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Additive metabolic costs of thermoregulation and pathogen infection

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Summary

1. Thermoregulation and pathogen resistance are two energetically demanding processes that co-occur during seasonal epidemics for many endothermic vertebrates. The ability of hosts to cope with these processes simultaneously may influence population-level disease dynamics.

2. In North American house finches (*Carpodacus mexicanus*), outbreaks of the bacterium *Mycoplasma gallisepticum* occur during fall and winter, when ambient temperatures across the host's range are often below thermoneutrality. Here, we examined how ambient temperature influences host energetics and susceptibility to this naturally occurring seasonal pathogen by experimentally infecting wild-caught house finches with *M. gallisepticum* at either thermoneutral or subthermoneutral temperatures in the laboratory. We quantified the metabolic costs of infection, measures of body condition, two components of the acute-phase response, disease expression and pathogen loads under both temperature regimes.

3. The metabolic costs of simultaneous infection with *M. gallisepticum* and thermoregulation were additive and significant (combined costs of 4·71 kJ per night; within the range of the daily energy requirements of passerine moult). Contrary to our predictions, house finches at subthermoneutral temperatures had lower disease expression and higher circulating levels of the cytokine interleukin-6 in response to experimental infection with *M. gallisepticum* than finches at thermoneutral. However, pathogen loads did not differ between the two temperature treatments. Finches from both treatments expressed fever in response to infection, but the magnitude of fever did not vary with ambient temperature.

4. Despite the significant energy costs of infection and thermoregulation, house finches from both temperature treatments maintained body mass and pectoral muscle condition, suggesting that birds housed at subthermoneutral consumed more food to maintain energy balance. In the field, competition for finite resources would be expected to exacerbate the effects found here and force infected birds to spend more time at feeders where *M. gallisepticum* is transmitted.

5. Overall, our results indicate that moderate cold stress alters house finch immunity, energetics and disease pathology, but does not alter infectiousness as measured by pathogen load. The effects of ambient temperature on host response and energy demands could directly or indirectly contribute to seasonal and geographical variation in disease dynamics in free-living house finches. More broadly, our results suggest that even subtle changes in abiotic factors such as temperature can alter host disease expression, with broad implications for disease dynamics.

Key-words: acute-phase response, climate, disease dynamics, fever, house finch, metabolic rate, *Mycoplasma gallisepticum*, temperature stress

Introduction

The extent to which abiotic factors such as temperature mediate host–pathogen dynamics is of growing interest (e.g. Martinez & Merino 2011), particularly given the potential for global climate change to alter the likelihood and severity of disease epidemics (Harvell *et al.* 2002; Ostfeld 2009). Although global climate change is generally characterized by warming, many areas of the United States, including the southeast where our study animals were collected, have

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experienced notable cooling (Karl *et al.* 1996). Furthermore, the magnitude of weather extremes is increasing globally with climate change (Jentsch, Kreyling & Beierkuhnlein 2007). Ambient temperature may influence the geographical range of a pathogen or its vector and may also alter the virulence or persistence of a pathogen in the environment (Harvell *et al.* 2009). A relatively less considered mechanism, particularly for endothermic vertebrates, is the effect of temperature-dependent changes in host immunity and pathogen susceptibility (Raffel *et al.* 2006; Harvell *et al.* 2009; Martin *et al.* 2010). The seasonal dynamics of many infectious agents in birds and mammals point to the potential for abiotic factors such as photoperiod and temperature to influence the outcome of host–pathogen interactions. However, the roles of host physiology and susceptibility in contributing to seasonal disease patterns and/or responses to global climate change are challenging to unravel (Dowell 2001; Nelson *et al.* 2002). Given the large number of zoonotic diseases transmitted by endothermic birds and mammals (Wolfe, Panosian Dunavan & Diamond 2007; Nunn *et al.* 2010), the role of temperature in altering endothermic host susceptibility to pathogens is particularly important to understand.

Thermoregulation is an important energetic cost for endothermic hosts that may directly trade-off with other energetically demanding processes such as host resistance to pathogens (reviewed in Schmid-Hempel 2011). For example, Liu *et al.* (2007) found that outbreaks of H5N1 virus in Eurasian wild birds were more likely to occur following severe temperature drops (below −8 °C), which they hypothesized to result from the combined stresses of migration and thermoregulation. A number of studies have examined the effects of cold temperatures on endothermic immunity in laboratory rodents and domesticated chickens, often with conflicting results. Demas & Nelson (1996) found that cold-stressed deer mice (*Peromyscus maniculatus*) housed at 8 °C had depressed basal IgG levels and lower splenic mass than conspecifics housed at a milder ambient temperature (20 °C). Interestingly, a follow-up study on the same system found comparable immune suppression in deer mice housed at low ambient temperatures (8 °C) only when animals were food restricted (Demas & Nelson 1998), but different immune parameters were measured in the latter study. Results from studies of the effect of cold temperatures on immune parameters in domesticated chickens vary markedly, with some showing immunosuppression (Regnier & Kelley 1981; Hester, Muir & Craig 1996), some finding no detectable effect (Regnier, Kelley & Gaskins 1980) and others indicating immunoenhancement (Subba Rao & Glick 1977; Hangalapura *et al.* 2003). Finally, Dabbert, Lochmiller & Teeter (1997) housed northern bobwhite (*Colinus virginianus*) at thermoneutral (21 °C) or subthermoneutral (cycles from 3·6 to −20 °C over 24 h) temperatures and measured immune competence at day 5. Although a large panel of immune characterizations were not influenced in cold-stressed bobwhite, survival in response to injection with the bacterium *Pasteurella multocida* was enhanced following cold stress, and the authors suggested that the activity of phagocytic leucocytes may have increased under cold stress, improving survival (Dabbert, Lochmiller & Teeter 1997). Few studies have examined how cold stress influences immunity in passerine bird species (Svensson *et al.* 1998; Burness *et al.* 2010), whose small body size make them particularly sensitive to heat loss at subthermoneutral temperatures. Svensson *et al.* (1998) compared antibody responses to diphtheria and tetanus vaccines in blue tits (*Parus caeruleus*) housed either at 20 °C both day and night or at 4 °C during the day and −15 °C at night. Cold-stressed birds had compromised antibody responses to both immune antigens, suggesting that cold temperatures may compromise adaptive immune responses for endothermic birds.

