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RESEARCH ARTICLE

Body temperature changes during simulated bacterial infection in a songbird: fever at night and hypothermia during the day

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ABSTRACT

Although fever (a closely regulated increase in body temperature in response to infection) typically is beneficial, it is energetically costly and may induce detrimentally high body temperatures. This can increase the susceptibility to energetic bottlenecks and risks of overheating in some organisms. Accordingly, it could be particularly interesting to study fever in small birds, which have comparatively high metabolic rates and high, variable body temperatures. We therefore investigated two aspects of fever and other sickness behaviours (circadian variation, dose dependence) in a small songbird, the zebra finch. We injected lipopolysaccharide (LPS) at the beginning of either the day or the night, and subsequently monitored body temperature, body mass change and food intake for the duration of the response. We found pronounced circadian variation in the body temperature response to LPS injection, manifested by (dose-dependent) hypothermia during the day but fever at night. This resulted in body temperature during the peak response being relatively similar during the day and night. Day-to-night differences might be explained in the context of circadian variation in body temperature: songbirds have a high daytime body temperature that is augmented by substantial heat production peaks during activity. This might require a trade-off between the benefit of fever and the risk of overheating. In contrast, at night, when body temperature is typically lower and less variable, fever can be used to mitigate infection. We suggest that the change in body temperature during infection in small songbirds is context dependent and regulated to promote survival according to individual demands at the time of infection.

KEY WORDS: Acute phase response, Circadian variation, Dose dependence, Heterothermy, LPS, Sickness behaviour, *Taeniopygia guttata*, Zebra finch

INTRODUCTION

Fever, a closely regulated increase in the body's set-point temperature in response to infection, is an evolutionarily conserved defence mechanism (Kluger et al., 1998; Blatteis, 2003) that is widely used across the animal kingdom in organisms ranging from invertebrates (Boorstein and Ewald, 1987; Adamo, 1998) to endotherms (Kurokawa et al., 1996; Escobar et al., 2007; Bingham et al., 2009). Two, not mutually exclusive, hypotheses have been proposed to explain the adaptive value of fever: (i) fever might cause a hostile environment for pathogens, which hampers their growth, proliferation and survival, and/or (ii) fever may enhance the efficiency of the host's immune system, thereby facilitating clearance of the infection (Kluger et al., 1998; Blatteis, 2003). However, the role of fever in disease is enigmatic because it is energetically costly (Kluger, 1991; Marais et al., 2011c), its occurrence or absence during infection is equivocal and its benefits are not always obvious (Kluger et al., 1998; Blatteis, 2003).

In vertebrates, fever is an integral part of the acute phase response – the first line of defence against a pathogen – that consists of a suite of physiological and behavioural adjustments (Hart, 1988; Blatteis, 2003). During an acute phase response, animals display typical 'sickness behaviours', that (besides fever) include reduced food intake (and even anorexia) and activity (lethargy). These adjustments collectively act to alleviate the effects of infections and facilitate the elimination of the pathogen (Hart, 1988; Kluger et al., 1998). However, because sickness behaviours affect metabolic rate and, hence, an animal's energy budget, they may ultimately constrain the amount of energy available for other activities (e.g. Sheldon and Verhulst, 1996). It is perhaps partly for this reason that empirical studies show variation in the strength of the fever response depending on, for example, pathogen load (Maloney and Gray, 1998; Koutsos and Klasing, 2001; Deak et al., 2005; Rudaya et al., 2005), ambient temperature (Rudaya et al., 2005), site of infection (Ashley and Wingfield, 2012) and circadian timing of infection (Nomoto, 1996). In other cases, an organism may respond to infection with hypothermia instead of fever, either as a natural part of the body's defence or as a result of septic shock (Romanovsky et al., 1996, 2005; Martin et al., 2008). Under the former scenario, fever and hypothermia have been suggested to be two alternative strategies to mitigate infection. Hypothermia would be favoured when resources are scarce and the energetic costs of a fever response cannot be supported (Romanovsky and Székely, 1998). This can be the case during very severe infections or in energetically demanding environments (Liu et al., 2012), or in cases where insufficient body insulation and/or small body size precludes any sustained increase in body temperature (such as in neonates) because the resultant heat loss would be detrimental (Jones et al., 1983; Frafield and Kaplanski, 1998).

