and life-history traits is the acute phase response (APR) (Bonneaud et al., 2003; Lee et al., 2005; Owen-Ashley and Wingfield, 2007). The APR is part of the innate arm of the immune system, and occurs at the onset of infection. The APR typically involves adjustments in body temperature (T_b) (e.g. fever), production of hepatic acute phase proteins, activation of the hypothalamo-pituitary-adrenal axis, suppression of the hypothalamo-pituitary-gonadal axis and expression of stereotypical sickness behaviours (Klasing, 2004; Owen-Ashley and Wingfield, 2007). Sickness

behaviours include reductions in food (anorexia) and water (adipsia) intake, decreased activity and mass loss; adaptations believed to limit nutrients to the pathogen and minimise energy expenditure on activities not immediately essential to survival (Hart, 1988).

Experimentally, an APR can be induced by injection of lipopolysaccharide (LPS), the immunogenic component of the cell wall of Gram-negative bacteria. Administration of LPS allows for an individual's behavioural and metabolic response to a simulated infection to be studied, independent of the actual damage caused by a pathogen. Responding to LPS appears costly and may result in physiological trade-offs within life-history stages (Owen-Ashley and Wingfield, 2007). For example, female house sparrows challenged with LPS lost mass, had decreased nestling feeding rates and reduced breeding success when compared with saline-injected controls (Bonneaud et al., 2003). Meanwhile, in northwestern song sparrows (*Melospiza melodia morphna*), the magnitude of an individual's response to LPS varies seasonally, probably in part due to variation in energy stores (Owen-Ashley and Wingfield, 2006).

In the current study, we explored whether variation in the magnitude of an individual's response to LPS was consistent with evidence of energetic-based trade-offs. We tested the combined effects of thermoregulation and LPS on the metabolic and behavioural response of zebra finches. We predicted that if responding to LPS entails significant costs (direct and indirect), individuals acclimated to temperatures below thermoneutrality (15°C) would display a weaker response to LPS (less of a reduction in food intake, less mass loss, less of an increase in MR) when compared with individuals held at thermoneutrality (34°C). We conducted two related experiments. First, we quantified food intake, body mass change and behavioural response to LPS challenge at each of two temperatures (15°C and 34°C). Second, we measured the overnight metabolic cost of responding to LPS at each temperature, using flow-through respirometry. Because individuals did not have access to food during their overnight measurements, we also had the opportunity to explore the cost of responding to LPS in terms of energy balance/mass loss.

MATERIALS AND METHODS

Animal care and housing

Male zebra finches (*Taeniopygia guttata* Vieillot 1817) were group housed in flight cages (45 cm × 45 cm × 90 cm) in a walk-in environmental chamber, set at either thermoneutral (34±0.5°C) (Calder, 1964) or sub-thermoneutral (15±0.5°C) temperatures. Different individuals were tested at each temperature. Individuals were allowed to acclimate to the temperature of the environmental chamber for a minimum of two weeks before any experimental procedures were conducted. To reduce capture and handling time, which has been shown to negatively affect indices of immunity (Ewenson et al., 2003; Berzins et al., 2008), each individual was removed from its group-housing cage and placed in an individual cage at least 24 h prior to beginning any experiments. We assumed, as we have previously (Berzins et al., 2008), that isolation in which individuals could still hear each other did not result in elevated stress in our study species.

Once an experiment at one temperature had been completed, all finches were returned to the Animal Care Facility (Peterborough, ON, Canada), the temperature of the environmental chamber was reset to the second temperature, and a new group of birds was introduced. Lights in the environmental chamber were set on a 14 h:10 h, light:dark schedule, with lights turning on at approximately 07:00 h.

While in the Animal Care Facility, food (Topcrop^{TM}, Essex, ON, Canada: 50% Budgie, 50% finch mix) and water were provided to finches *ad libitum*; supplemented with Vita-Sol multi-vitamins (Eight in One Pet Products, Inc., Islandia, NY, USA) (added to the water), egg-meal or lettuce once per week. Once experiments began, and individuals were transferred to the environmental chamber, we ceased feeding egg-meal and lettuce. Multi-vitamins were provided every 1–2 weeks in the drinking water. All experiments followed the guidelines of the Canadian Council on Animal Care (CCAC), and were approved by Trent University's Animal Care Committee.

Injection procedures

Zebra finches were injected into the abdominal cavity with a warmed, sterile solution of either 100 µl of 0.1 mg ml^{-1} LPS (Sigma-Aldrich #L4005, Serotype 055:B5, Oakville, ON, Canada) or 100 µl of 10 mmol l^{-1} phosphate-buffered saline (Sigma-Aldrich #3813). Prior to injection, the skin surrounding the injection site was sterilised with ethanol. The particular dose of LPS chosen was *ca*. 1 mg kg^{-1} body mass [following Owen-Ashley et al. (Owen-Ashley et al., 2006)]. Individuals acted as their own controls and were injected seven days later with the opposite solution. To eliminate order effects, injection solutions were randomised at each temperature.