One component of the innate immune system thought to be particularly energetically costly in vertebrates is the acute-phase response (APR), a suite of physiological and behavioural changes that occur at the onset of infection and include fever, the release of pro-inflammatory cytokines, the expression of sickness behaviours, and activation and suppression of the hypothalamo-pituitary-adrenal and hypothalamo-pituitary-gonadal axes, respectively (Adelman & Martin 2009). As such, the acute-phase response may be more likely to trade-off with energetically expensive activities such as reproduction and thermoregulation than adaptive responses (Bonneaud *et al.* 2003). Burness *et al.* (2010) examined acute-phase responses of zebra finches (*Taeniopygia guttata*) at two ambient temperatures – one in the range of thermoneutrality (34 °C) and one subthermoneutral temperature (15 °C). Acute-phase responses as measured by changes in food consumption, sickness behaviours and changes in body temperature in response to injection with lipopolysaccharide (LPS) did not differ across the two ambient temperatures. However, zebra finches housed at 15 °C lost significantly more mass and used significantly more energy (as measured by metabolic rate) in response to LPS vs. saline injection (Burness *et al.* 2010). Therefore, although a direct trade-off was not detected between thermoregulation and the strength of the acute-phase response, mounting an immune response of equal magnitude at lower ambient temperatures placed zebra finches in a negative energy balance.

Taken together, prior work suggests that subthermoneutral temperatures can influence the strength of the endothermic vertebrate immune response or the energetic costs experienced by the host, but that effects likely differ both among immune components and the species being studied. Here, we examined how subthermoneutral temperatures influence host response to an ecologically relevant infectious pathogen – *Mycoplasma gallisepticum* – a bacterium that causes annual epidemics of debilitating conjunctivitis in North American house finches (*Carpodacus mexicanus*) (Ley, Berkhoff & McLaren 1996; Dhondt, Tessaglia & Slothower 1998). Because house finches are infected with this bacterial pathogen primarily during the fall and winter (Hartup *et al.* 2001; Altizer, Hochachka & Dhondt 2004) when ambient temperatures are frequently subthermoneutral, the role of ambient temperature on host response is particularly interesting and important to understand. We housed wild-caught house

finches at two ambient temperature regimes – thermoneutral or subthermoneutral – and examined the course of experimental infection with *M. gallisepticum* via disease expression (measured as conjunctival lesion scores) and pathogen load. We measured two aspects of the acute-phase response in response to *M. gallisepticum* infection and subthermoneutral temperatures – the expression of fever and circulating levels of interleukin-6 (IL-6), a pro-inflammatory cytokine associated with high energy expenditure in humans (Tsigos *et al.* 1997). We simultaneously measured metabolic rate to quantify the energetic costs of *M. gallisepticum* infection at distinct temperature regimes. We predicted that both responding to infection with *M. gallisepticum* and thermoregulating at cooler temperatures would place significant and additive energy demands on house finches such that metabolic rate would scale with these energetic challenges (i.e. metabolic rate of house finches in descending order: infected, subthermoneutral > control, subthermoneutral > infected, thermoneutral > control, thermoneutral). Second, because *M. gallisepticum* is most prevalent in wintering house finches, we predicted that birds subjected to the simultaneous energetic demands of thermoregulation and infection would experience lower body condition, lower expression of the acute-phase response, higher disease expression and higher pathogen load compared to birds not subjected to these treatments.

Materials and methods

EXPERIMENTAL DESIGN

We used a two-by-two factorial design, manipulating both ambient temperature and infection status (inoculation with *M. gallisepticum* or media alone). The final sample size for each cell (temperature treatment*infection status) was 7–8 individuals, for a total of 30 house finches. To more closely mimic natural conditions, both temperature treatments experienced a 6 °C difference in ambient temperature between daytime (07.00–19.00) and night-time (19.00–07.00). For the subthermoneutral treatment [19 °C (day), 13 °C (night)], we selected temperatures below the apparent thermoneutral zone of house finches (Dawson, Buttemer & Carey 1985; Root, O'Connor & Dawson 1991). In contrast, for the thermoneutral treatment [28 °C (day), 22 °C (night)], we selected temperatures within the thermoneutral zone (Dawson, Buttemer & Carey 1985; Root, O'Connor & Dawson 1991).

EXPERIMENTAL TIMELINE

Prior to and following inoculation (days −2, 1, 4, 7, 10 and 13 post-inoculation), we measured metabolic rate, disease expression (conjunctival lesion score), pathogen load, cloacal body temperature, body mass and pectoral muscle condition for all individuals (see details below). Furthermore, we bled all individuals on day 2 and extracted plasma to quantify circulating levels of the pro-inflammatory cytokine IL-6 (see details below). We staggered the inoculation date of each group of 7–8 individuals so that metabolic rate measurements could be taken at the same time point prior to or following inoculation. Therefore, our experiment was divided into two rounds, which included equivalent distributions of all four treatment cells. Round 1 was completed from November 1 to 18 and Round 2 from November 30 to December 16, 2010.