Patterns in the presence or absence of fever during an acute phase response in birds are equivocal. Even in response to a challenge with similar or identical doses of the same artificial endotoxin, large non-passerine birds such as fowl typically demonstrate fever (Maloney and Gray, 1998; Koutsos and Klasing, 2001; Leshchinsky and Klasing, 2001; Marais et al., 2011a), whereas small passerines sometimes respond with fever (Adelman et al., 2010a,b; Coon et al., 2011; Nord et al., 2013) and sometimes with hypothermia (Owen-Ashley et al., 2006; Burness et al., 2010; King and Swanson, 2013), the latter being observed more often during the day (but see Adelman et al., 2010b). The reasons for this variation in the body temperature response to an endotoxin challenge among small

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passerine birds, and between small and large (e.g. between passerine and non-passerine) birds, are not known. Nor is it currently known whether endotoxin-induced hypothermia in birds is adaptive or simply a consequence of improper dosage (Gray et al., 2013). These circumstances make birds interesting study subjects when testing hypotheses on the functionality and trade-offs involved in fever responses.

In this study, we used a small bird model (the zebra finch, *Taeniopygia guttata* Vieillot, ca. 14 g) and an endotoxin challenge (*Escherichia coli* lipopolysaccharide) to better understand why the fever response varies among and within birds. Lipopolysaccharide (LPS) is a pyrogenic component of the cell walls of gram-negative bacteria that triggers the host's immune system to react largely as it would do when infected by live, replicating bacteria (Ashley and Wingfield, 2012). LPS injection is frequently used to induce an acute phase response and stimulate fever in animals (Nomoto, 1996; Harden et al., 2006; Owen-Ashley et al., 2006; Marais et al., 2011c). Specifically, we assessed the extent to which fever (i.e. a regulated rise in deep body temperature; Blatteis, 2003) and other physiological and behavioural responses that might affect energy expenditure and/or thermoregulation (food intake, body mass changes) (i) showed circadian variation, and (ii) were dose dependent during the day. The latter would provide insight into how the body responds to variation in the strength of an endotoxin challenge, which is important to better understand the presence or absence of endotoxin-induced hypothermia. We first challenged birds with different doses of LPS in the morning, and subsequently measured body temperature during the day of the challenge, as well as body mass changes and food intake during the next 2 days. We predicted that low doses of LPS would trigger a (dose-dependent) fever response as has previously been found for larger birds (e.g. Maloney and Gray, 1998), whereas higher doses may result in hypothermia (cf. Owen-Ashley et al., 2006; Burness et al., 2010; King and Swanson, 2013). This would be compatible with the idea that fever is not a viable option during severe infection in small birds because of its high energy costs, or that hypothermia is a sign of sepsis caused by severe infection. We further expected body temperature changes to be mirrored by changes in food intake, body mass gain and overnight body mass loss (the last of these being attenuated for doses that resulted in the use of hypothermia where energy costs of the immune response should be lower). Two months after the first experiment, we administered a single, moderately strong, dose of LPS (that has previously been used to trigger nocturnal fever in passerines; Nord et al., 2013) in the evening and measured the body temperature response and body mass loss at night, in order to study any variation in these responses that could be related to the circadian timing of the challenge. We predicted that the nocturnal fever response and associated body mass loss should be similar to those observed during the day, which would be compatible with the notion that endotoxin-induced hypothermia develops only during severe infection or sepsis. The results of our study offer important new insights into circadian and functional variation in fever and sickness behaviours, with important implications for our general understanding of the costs and benefits of body temperature regulation during infection in homeotherms.

RESULTS

Test statistics for all final models are reported in supplementary material Table S1.

Responses to a LPS challenge during the day

LPS-injected birds decreased their body temperature in a dose-dependent manner starting 20 min after injection (Fig. 1, Table 1), although the mean body temperature during this initial period of the acute phase response was not significantly affected by LPS dose (P=0.11; Fig. 2A). Birds in all treatment categories reached the lowest body temperature after 3.0±0.33 h (Fig. 1), during which time there was a significant negative relationship between mean body temperature and LPS dose (P=0.039; Fig. 2B). After the maximum body temperature response, birds in all four experimental groups (see Materials and methods) steadily increased their body temperature, with most LPS-injected birds having body temperatures that converged on those of the control birds 5 h after injection (Fig. 1). However, birds injected with the second to highest LPS dose (100 µg LPS kg^{-1}) maintained a lower body temperature for an additional 2 h (i.e. until 7 h after injection). There was no relationship between LPS dose and mean body temperature during the last period of the day (12 h after injection, P=0.26; Fig. 2C).