Experiment I: behavioural and thermoregulatory response to LPS

Mass change and food consumption

Body mass was measured 1 h prior to injection and 24 h following injection (±0.1 g; Table 1). Although measurements of mass change were conducted over 25 h, data were recalculated and expressed per 24 h for clarity. To determine rate of food consumption, we placed 30 g of seed into an open-top 946 ml Ziploc container on the floor of each individual's cage immediately before injection. Twenty-four hours following injection, the food remaining in the container was reweighed; there was always food remaining 24 h post-injection. Due to the depth of the containers virtually no food was scattered throughout the cages; however, any food scattered was assumed to be random across treatments.

Thermoregulation and behaviour

To monitor each individual's core T_b, we used a Physitemp BAT-12 digital thermocouple thermometer (Physitemp Instruments, Inc., Clifton, NJ, USA). We measured cloacal temperature 1 h prior to and 1, 4, 6 and 8 h following injection using a sterilised 18-guage Physiotemp thermocouple inserted 1 cm into the cloaca. T_b was recorded once the reading on the digital thermometer was stable for 10 s [following Owen-Ashley et al. (Owen-Ashley et al., 2006)].

Table 1. Experimental time line for measuring the effects of lipopolysaccharide (LPS) injection on behaviour, body temperature and body mass of male zebra finches

Time (h)	Day 1	Day 2
07:00	Lights on	Lights on
08:30	Pre-injection behaviour	–
09:00	Initial body mass, temperature	–
10:00	Inject birds with LPS or saline, food mass	Final body mass, temperature, food mass
11:00	Temperature	–
14:00	Temperature	–
16:00	Temperature	–
18:00	Temperature	–
18:30	Post-injection behaviour	–
21:00	Lights off	Lights off

Two birds were measured simultaneously.

Behaviour was recorded for 30 min, 1.5 h prior to injection and again 8.5 h following injection using a Canon digital video camera (Canon Canada, Inc., Mississauga, ON, Canada). To minimise disturbance, we turned the camera on 10 min before data were to be collected, and then left the environmental chamber while videotaping occurred. Two individual birds were filmed simultaneously. Video footage was transferred to a computer and analysed manually. Each individual bird was assigned to one of five behaviours every 30 s, for 30 min: resting, hopping, preening, eating and drinking. The maximum value for a given behaviour was 60, if the individual performed only a single behaviour for the entire 30 min. Due to the low number of occurrences of drinking (four out of 2760 possible observations), drinking was not considered further.

Experiment II: overnight oxygen consumption

Respirometry

Experiment II used different individuals from those used in Experiment I. As in Experiment I, individuals were acclimated to the environmental temperature for a minimum of two weeks before any measurements began. The metabolic cost of responding to LPS was measured indirectly as $\dot{V}_{O_2}$ using flow-through respirometry. Each measurement series period took place over three days (days –1, 0, 7). Day –1 consisted of acclimating the bird to the metabolic chamber and other procedures but involved no injection. On day 0, each individual received an injection of either LPS or saline (in random order). Seven-days post-injection, each individual was injected with the alternative solution. Food was removed at 19:00 h, 2 h before lights went off and 3 h before any injection. Shortly before 22:00 h, each experimental zebra finch was removed from its cage and weighed (±0.1 g). Each individual was then injected with either LPS or saline and transferred to a 700 ml Plexiglas metabolic chamber (model G114, QUBIT systems, Kingston, ON, Canada). The metabolic chamber was placed in a Styrofoam cooler (to keep the bird in the dark), and rapidly transported to the respirometry lab *ca*. 150 m away. The metabolic chamber containing the bird was then placed in a temperature-controlled incubator (Thermo Low Temperature Incubator, Model 815, Fisher Scientific Ltd, Nepean, ON, Canada), set at either 15°C or 34°C (±1°C). At 07:00 h, each individual was transported back to the environmental chamber, reweighed and placed back into its respective cage.

To measure $\dot{V}_{O_2}$, external air was scrubbed of water and CO_2 using columns of Drierite, soda lime and Ascarite, and then split into two lines. Air in one line went into the purge valve of the valve multiplexer (TR-RM, Sable Systems, Las Vegas, NV, USA). Air in the second line passed through the multiplexer into a mass flow meter (model 840, Sierra Instruments, Amsterdam, The Netherlands), controlled by an attached mass-controller (MFC-2, Sable Systems), and then into one of the three metabolic chambers (each containing a bird) or into a piece of Bev-A-Line tubing (Cole-Palmer Canada, Inc., Montreal, QC, Canada) to measure baseline. Flow of air entering each chamber and the baseline loop was set at 500 ml min^{-1}. Excurrent air leaving the multiplexer was sub-sampled (TR-SS3, Sable Systems) from a 10 ml syringe barrel, scrubbed of water and CO_2 using magnesium perchlorate and Ascarite, respectively, and drawn into an oxygen analyzer (FC-10a O_2 Analyzer, Sable Systems). When not being sampled, chambers were supplied with dry, CO_2-free air through use of the purge valve on the multiplexer.

Our set-up allowed us to measure the $\dot{V}_{O_2}$ of three birds per night. Each recording sequence began with measuring 5 min of baseline air, followed by 15 min of air from a metabolic chamber and then another 5 min of baseline. The system would then automatically switch to begin recording O_2 in the next chamber in the series. Our set-up allowed for collection of at least 135 min of O_2 measurements from each bird over the course of the 9 h night. To make $\dot{V}_{O_2}$ measurements fully comparable between treatments, individuals were always measured in the same sequence and time of night. Data collection stopped at 07:00 h. As an index of resting metabolic rate (RMR) we estimated the minimum $\dot{V}_{O_2}$ for each individual by identifying the lowest 5 min of continuous O_2 consumption per night using LabAnalyst X (Warthog Systems, www.warthog.ucr.edu). To estimate each individual's total O_2 consumption per night (as an index of total overnight MR), we calculated the total O_2 consumed by each individual during the 135 min of actual measurement, and expressed it per hour of measurement.