BIRD CAPTURE AND HOUSING

We captured 30 house finches using cage traps in Auburn, Alabama, USA and transported them to Virginia Tech where they were housed in indoor animal rooms in groups of 2–3 individuals. A *M. gallisepticum*-specific enzyme-linked immunosorbent assay (ELISA) indicated that all birds were seronegative for exposure to this pathogen at the time of capture (serological methods as per Hawley *et al.* 2011). Two weeks prior to inoculation (day 0), we transported all birds to one of two walk-in environmental chambers and housed them in individual cages (30″ × 18″ × 18″) on a 12L : 12D light cycle for the duration of the experiment. Birds were fed *ad libitum* (Roudybush Maintenance Diet) and provided with two wooden perches. All procedures for animal care and use were approved by Virginia Tech Institutional Animal Care and Use Committees.

TEMPERATURE TREATMENT

All individuals were housed at 28 °C during their first 3 days in the environmental chamber. To minimize stress while birds adjusted to experimental temperatures, the ambient temperature of the environmental chambers was incrementally lowered as follows: the night-time temperature for the thermoneutral treatment group was lowered by 2 °C increments on days −10, −8 and −6 until birds were housed at 22 °C overnight for the remainder of the experiment. The daytime temperature for the subthermoneutral treatment group was lowered by 3 °C increments and the night-time temperatures by 5 °C increments on days −10, −8 and −6 until birds were housed at 19 °C during the daytime and 13 °C overnight for the remainder of the experiment.

EXPERIMENTAL INOCULATION

On day 0, we inoculated all individuals bilaterally in the palpebral conjunctiva with either 0·025 mL of Frey's media (negative control) or 0·025 mL of an isolate of *M. gallisepticum* obtained from a free-living house finch. This isolate (NC2006) was obtained from a male house finch with conjunctivitis in North Carolina in September 2006. The fourth passage of NC2006 (2006·080-5 4P 1/9/09) was expanded and quantified for use as inoculum (David H. Ley, NC State University, College of Veterinary Medicine, Raleigh, NC, USA 27606). The batch of inoculum used in this experiment had a viable count of $3{\cdot}04 \times 10^8$ colour changing units per mL of Frey's media as determined by the most probable number method (Meynell & Meynell, 1970). Because of the staggered nature of inoculation, a single 1 mL tube of inoculum was thawed once, aliquoted into four equal volumes and refrozen at −80 °C prior to use. Therefore, viable counts were likely lower than $3{\cdot}04 \times 10^8$ colour changing units at the time of inoculation, though equivalent for each round. The selected inoculation dose falls within the range of pathogen load values harboured by free-living infected house finches captured in Auburn, Alabama (D. M. Hawley *et al.*, unpublished data).

ENERGY EXPENDITURE

We determined house finch metabolic rates using open-flow respirometry (Microoxymax; Columbus Instruments, Columbus, OH, USA). The instrument is computer-controlled, allows monitoring of multiple (n = 10) independent respiratory chambers simultaneously and is interfaced with a large environmental chamber that allows control of temperature. Incurrent air passed through columns of Drierite® to

absorb water vapour before passing into individual respirometry chambers. Air leaving respirometry chambers was dried again using a hygroscopic Nafion tube drier (Columbus Instruments) to remove water vapour before determining oxygen consumption rates using an electrochemical fuel cell. We simultaneously determined CO_2 production rates in each chamber, and oxygen consumption values were corrected for CO_2 concentrations using the MicroOxymax software. Additional details on respirometry procedures are outlined in recent publications (Dorcas, Hopkins & Roe 2004; Hopkins *et al.* 2004; DuRant *et al.* 2008).

We conducted all metabolic measurements at night (~17.45–08.00) at the night-time temperature for a given treatment. House finches were weighed prior to placement in individual 1-L, covered respiratory chambers (n = 8/trial) and held in the dark in the environmental chamber for the duration of metabolic measurements. Trials began at 17.30–17.55, and oxygen consumption rates (mL h^{-1}; VO_2) of finches were measured ~45 min after the start of the trial and every 27 min thereafter resulting in 29 measurements of VO_2 per house finch per trial. The first three data points were discarded from the data set because birds were acclimating to the chamber conditions and recovering from handling during this time. After the acclimation period, finches exhibited very little activity during metabolic measurements as evidenced by their stable respiratory rates. Trials ended at 07.50–08.15. Metabolic chambers were cleaned thoroughly with bleach to prevent cross-contamination between trials.

For each trial, we removed activity from our estimates of each individual's metabolic rate (hereafter MR), by ranking oxygen consumption rates in ascending order and designating the lowest quartile value as each finch's MR (Hopkins *et al.* 2004). Thus, the MR of control birds at thermoneutral represents resting metabolic rate (RMR). In contrast, MRs of birds exposed to subthermoneutral temperatures provide an estimate of RMR plus the additional cost of thermoregulating. Similarly, MRs of birds exposed to *M. gallisepticum* provide an estimate of RMR plus the additional cost of infection. In addition to quantifying MR, we estimated the total volume of oxygen consumed (mL) by each inactive finch at night (12 h) during experimental infection. To accomplish this, we assumed that finches maintained stable MRs for each 12-h measurement period and then linearly interpolated between each of the five evenings post-inoculation that we quantified MR. We integrated the area of the curve produced by plotting MR against sample date. Because our estimates of MR were only generated for the 12 h of night-time, we divided this integral into half to produce estimates of night-time oxygen consumption. We then converted oxygen consumed into energy equivalents by assuming that 1 L of oxygen is equivalent to 19·8 kJ (Gessaman & Nagy 1988). We eliminated three individuals that died between day 10 and 13 (see Statistical analysis) from our calculations of total volume of oxygen consumed.

DISEASE EXPRESSION

Disease expression (e.g. the extent of conjunctival lesions) was scored by an individual blind to each bird's temperature treatment (A.W. or T.L.) on a 0–3 scale (as per Hawley *et al.* 2011) as follows: an eye received a score of 0 for no swelling, 1 for minor swelling around the eye ring, 2 for moderate swelling and eversion of the conjunctival tissue, and 3 if the eye was nearly hidden by swelling and crusted exudate. Lesion scores for each individual represent the sum of scores for both eyes as measured on a given sampling day.

PATHOGEN LOAD

We quantified *M. gallisepticum* presence and load using quantitative PCR as described in Grodio *et al.* (2008). Sterile forceps (Fisher Scientific NC9655622) were used to insert a sterile cotton swab predipped in tryptose phosphate broth (TPB) into each conjunctiva. Following 5 s of swabbing, the end of the swab was placed directly into 300 μL of sterile TPB. Swabs were swirled and wrung out on the inside of tubes to remove liquid from them before they were discarded. Samples from each eye (left and right) were pooled for a given bird and sampling day. Samples were kept on ice and then frozen at −20 °C prior to DNA extraction.

We extracted genomic DNA from all conjunctival swabs using Qiagen DNeasy 96 Blood and Tissue kits (Qiagen, Valencia, CA, USA). As per Grodio *et al.* (2008), we used primers and a probe that targeted the *mgc2* gene of *M. gallisepticum*. Each 25 μL reaction contained 12·5 μL iQ™ Supermix (2X), 0·65 μL each of 10 μM forward and reverse primers, 0·35 μL of 10 μM probe, 5·85 μL DNase-free water and 5 μL of DNA sample. Cycles of reaction were performed using an MyiQ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the following parameters: 95 °C for 3 min and 40 cycles of: 95 °C for 3 s and 60 °C for 30 s with a ramp rate at 0·5 ° per second. Standard curves were generated for each run. The standard was based on 10-fold serial dilutions of plasmid containing a 303-bp *mgc2* insert (Grodio *et al.* 2008). The curve was created using $1{\cdot}15 \times 10^2$–$1{\cdot}15 \times 10^8$ copy numbers.

PLASMA IL-6 LEVELS

We assayed plasma IL-6-like bioactivity using a cell proliferation assay, following the methods of Adelman *et al.* (2010) and Van Oers, Van Der Heyden & Aarden. (1988), with several minor modifications. First, as LPS was not used in the experiment, no polymyxin B was added to the cell suspension in this assay. Second, initial plasma dilutions started at 1 : 2 rather than 1 : 4. Finally, instead of confining absorbance measurements to 595 nm, we controlled for non-specific turbidity by subtracting absorbance values at 630 nm from those at 595 nm on a Bio-Rad iMark plate reader (168–1135; Life Science Research, Hercules, CA, USA). For statistical analyses, we extracted a single value of absorbance for each bird by averaging absorbance at 595–630 nm across all six dilutions and both replicates (Adelman *et al.* 2010).

BODY TEMPERATURE

We measured body temperature using a cloacal thermometer (Model 3K4054; Miller & Webber Inc., New York, NY, USA) between 07.50 and 08.30 as birds were removed from their metabolic chambers. Fever measurements were taken within 1 min of removing each individual from their metabolic chamber to minimize variation in body temperature because of handling stress. To avoid cross-contamination, we used a single thermometer for all infected birds and a single thermometer for all control birds. To control for potential differences between the two thermometers, we quantified fever as the change in temperature for an individual relative to its body temperature at day −2 prior to inoculation.

BODY CONDITION INDICES

We measured body mass to 0·1 g using an electronic balance prior to placing each finch in the metabolic chamber (all mass measurements

were taken between 17.00 and 17.45). We used a modification of Gosler (1991) to score the amount of pectoral muscle around the keel on a 1–4 scale as follows: 1 = poor pectoral muscle condition, concave muscle and prominent keel; 2 = moderate pectoral muscle condition, keel detectable but not prominent, muscle slightly concave; 3 = good pectoral muscle condition, keel only slightly detectable by touch, muscle not concave or slightly convex; and 4 = excellent pectoral muscle condition, keel not detectable by touch, muscle significantly convex. For statistical analyses, we examined changes in body mass and pectoral muscle condition relative to each individual's value at day −2 prior to inoculation.

STATISTICAL ANALYSIS

We performed all statistical analyses in SAS 9.1 (SAS Institute, Cary, NC, USA). We used mixed linear models (PROC MIXED) to examine the effect of temperature treatment, infection status (control or *M. gallisepticum*) and their interaction on metabolic rate, disease expression, pathogen load, body temperature and body condition indices over time. As a result of unequal samples sizes across groups, we used the Satterthwaite correction to better approximate denominator degrees of freedom.

To incorporate how our dependent variables changed over the course of experimental infection, all models included day post-inoculation (PI) as both a main effect and in interaction with infection status and temperature treatment. Body mass was included as a covariate and metabolic chamber as a random effect in all analyses of metabolic rate. Experimental round and bird ID were included as random effects in all models, and observer identity was included as an additional random effect for all disease expression models.

We compared total energy expended during the course of the study using a general linear model (PROC GLM) with finch body mass at the start of the study as a covariate. Both temperature treatments, infection status and their interaction were included as main effects.

Three individuals (one control from each temperature treatment, one infected from the thermoneutral treatment) died of undetermined causes between day 10 and 13 (the penultimate and final day of measurement). With the exception of the calculations of total energy expenditure throughout the course of infection (see Energy expenditure, above), we retained these three birds in all statistical analyses. Removal of these three individuals from the analyses did not alter our results or conclusions.