Statistical analysis

Within each environmental temperature, each zebra finch acted as its own control, receiving both an LPS and a saline injection. As such, we used a restricted maximum likelihood mixed model two-way, repeated-measures analysis of variance (ANOVA) or analysis of covariance (ANCOVA) to analyse the effects of treatment (LPS or saline), environmental temperature (15°C and 34°C) and injection order (whether LPS or saline was injected first) on parameters of interest. Individual identification was treated as a random effect, and when appropriate, body mass was included as a covariate in analyses. Initially, all biologically relevant two-way interaction terms were included in the model but these were subsequently excluded if not significant, and the model was re-run. All data were untransformed, with the exception of mass loss in Experiment II, which was $\log_{10}$-transformed.

Behavioural data were analysed using non-parametric statistical tests. We initially tested for an effect of environmental temperature on behaviour by comparing median activity at each temperature for saline-injected birds using a Wilcoxon sign-rank test. To explore the impact of LPS on each individual's behaviour, we calculated relative activity as the percentage of observation sessions (of 60 sessions) in which a given activity was observed following saline injection, subtracted from the percentage of observations in which the activity was observed following LPS injection. We tested the median relative activity at each temperature against a median of 0, using a Wilcoxon signed-rank test. A lack of significance (i.e. median relative activity not different from 0) was interpreted to mean that individuals responded behaviourally in a similar fashion to injections of LPS and saline. Statistical significance is claimed at $P<0.05$. All analyses were performed using JMP 5.0.1a (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Experiment I

Food consumption and mass loss

Initial body mass did not differ between members of the two treatment groups (LPS *versus* saline: $F_{1,21}=0.24$, $P=0.63$) or between individuals housed at each of the two environmental temperatures (15°C *versus* 34°C: $F_{1,21}<0.01$, $P=0.99$). Birds were *ca*. 0.29 g heavier before their first injection than before their second injection (injection order: $F_{1,21}=4.63$, $P=0.043$).

Zebra finches injected with LPS ate less food compared with when they had been injected with saline ($F_{1,21}=21.05$, $P<0.001$; Fig. 1A). Individuals housed at 15°C ate significantly more food than individuals housed at 34°C ($F_{1,21}=21.82$, $P<0.001$; Fig. 1A). The absolute reduction in food intake following LPS injection was similar between individuals housed at each temperature (temperature × treatment: $F_{1,21}=0.63$, $P=0.44$).

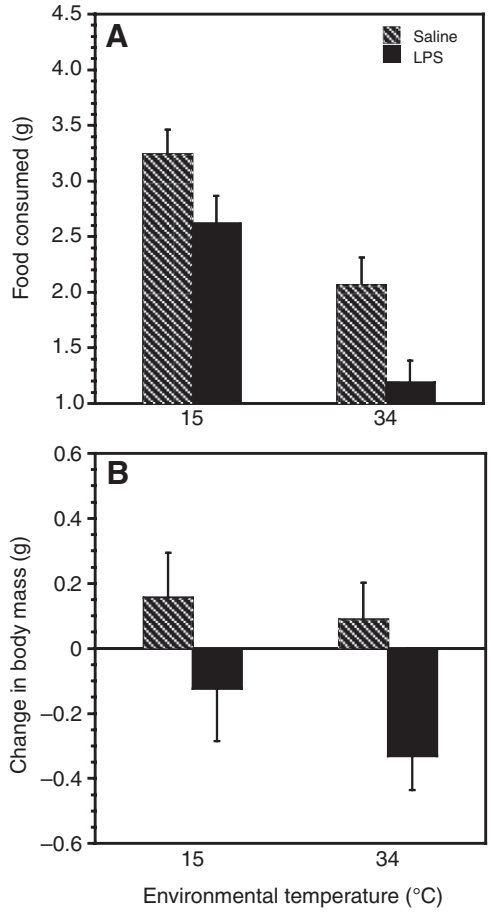

Fig. 1. Twenty-four hour change in (A) food consumption and (B) body mass of male zebra finches housed at 15°C or 34°C and challenged with lipopolysaccharide (LPS) or saline. Bars represent standard errors. *N*=12 per temperature.