Results

METABOLIC RATE AND ENERGY EXPENDITURE

Infection with *M. gallisepticum* and subthermoneutrality had significant and additive effects on house finch MR. When controlling for body mass, which had a strong positive influence on MR ($F_{1,77·2}$ = 25·7, P < 0·001), house finches experimentally infected with *M. gallisepticum* had significantly higher MRs (infection status: $F_{1,25·8}$ = 8·17, P = 0·008; days PI*infection status: $F_{5,125}$ = 5·27, P < 0·001) than control finches. Infected finches in the thermoneutral treatment showed on average an 8·6% increase in MR relative to control finches within the same temperature treatment, while infected finches in the subthermoneutral treatment showed a 5·7% increase in MR relative to control finches within the same temperature treatment (Fig. 1). Consistent with Dawson, Buttemer & Carey (1985), subthermoneutral temperature had a strong effect on house finch MR (temperature: $F_{1,25·6}$ = 271·85, P < 0·001), resulting in 33·8% higher MRs relative to house finches held at thermoneutrality (Fig. 1). There was no significant interaction between experimental infection and temperature on MR (temperature*infection status: $F_{1,25·6}$ = 0·09, P = 0·77), indicating that effects of *M. gallisepticum* infection and thermoregulation on house finch energetics were additive.

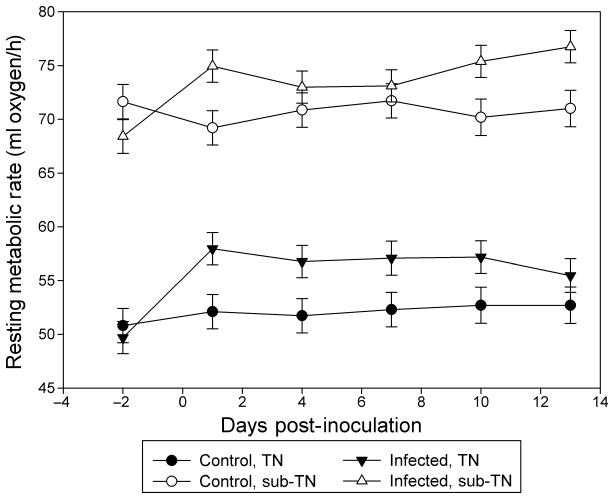

Fig. 1. Metabolic rates of house finches held at thermoneutral (=TN, closed symbols) or subthermoneutral (open symbols). On day 0, infected individuals (triangles) were inoculated with *Mycoplasma gallisepticum*, while control individuals (circles) were given media alone. Data (mean ± SE) are predicted means of a mixed model controlling for body mass, experimental round and respiratory chamber.

Infection with *M. gallisepticum* increased night-time energy expenditure of finches by 10·1–10·6 kJ relative to uninfected finches at their respective temperature regime ($F_{1,22}$ = 8·02; P = 0·01; mass covariate: $F_{1,22}$ = 5·61; P = 0·03; Table 1). Subthermoneutral temperature is predicted to have resulted in 49·9 kJ greater night-time energy expenditure during the 13 days of this study relative to finches housed at thermoneutral ($F_{1,22}$ = 158; P < 0·001; Table 1). As with metabolic rate, there was not a significant interaction between infection and thermal treatment on oxygen consumption (P = 0·98). The additive effects of infection and thermoregulation at cooler temperatures are predicted to have resulted in 61·3 kJ greater night-time total energy expenditure compared to uninfected finches at thermoneutral temperatures over the course of the study (Table 1).

DISEASE EXPRESSION

Conjunctival lesion scores varied significantly with days since inoculation (days PI: $F_{1,143}$ = 157·95, P < 0·001; Fig. 2). As expected, experimentally inoculated individuals had significantly higher eye lesion scores than control individuals (infection status: $F_{1,39·1}$ = 5·44, P = 0·03; infection status*days PI: $F_{1,143}$ = 158·72, P < 0·001). However, contrary to our

Table 1. Energetics of house finches (*Carpodacus mexicanus*) challenged simultaneously with subthermoneutral (sub-TN) temperatures and infection with *Mycoplasma gallisepticum*. The projected night-time energy costs (kJ) for the course of *M. gallisepticum* infection are mass-corrected and were calculated by assuming an average infection duration of 72·8 days (Kollias *et al.* 2004) and equivalent nightly energetic costs throughout that time period

	Relevant comparison	kJ total (over 13-day study duration*)	kJ per night	Projected night-time cost (kJ) for course of infection
Cost of infection at thermoneutrality	Infected TN – control TN	11·2	0·86	62·8
Cost of infection at subthermoneutrality	Infected sub-TN – control sub-TN	11·4	0·87	63·7
Cost of subthermoneutrality alone	Control sub-TN – control TN	49·9	3·84	279·4
Cost of simultaneous infection and subthermoneutrality	Infected sub-TN – control TN	61·3	4·71	343·1

*Only post-inoculation oxygen consumption values were used to calculate energetic costs.

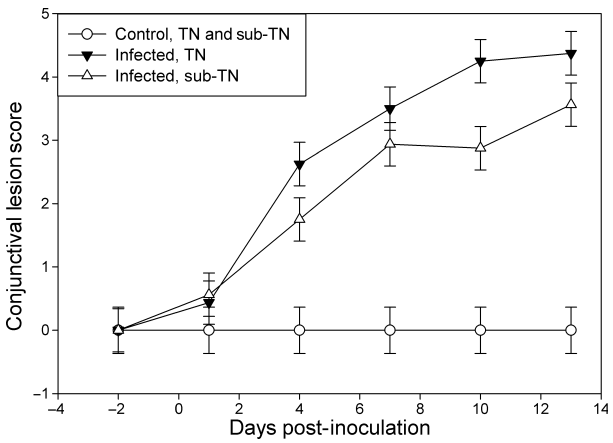

Fig. 2. Conjunctival lesion scores in response to inoculation with *Mycoplasma gallisepticum* for house finches held at thermoneutral (=TN, closed symbols) or subthermoneutral (open symbols). On day 0, infected individuals (triangles) were inoculated in the conjunctiva with *M. gallisepticum*, while control individuals (circles) were given media alone. Data (mean ± SE) are predicted means of a mixed model controlling for experimental round.