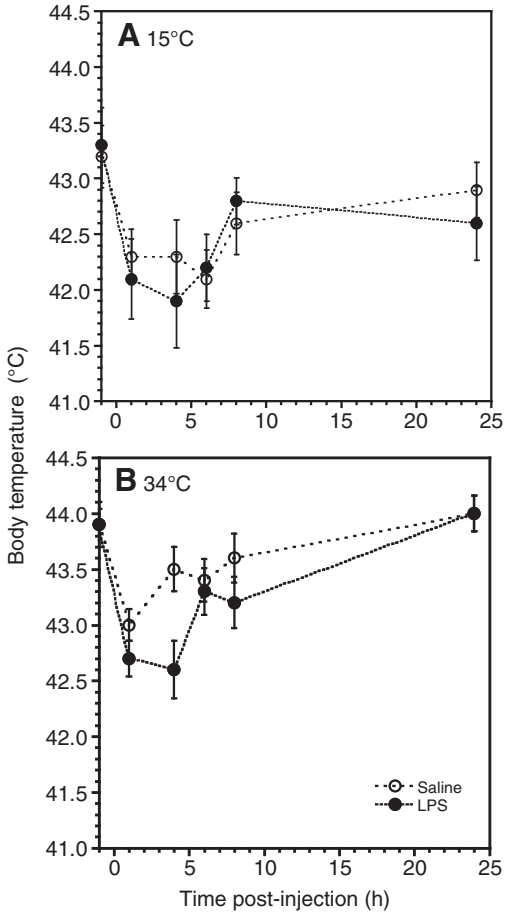

Fig. 2. Core body temperature (°C) of male zebra finches housed at (A) 15°C or (B) 34°C and challenged with lipopolysaccharide (LPS) or saline. Challenge occurred at time=0. Bars represent standard errors. *N*=12 per temperature.

Individuals injected with LPS lost significantly more mass than when they had been injected with saline ($F_{1,21}$=7.90, P=0.011; Fig. 1B), presumably due to decreased food intake. Environmental temperature did not result in significant mass loss ($F_{1,21}$=1.00, P=0.33; Fig. 1B); thus, individuals remained in energy balance irrespective of environmental temperature. Temperature had no effect on the amount of mass an individual lost when responding to LPS (treatment × temperature: $F_{1,21}$=0.32, P=0.58). There was no effect of initial body mass on mass loss ($F_{1,20}$=1.63 P=0.21), indicating that body condition did not play a role in an individual's response. Finally, there was no effect of injection order on either food consumption or mass loss (each P>0.20). Together, these data suggest independent effects of temperature and immune challenge on food intake and body mass dynamics, and no evidence of energetic trade-offs.

Thermoregulation

The core T_b of zebra finches varied significantly with time following injection of either LPS or saline ($F_{5,256}$=15.30, P<0.0001; Fig. 2A,B), dropping initially. The mean T_b of birds injected with LPS was lower than when injected with saline, although this difference failed to attain statistical significance (least squares mean ± 1 s.e.m.; LPS: 42.9±0.11°C; saline: 43.1±0.11°C; $F_{1,256}$=3.56, P=0.060). The mean T_b of zebra finches housed at 15°C was significantly lower (by 0.9°C) than zebra finches at 34°C ($F_{1,256}$=20.99, P<0.0001). A lack of treatment × temperature interaction ($F_{1,256}$=1.20, P=0.28) indicated that individuals responded similarly to LPS, irrespective of the environmental temperature at which they were acclimatised. The order of injection did not significantly affect T_b (P=0.080).

Behavioural responses

Due to a camera failure at 15°C, two birds (one LPS-injected and one saline-injected) have incomplete behavioural data. For statistical analyses of behaviours involving 15°C birds, sample sizes were 10 in paired statistical tests and 11 for unpaired tests. These two individuals were included in all other analyses (e.g. food consumption and thermoregulation).

Resting

Saline-injected individuals spent approximately 60% of their time resting; this did not differ with temperature (Z=0.40, P=0.69; Fig. 3). Following injection of LPS, individuals at 15°C did not increase the amount of time they spent resting (t=–1.5, d.f.=9, P=0.92). Individuals injected with LPS at 34°C increased their time resting but not significantly so (t=–22.0, d.f.=11, P=0.09).

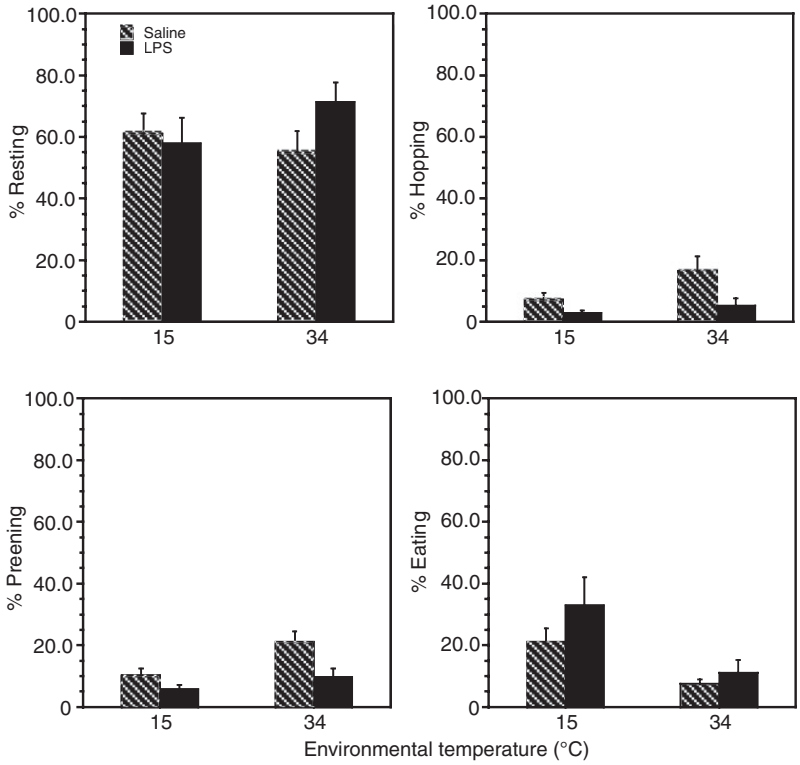

Fig. 3. Behaviour of male zebra finches housed at 15°C or 34°C and challenged with lipopolysaccharide (LPS) or saline. Histograms represent the percentage of observation sessions (of a possible 60 sessions) in which a given activity was observed during 30-min observation period. Bars represent standard errors. Sample sizes: 34°C, N=12; 15°C, N=11.