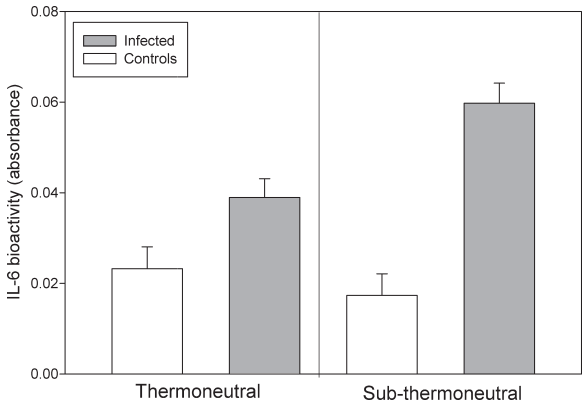

Fig. 3. Plasma IL-6 bioactivity of house finches held at thermoneutral (left) or subthermoneutral (right). Plasma samples were taken 44 h after finches were inoculated with either media (open bars) or *Mycoplasma gallisepticum* (closed bars). Data (mean ± SE) are predicted means of a mixed model controlling for experimental round.

predictions, house finches in the subthermoneutral treatment had lower eye lesion scores over time than finches held at thermoneutral temperatures (treatment*days PI: $F_{1,140} = 3{\cdot}88$, $P = 0{\cdot}05$; treatment*days PI*infection status: $F_{1,139} = 3{\cdot}36$; $P = 0{\cdot}07$ Fig. 2).

PATHOGEN LOAD

Pathogen load increased significantly over time in birds inoculated with *M. gallisepticum* (days PI: $F_{1,156} = 24{\cdot}05$, $P < 0{\cdot}001$; infection status: $F_{1,156} = 48{\cdot}54$, $P < 0{\cdot}001$; days PI*status: $F_{1,156} = 29{\cdot}98$, $P < 0{\cdot}001$). However, infected birds in the subthermoneutral (mean and SE: 5·85 ± 0·27 $\log_{10}$ copies) and thermoneutral (mean and SE: 5·91 ± 0·27 $\log_{10}$ copies) treatments had equivalent pathogen loads (treatment, treatment*days PI, treatment*infection status: all $F < 0{\cdot}16$, all $P > 0{\cdot}69$). The low variance in pathogen load (0·11–0·30) within the inoculated birds prevented us from quantifying relationships between pathogen load and other response variables such as metabolic rate, fever or oxygen consumption.

PLASMA IL-6 LEVELS

Infected house finches held at both temperatures showed higher levels of IL-6 activity than uninfected controls (Fig. 3; infection status: $F_{1,22} = 40{\cdot}91$, $P < 0{\cdot}001$). Contrary to our predictions, IL-6 levels were highest for infected birds housed at subthermoneutral temperatures (Fig. 3; infection status*treatment: $F_{1,22} = 8{\cdot}65$, $P = 0{\cdot}008$).

BODY TEMPERATURE

Infection status significantly influenced body temperature (Fig. 4; $F_{1,26{\cdot}1} = 9{\cdot}56$; $P = 0{\cdot}005$), with infected birds from both thermal treatments increasing their body temperature post-inoculation relative to uninfected birds. The subthermoneutral treatment tended to result in lower average body temperatures regardless of infection status ($F_{1,26{\cdot}1} = 3{\cdot}44$; $P = 0{\cdot}08$). There was no interaction between treatment and infection status on body temperature ($P > 0{\cdot}92$); though

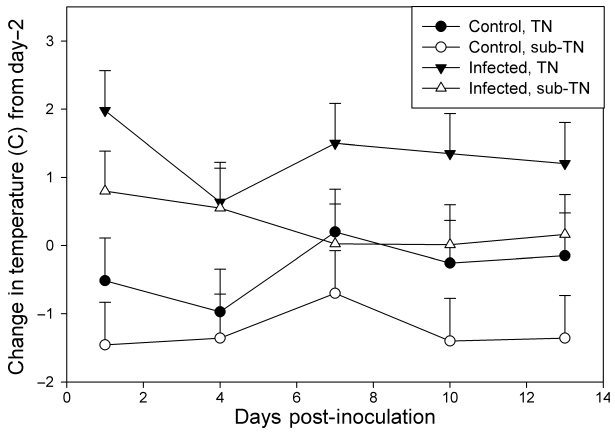

Fig. 4. Change in body temperature of house finches held at thermoneutral (=TN, closed symbols) or subthermoneutral (open symbols). On day 0, infected individuals (triangles) were inoculated with *Mycoplasma gallisepticum*, while control individuals (circles) were given media alone. Temperatures were calculated relative to baseline body temperatures recorded on day −2, prior to inoculation. Data (mean ± SE) are predicted means of a mixed model controlling for experimental round. Standard error bars are shown in only one direction to facilitate visual comparisons among groups.

qualitatively, infected birds in the two treatments appear to behave distinctly. Birds in the thermoneutral treatment experienced a 2 °C increase in body temperature at day 1 post-inoculation (Fig. 4), and body temperatures remained high (on average >1 °C) for most days of infection. In contrast, infected individuals in the subthermoneutral treatment showed a moderate increase in body temperature (average of 0·8 °C on day 1) that rapidly declined over the first few days of infection.