Hopping

Saline-injected individuals spent a similar percentage of their time hopping at 15°C and 34°C (Z=–1.76, P=0.078, Fig. 3). Following injection of LPS, there was a significant reduction in % hopping activity at both temperatures (15°C: t=12.0, d.f.=9, P=0.047; 34°C: t=26.0, d.f.=11, P=0.041). The amount by which individuals reduced their % hopping activity (i.e. % hopping following saline injection – % hopping following LPS injection) did not differ with temperature (Z=1.32, P=0.19). Thus, following LPS injection, all individuals reduced their hopping activity to a similar degree, irrespective of temperature.

Preening

Individuals held at 15°C spent less time preening than individuals held at 34°C (Z=–2.13, P=0.03, Fig. 3). Following injection of LPS, there was a significant reduction in preening activity among individuals at 34°C (t=29.0, d.f.=11, P=0.02) but not significantly so among those held at 15°C (t=15.0, d.f.=9, P=0.14). The amount by which individuals reduced preening activity (i.e. % preening following saline injection – % preening following LPS injection) did not differ with temperature (Z=–1.58, P=0.11).

Eating

Control individuals were observed eating significantly more times per observation session at 15°C than at 34°C (Z=2.81, P=0.005, Fig. 3). Following LPS injection, there was no significant reduction in eating behaviour at either temperature (15°C: t=–11.5, d.f.=9, P=0.28; 34°C: t=–5.5, d.f.=11, P=0.66); individuals at 15°C continued to be observed eating more frequently than individuals at 34°C (Z=2.23, P=0.03).

Experiment II

Overnight mass loss and MR

LPS and saline-injected birds weighed the same upon being placed in the metabolic chambers ($F_{1,21}$<0.02, P=0.89). However, individuals held at 15°C were *ca*. 2.4 g heavier in the evening than individuals held at 34°C, mean ± s.e.m. (15°C: 16.7±0.27 g; 34°C: 14.3±0.29 g; $F_{1,21}$=18.26, P<0.001). Birds weighed *ca*. 0.26 g more before their first injection than before their second injection (injection order: $F_{1,21}$=11.18, P=0.003).

Individuals injected with LPS lost more mass overnight than when injected with saline (treatment: $F_{1,19}$=61.34, P<0.001). Similarly, individuals exposed to 15°C lost more mass overnight than those held at 34°C (temperature: $F_{1,19}$=18.00, P<0.001). Consistent with an energetic-based trade-off between immunity and thermoregulation, an individual's mass loss in response to LPS varied with environmental temperature (treatment × temperature: $F_{1,19}$=10.17, P=0.005; Fig. 4). Individuals at 34°C lost 0.39 g (±0.05 g) more mass responding to an injection of LPS than they did when responding

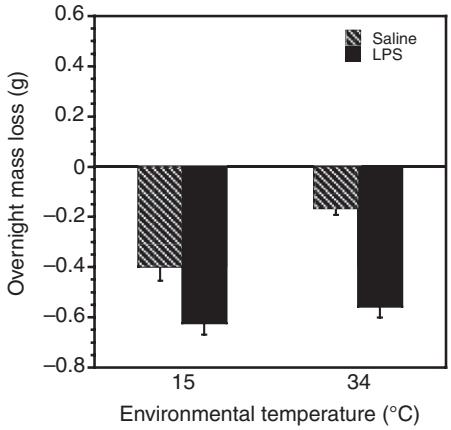

Fig. 4. Overnight mass loss of male zebra finches housed at 15°C or 34°C and challenged with lipopolysaccharide (LPS) or saline. Bars represent standard errors. N=12 per temperature.

to saline. Meanwhile, individuals at 15°C lost 0.23 g (±0.08 g) more mass when responding to LPS; this difference in mass loss between temperatures was significant (t=3.00, d.f.=22, P=0.007). Initial body mass did not influence subsequent overnight mass loss ($F_{1,18}$<0.01 P=0.93), suggesting little role for body condition in determining an individual's response to LPS or saline.

The RMR of individuals injected with LPS was *ca*. 10% higher than when those same individuals were injected with saline (treatment: $F_{1,20}$=35.36, P<0.001; mass as covariate: $F_{1,20}$=8.33, P=0.009; Fig. 5A). As expected, individuals at 15°C had a higher RMR than individuals at 34°C (temperature: $F_{1,20}$=291.23, P<0.001; Fig. 5A). An individual's metabolic response to LPS depended on the environmental temperature (temperature × treatment: $F_{1,20}$=4.46, P=0.048). To explore this interaction, for each individual we subtracted the $\dot{V}_{O_2}$ following saline injection from the $\dot{V}_{O_2}$ following injection of LPS. Contrary to predictions of an energetic trade-off, and contrary to expectations based on mass loss (above), individuals at 15°C put significantly more energy into responding to LPS than did individuals housed at 34°C (temperature: $F_{1,21}$=7.92, P=0.01; mass: $F_{1,20}$=3.39, P=0.080). That is, birds at 15°C showed evidence of an energetic trade-off with respect to one measure of energy expenditure (mass loss) but no-evidence of an energetic trade-off with respect to an alternative measure of expenditure (RMR).

The total overnight energy expenditure was 16% higher in birds injected with LPS than with saline ($F_{1,20}$=40.70, P<0.0001; mass a covariate: $F_{1,20}$=2.62, P=0.12; Fig. 5B). The total energy expenditure of individuals at 15°C was 136% greater than for individuals at 34°C ($F_{1,20}$=121.83, P<0.0001; Fig. 5B). The magnitude of an individual's metabolic response to LPS did not vary with temperature (temperature × treatment, $F_{1,20}$=0.27, P=0.61), providing no evidence of an energetic-based trade-off. There were no significant effects of injection order (each P>0.2) nor any significant injection order × treatment interactions (each P>0.05) on mass loss, RMR or total MR.

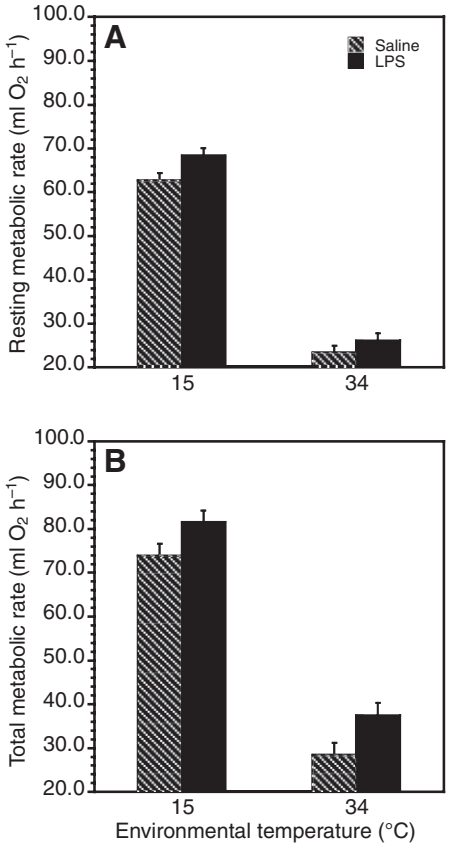

Fig. 5. (A) Resting metabolic rate (ml O_2 h^{-1}) and (B) total metabolic rate (ml O_2 h^{-1}) of male zebra finches housed at 15°C or 34°C and challenged with lipopolysaccharide (LPS) or saline. Histograms and bars represent least squares means ± 1 s.e.m., with body mass included as a covariate. N=12 per temperature.

DISCUSSION

In this study, we tested whether zebra finches exposed to cold and simultaneously challenged with a simulated bacterial infection would display an attenuated response when compared with individuals exposed to thermoneutral temperatures. We reasoned that given the costly nature of responding to LPS (Bonneaud et al., 2003), individuals at 15°C would be less likely to have sufficient energy reserves to mount a robust response. Although responding to LPS resulted in a *ca*. 10% elevation in RMR and a 16% increase in total MR, for the most part, we could detect few obvious energetic trade-offs.

Thermoregulatory response to LPS and environmental temperature

In endotherms, an elevation of T_b is typically an integral part of an APR (Owen-Ashley and Wingfield, 2007). However, at both 15°C and 34°C, the anticipated fever was not seen in zebra finches and, instead, an initial hypothermia occurred in birds injected with LPS and saline (Fig. 2). Although this initial drop could be due to diurnal variation, the observation that LPS birds had lower T_b than control birds (albeit not significantly so, P=0.06) suggests that the LPS played some role in the generation of the hypothermic response.

In rodents, the thermoregulatory response to LPS is dose-dependent; low doses result in hyperthermia (Blatteis, 2006) whereas high doses can result in hypothermia, particularly if administered at sub-thermoneutral temperatures (Rudaya et al., 2005). We chose our dose based on Owen-Ashley et al. (Owen-Ashley et al., 2006), and thus we cannot evaluate the influence of dose on the metabolic response of zebra finches. We can, however, exclude environmental temperature as an important factor because individuals became hypothermic even when held at thermoneutrality (Fig. 2B).

The response to LPS is not consistent across birds; hyperthermia is frequently reported in domestic species [e.g. Pekin ducks (Gray et al., 2008)] while hypothermia is common in passerines (Owen-Ashley and Wingfield, 2007). Zebra finches (and passerines in general) have very high T_b, which may preclude any further rise in response to administration of LPS (Martin et al., 2008a). Additionally, further elevations in T_b may simply not be possible due to high surface area to volume ratios and the resultant heat loss (Owen-Ashley and Wingfield, 2007).

High T_b and hypothermia may be evolutionarily adaptive. For example, elevated T_b have been hypothesised to act as a natural defence against bacterial infections in free-living California ground squirrels [*Spermophilus beecheyi* (Muchlinski et al., 2000)] and captive mice [*Peromyscus melanophrys* (Martin et al., 2008a)]. Additionally, hypothermia may be a defence against pathogens under conditions of low resource availability (Romanovsky and Szekely, 1998). Thus, hypothermia may be an adaptive response to infection in zebra finches, as hypothesised previously for rodents that originate in regions of low productivity (Martin et al., 2008a).