BODY CONDITION INDICES

Birds in both temperature treatments maintained their body mass over time (treatment as main effect or in interaction with infection or time: all $F < 0{\cdot}29$, $P > 0{\cdot}59$). Although body mass generally decreased in infected birds during the initial stages of infection (days PI*infection status: $F_{1,143} = 4{\cdot}75$, $P = 0{\cdot}03$), this effect did not vary with temperature treatment. Pectoral muscle condition also decreased in infected birds over time (days PI*infection status: $F_{1,143} = 13{\cdot}3$, $P < 0{\cdot}001$), but there was no effect of temperature treatment as a main effect or in interaction with infection (all $F < 0{\cdot}01$, $P < 0{\cdot}94$). Although the three-way interaction between treatment, time and infection status on pectoral muscle condition was statistically significant (treatment*days PI*infection status: $F_{1,143} = 3{\cdot}87$, $P = 0{\cdot}05$), there was no detectable directional effect of temperature treatment over time.

Discussion

We found that thermoregulation and pathogen infection have significant and additive effects on energy expenditure in house finches. Both subthermoneutral temperatures and experimental infection significantly increased night-time energy expenditure of house finches in our study. However, we did not detect an interactive effect of thermoregulation and pathogen infection on metabolic rate, indicating that the energetic costs of infection with *M. gallisepticum* are comparable at thermoneutral and subthermoneutral temperatures. Our results are consistent with previous work in endothermic rodents, which detected additive but not interactive effects of cold exposure and nematode parasitism on RMR (Kristan & Hammond 2000, 2003), but in contrast to work by Meagher & O'Connor (2001), which found that nematode infection resulted in increased metabolic rates in deer mice only under short-term but severe cold stress (2 °C plus Heliox gas flow).

The simultaneous, additive demands of thermoregulating and combating infection resulted in an average 42·2% increase in metabolic rate and a night-time energy requirement of 4·71 kJ per night compared to birds not experiencing either of these challenges (Table 1). The average night-time energy expenditure associated with infection and thermoregulation (4·71 kJ per night) in house finches is comparable to the daily cost of moult for some, but not all, small birds (Hoye & Buttemer 2011). Given that house finches in North America regularly face the demands of *M. gallisepticum* infection and thermoregulation simultaneously (Hartup *et al.* 2001; Altizer, Hochachka & Dhondt 2004) and that they typically face much colder temperatures than in our study, the detected energetic costs of infection and thermoregulation are biologically meaningful for free-living house finches and likely to vary along latitudinal gradients with ambient temperature. Importantly, the *ad libitum* food in our study allowed house finches to compensate for the energy deficits caused by thermoregulation and infection, as body mass and pectoral muscle condition were unaffected by temperature treatment. In the wild, competition for limited resources and the potentially lower competitive ability of infected individuals (Bouwman & Hawley 2010) are likely to make it more difficult for finches to compensate for the energy deficits associated with infection and thermoregulation.

To our knowledge, this is the first study to estimate the energetic cost of experimental infection with an acute, naturally occurring infectious pathogen in an ecologically relevant host. Although many studies have estimated the metabolic costs of the chronic infections caused by ecologically relevant ectoparasites, microsporidia and macroparasitic worms (reviewed in Robar, Murray & Burness 2011), the acute nature of bacterial, viral and some protozoal infections may result in distinct metabolic responses within hosts. Quantifying the energetic costs of pathogen infection is critical as these costs can shed light on the evolution of optimal host immune defenses (Romanyukha, Rudnev & Sidorov 2006), an overarching goal of the field of ecological immunology (Viney, Riley & Buchanan 2005). Experimental infection with *M. gallisepticum*, which causes reduced overwinter survival in free-living house finches (Faustino *et al.* 2004), resulted in an average 6·6% increase in metabolic rate, equivalent to an

additional 0·86–0·87 kJ per night (Table 1). Several studies have measured the energetic costs of immune responses via novel antigen injection, with somewhat variable results (reviewed in Schmid-Hempel 2011). In passerine birds, cutaneous injections with phytohemagglutinin, which stimulates aspects of innate and adaptive immunity, resulted in a 4·5% increase in RMR in great tits (*Parus major*) (Nilsson, Granbom & Råberg 2007), but a much higher 29% increase in resting oxygen consumption in house sparrows (*Passer domesticus*) (Martin, Scheuerlein & Wikelsi 2003). The acute-phase response to systemic injection with lipopolysaccharide, which may be more comparable to the host inflammatory response generated by infection with *M. gallisepticum*, resulted in a 10% average increase in metabolic rate in zebra finches (Burness *et al.* 2010). Overall, the increase in metabolic rate associated with *M. gallisepticum* infection detected in this study falls below that typically measured for immune antigen injection alone, which suggests that immune activation owing to antigen injection may be stronger but perhaps of shorter duration than that associated with an active pathogen infection. The lower metabolic costs associated with *M. gallisepticum* infection relative to immune antigen injection may result from actively infected individuals downregulating certain physiological processes to counteract the energetic demands of fighting a prolonged infection. Given that experimental *M. gallisepticum* infection lasts an average of 10·4 weeks in house finches (Kollias *et al.* 2004), the overall night-time energetic costs of responding to this infectious pathogen may be as high as 62·8 kJ at thermoneutrality and 63·7 kJ at subthermoneutrality (Table 1). Further work is needed to determine the diurnal costs of infection with *M. gallisepticum*, which will allow more robust estimates of total energy costs experienced by infected finches.