Sickness behaviours and thermoregulation

Following injection of LPS, individuals decreased hopping and preening behaviours, and tended to increase their time at rest (Fig. 3). Such responses are considered to be stereotypical sickness

behaviours (Hart, 1988), and have been reported previously in birds treated with LPS (e.g. Bonneaud et al., 2003; Owen-Ashley et al., 2006). Evidence for a trade-off between thermoregulation and sickness behaviour was in our study equivocal. Preening was the only behaviour supportive of an energetic trade-off; individuals injected with LPS reduced preening at 34°C but not at 15°C. Because individuals at 15°C preened less overall than individuals at 34°C, further reductions in preening at 15°C may simply not have been possible. Whether preening is energetically costly is not clear, although small mammals experiencing increased energetic burdens due to lactation reduce time spent grooming, and may suffer increased ectoparasite burdens as a result (Speakman, 2008). Interestingly, we observed no reduction in eating in response to LPS injection at either temperature (Fig. 3), which contrasts with the predicted anorexia typical of sickness behaviours (Hart, 1988). However, based on the reduced food consumption and loss of body mass we measured in LPS-injected individuals (Fig. 1A,B), we suspect there was in fact a reduction in eating behaviour but we were unable detect it. Our inability to detect an effect of LPS on eating behaviour probably reflects the relatively short timeframe over which our behavioural observations were made (30 min). Although this suggests our behavioural observations may not have been long enough, 30 min. observation sessions were sufficient to detect a reduction in eating/feeding behaviour following LPS injection in studies of other species (Owen-Ashley et al., 2006).

Energy balance

We predicted that if the expression of sickness behaviours is energetically costly, individuals housed at 34°C would have a greater reduction in food intake and greater mass loss in response to LPS injection than would individuals at 15°C. Instead, following injection of LPS, individuals at both temperatures showed similar reductions in the amount of food consumed and lost similar amounts of body mass (Fig. 1). However, the food consumption of individuals injected with LPS at 15°C was still significantly greater than individuals at 34°C following LPS injection, with the bulk of this energy presumably allocated toward thermoregulation. Zebra finches apparently maintain a constant body mass in the presence of *ad libitum* food, irrespective of temperature (Salvante et al., 2007). As such, all individuals had similar energetic reserves to allocate to mounting an immune response. In our study, in response to an endotoxin challenge all individual zebra finches let their body mass fall by approximately the same degree (Fig. 1B), and once this set point was reached, body mass was maintained. Although we detected a reduction in food consumption following LPS administration, the extent of the reduction may represent a minimum estimate. We measured the food consumption over a period of 25 h; however, if the effects of LPS occur over a shorter time course in zebra finches, then individuals may be in a compensatory food consumption phase by the time we reweighed the food dishes.

In contrast to patterns of mass loss in the presence of *ad libitum* food, individuals held at 15°C and simultaneously challenged with LPS overnight had an attenuated response to LPS (Fig. 4). That is, individuals at 15°C lost less mass, when compared with saline-injected controls, than did individuals at 34°C. We do not consider mass loss in itself to be adaptive but rather we consider mass loss to be an index of the strength of an individual's response to LPS. Our results are consistent with an energetic-based trade-off. Previous studies have shown relationships between the magnitude of LPS-induced mass loss and energy reserves across various taxa [mammals (Bilbo et al., 2002; Lennie, 1998); birds (Owen-Ashley et al., 2006; Owen-Ashley and Wingfield, 2006); reptiles (Deen and Hutchison, 2001)]. Despite the attenuated response of zebra finches held at 15°C, these individuals did not have lower energy reserves than those held at 34°C; they were in fact 2.4 g heavier, and were still heavier upon removal from the metabolic chamber the following morning. Given their extra body reserves, it is not clear why individuals at held at 15°C did not mount a stronger response and lose more mass. Perhaps when individual zebra finches are acclimated to cold overnight temperatures they maintain an energetic safety margin in case of unexpected demands (Diamond, 2003). Attenuation of an immune response during cold exposure has been reported previously. For example, blue tits reduce antibody production to Diptheria–Tetanus when exposed to cold temperatures, relative to individuals maintained at higher temperatures (Svensson et al., 1998).

Responding to LPS resulted in an average 10% increase in RMR of individual finches at both environmental temperatures (Fig. 5A). This is slightly lower than detected for young rats challenged with LPS (Buchanan et al., 2003) but similar to the costs of generating a humoral immune response to sheep red blood cells in birds (Ots et al., 2001; Eraud et al., 2005). Interestingly, both sheep red blood cells and PHA (used in studies of cutaneous immunity) generate an APR (Klasing, 2004). As such, part of the elevation of RMR reported previously during an adaptive immune response may include the costs of generating an APR (Martin et al., 2008b).

Our RMR data suggest that zebra finches held at 15°C allocate more energy toward responding to LPS than do individuals at 34°C. This is inconsistent with an energetic-based trade-off between immunity and thermoregulation. We did not monitor overnight T_b in the zebra finches; thus, we do not know whether individuals displayed a febrile response to LPS or whether they became hypothermic as they did during the day. Generating a fever is energetically costly; there is a 10% increase in MR for each 1°C rise in T_b (Kluger, 1991). Given that zebra finches at 15°C had lower pre-injection body temperatures than individuals at 34°C, to raise their T_b to the same final temperature would require greater heat production and/or increased heat conservation. Our data are consistent with this hypothesis. Nonetheless, the elevation in RMR and total MR we detected may have been due more to the production of acute phase proteins than generating a febrile response (Klasing, 2004). In humans, for example, it is estimated that *ca*. 50% of the increase in MR associated with an infection is due to protein synthesis (Borel et al., 1998).

Our data for MR are difficult to reconcile with our data on overnight mass loss. Why individuals at 34°C lost relatively more mass responding to LPS than individuals at 15°C, but did not have a proportionally higher RMR or total MR, is not clear. Perhaps individuals differed in their metabolic fuel when at rest and when responding to LPS. Birds use fats as the predominant metabolic substrate when at rest (Suarez et al., 1990). By contrast, generation of an APR results in a complex metabolic response, including increased degradation of muscle protein and synthesis of hepatic acute phase proteins (Lochmiller and Deerenberg, 2000). In future studies it would be interesting to use non-invasive techniques such as magnetic resonance imaging to measure changes in body composition (e.g. lean mass, fat mass, water content) following LPS challenge. By accurately knowing body composition changes it would be possible to assess directly the energetic consequences of a response to LPS.

Maintaining zebra finches in the cold may be perceived as a stressor, potentially limiting an individual's ability to mount the same kind of immune response as it would in an unstressed state (i.e. thermoneutrality). An inability to mount the same response in the

cold may not be due to an energetic constraint but could be due to competition between the immune system and other physiological systems for the same resources. For example, in insects both lipid transport during flight and the immune system require the protein apolipophorin III, and competition between metabolic pathways for this protein can result in post-flight immunosuppression (Adamo et al., 2008). If in zebra finches the pathways involved in thermogenesis and immune function compete for the same compounds, non-energetic trade-offs may also be expected. Future investigation of potential non-energetic-based constraints may help reconcile our data on overnight mass loss with those of MR.

It is increasingly recognised that animals are able to adjust the relative balance between their resistance and tolerance to pathogens (Schneider and Ayres, 2008). Resistance is the mechanism by which a host reduces the pathogen burden whereas tolerance refers to the ability of a host to limit the health impact of a given pathogen burden (Schneider and Ayres, 2008; Råberg et al., 2009). Tolerance and resistance may vary in their energetic costs, and it may be cheaper to limit the damage inflicted by a pathogen than to eliminate the pathogen all together (Schulenburg et al., 2009). Whether individuals of different body condition or experiencing different environmental conditions vary the relative contributions of these two components of defence has been little studied (Råberg et al., 2009). Such plasticity would presumably be adaptive however in that it would allow the immune system to maximise function under different circumstances.

Although we detected a 10% increase in RMR and 16% increase in total MR when individuals were challenged with LPS, this may in fact be an underestimate of the actual energetic cost of generating an APR to LPS. First, like most previous studies of passerines, our control group received an injection of saline, designed to control for negative effects of the needle prick (e.g. Bonneaud et al., 2003; Owen-Ashley et al., 2006). By using saline-injected controls our estimated costs of responding to LPS can be applied to previous studies; however, saline-injections do result in local tissue damage, which may have generated an APR (e.g. van Gool et al., 1990). Future studies may wish to incorporate uninjected controls to explore the energetic costs associated with the saline injections alone. Additionally, handling animals, such as during weighing or taking body-temperature measurements, may result in short-term stress, which may be either immunosuppressive (Berzins et al., 2008) or immunoenhancing (Viswanathan et al., 2005). Although weighing animals before placing them in the metabolic chambers was unavoidable, given that metabolism trials were run overnight, the extent of such carry over effects was assumed to be minimal. Finally, although our saline solutions were sterile they were not necessarily pyrogen-free. This may have resulted in a mild inflammatory response in the control group, with individuals potentially having a MR higher than if they had been uninjected. As such, we can conclude that LPS results in at least a 16% increase in an individual's total overnight energy expenditure; however, it is premature to claim that an APR results in at most a 16% increase.

Conclusions

Responding to LPS injection resulted in a significant rise in energy expenditure in zebra finches; however, this was relatively small when compared with the costs of thermoregulation. Although there was a general tendency for individuals to mount a stronger response at 34°C than 15°C, for the most part, these results were not statistically significant. Individuals challenged overnight at 34°C had significantly greater mass loss when responding to LPS than did individuals held at 15°C; however, this mass loss of *ca*. 0.6 g seemed to represent a ceiling on what individuals appeared willing to lose, despite their energy stores. Taken together our results support recent suggestions that energetic savings may not be the central mechanism determining levels of immunocompetence. Instead, competition between the immune system and other physiological systems for shared resources [e.g. proteins (Adamo et al., 2008)] or degradation of body condition resulting from immune system activation (e.g. Eraud et al., 2005), may be more important factors.

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LIST OF SYMBOLS AND ABBREVIATIONS

APR	acute phase response
LPS	lipopolysaccharide
MR	metabolic rate
PHA	phytohaemagglutinin
RMR	resting metabolic rate
T_b	body temperature
$\dot{V}_{O2}$	rate of oxygen consumption

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