We found that house finches at subthermoneutral temperatures had higher plasma IL-6 bioactivity and moderately lower severity of the conjuctival pathology characteristic of infection with *M. gallisepticum* (Kollias *et al.* 2004). These results suggest that mild cold stress enhances pro-inflammatory cytokine levels in house finches, but paradoxically, does not appear to result in enhancement of conjunctival lesions, which are largely inflammatory (Hawley *et al.* 2011). Transcription of IL-6 and other pro-inflammatory cytokine genes, including TNF-α and IFN-γ, is upregulated during the early stages of *M. gallisepticum* infection in chickens (Mohammed *et al.* 2007), though the role each cytokine plays in disease progression remains unclear. An enhancing effect of cold stress on pro-inflammatory cytokine protein levels or gene expression has been documented in mice (Monroy *et al.* 1999), rats (Yildirim & Yurekli 2010), humans (Rhind *et al.* 2001) and chickens (Hangalapura *et al.* 2006), a result hypothesized to stem from attempts to maintain homeostasis, perhaps via cytokine-mediated recruitment of immune cells, in response to an environmental challenge. Both cold stress and other types of stressors have also been associated with enhanced resistance to bacterial pathogens in chickens (Gross 1962; Gross & Siegel 1965) and in bobwhite quail (Dabbert, Lochmiller & Teeter 1997). Although we did detect moderately lower conjunctival pathology in cold-stressed house finches, bacterial pathogen load did not differ, suggesting that resistance *per se*, or the ability to clear *M. gallisepticum* infection, did not differ across the two temperature treatments. This discrepancy suggests that conjunctival lesions are more dependent on host responses than on pathogen load *per se*. This result is consistent with histopathological analysis of conjunctival lesions in *M. gallisepticum*-infected house finches, which reveal inflammatory infiltration and epithelial hyperplasia, characteristics of host immune-mediated pathology (Luttrell *et al.* 1996, 1998; Hawley *et al.* 2011).

The mechanistic relationship between pro-inflammatory cytokine activity and conjunctival inflammation during *M. gallisepticum* infection is not yet known in house finches, but further study may reveal whether the higher IL-6 levels detected in the subthermoneutral treatment indicate protection from the severe inflammation characteristic of this disease (Kollias *et al.* 2004), perhaps via avoidance of chronic, disregulated inflammation. One potential explanation for the apparent discrepancy between IL-6 levels and pathology is that IL-6 may act as a transitional molecule between pro- and anti-inflammatory signals during the acute-phase response. In mammals, as the acute-phase response progresses, IL-6 facilitates the upregulation of anti-inflammatory molecules such as IL1-ra, IL-10 and sTNF-R (Tilg, Dinarello & Mier 1997; Steensberg *et al.* 2003). As our samples were taken at 44 h post-infection, it is possible that at this time point, IL-6 is playing more of an anti-inflammatory role. If this is the case, the elevated levels we see in subthermoneutral birds would be consistent with the reduced severity of conjunctival lesions in that group.

The differences in host response associated with ambient temperature may play an important and complex role in population-level dynamics of *M. gallisepticum*. The additive energetic costs associated with thermoregulation and *M. gallisepticum* infection likely contribute to the higher overwinter mortality rate of infected finches (Faustino *et al.* 2004). More interestingly, the energetic costs observed here may indirectly contribute to seasonal epidemics of *M. gallisepticum* in house finches (Altizer, Hochachka & Dhondt 2004; Hosseini, Dhondt & Dobson 2004), which are largely limited to periods of subthermoneutral temperatures. Visibly diseased house finches spend significantly more time on bird feeders than healthy conspecifics where they deposit and transmit *M. gallisepticum* (Hawley, Davis & Dhondt 2007), but the role of ambient temperature in mediating the extent of infection-induced changes in behaviour has not been examined. Because infected birds in the subthermoneutral treatment were able to maintain body mass and condition equivalent to birds housed at thermoneutral despite the high energetic costs of infection and thermoregulation, infected individuals exposed to subthermoneutral temperatures must be eating more and/or reducing their activity to maintain energy balance. Infected house finches show reductions in activity both in captivity (Kollias *et al.* 2004) and in the wild (Hawley, Davis & Dhondt 2007), which may help to

compensate for the increased energetic demands of infection. However, in our laboratory experiment, activity of all birds was greatly restricted because they were confined in individual cages. Thus, increased feeding under *ad libitum* conditions is probably the simplest mechanism by which finches compensated for the energy costs of infection. During colder months in nature, it seems probable that energy deficits force infected birds to spend more time on bird feeders where they deposit *M. gallisepticum* (Dhondt *et al.* 2007). Increased foraging to compensate for energetic deficits is therefore one mechanism by which cooler ambient temperatures may indirectly augment transmission of *M. gallisepticum* and contribute to the seasonality of *M. gallisepticum* epidemics.

Our work also sheds light on whether ambient temperature may influence geographical variation in *M. gallisepticum* dynamics. The effect of subthermoneutrality on host disease expression, whereby house finches housed at cooler temperatures showed less severe conjunctival lesions, is consistent with documented latitudinal differences in conjunctivitis prevalence in eastern North American finches. House finches wintering in the warmer southeastern United States (minimum January temperatures: −3 to 15 °C) show significantly higher prevalence of mycoplasmal conjunctivitis than do house finches in the cooler northeast (minimum January temperatures: −22 to −9 °C) at the same time of year (Altizer, Hochachka & Dhondt 2004). Although statistical modelling shows that host life-history and density can explain latitudinal patterns of disease pathology (Hosseini, Dhondt & Dobson 2004), these biotic factors covary with ambient temperature in the wild. While the difference in conjunctival pathology across our temperature treatments was subtle (an average of 0·5 lower eye score in finches at subthermoneutrality), these effects could be more pronounced at ambient temperatures characteristic of those experienced by wintering house finches across their range, such that birds exposed to realistic northeastern minimum temperatures (−22 to −9 °C) would exhibit even less pathology. By isolating the effect of ambient temperature, our results suggest that this abiotic factor must be considered in concert with other biotic factors as a potential driver of latitudinal differences in house finch disease dynamics. Overall, further study on the role of abiotic factors in driving host response to wildlife pathogens is critical in order to understand what role host immunity and susceptibility may play in seasonal and geographical variation in population-level disease dynamics (Martin *et al.* 2010; Hawley & Altizer 2011).

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