Daytime food consumption and body mass gain decreased with increasing LPS dose during the 2 days after injection

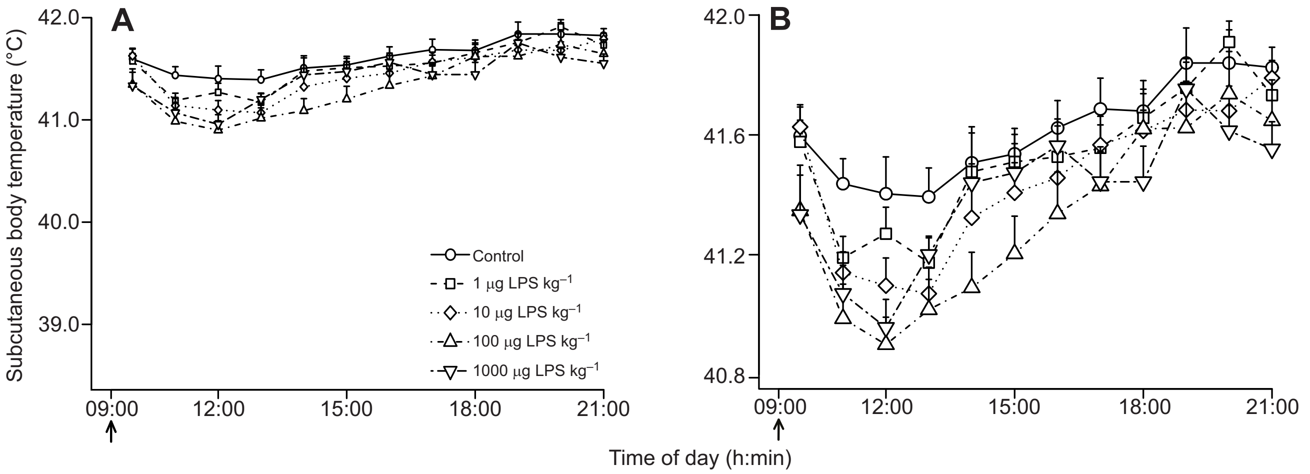

Fig. 1. Body temperature response to LPS challenge as a function of time of day. (A) Mean (+s.e.) body temperature in male zebra finches as a function of time of day subsequent to injection with saline (control) or an immune challenge with different doses of the bacterial endotoxin lipopolysaccharide (LPS; 1, 10, 100 or 1000 µg kg^{-1}). Each data point represents the mean of three consecutive measurements, which were obtained 20 min apart. (B) The same data plotted on a finer scale to more clearly illustrate the dose–response to LPS. The arrow indicates the time of injection. N=10 for all treatment groups.

Table 1 . Maximum body temperature response, seed consumption, body mass gain and overnight mass loss in male zebra finches challenged with LPS in the morning or in the evening

		LPS (µg kg^{-1})			
	Control	1	10	100	1000
Mean body temperature at maximum response during the day (3±0.33 h; °C)	41.4±0.2	41.2±0.1	41.1±0.2	40.9±0.2	41.0±0.1
Mean body temperature at maximum response during the night (3±0.33 h; °C)	39.3±0.1	–	–	40.5±0.1	–
Day 1: seed consumption (g)	2.9±0.1	3.0±0.2	2.4±0.2	2.3±0.2	2.0±0.2
Day 1: body mass gain (g)	0.9±0.0	0.7±0.1	0.5±0.2	0.5±0.1	0.5±0.2
Day 2: seed consumption (g)	2.6±0.1	3.0±0.2	2.7±0.1	2.7±0.1	1.8±0.2
Day 2: body mass gain (g)	0.7±0.0	0.7±0.1	0.9±0.1	0.8±0.1	0.4±0.2
Overnight mass loss after morning injection (night 1; g)	0.7±0.1	0.7±0.1	0.6±0.1	0.7±0.1	0.6±0.1
Overnight mass loss after evening injection (night 1; g)	0.8±0.0	–	–	0.8±0.1	–

Data are means±s.e. Control finches were injected with saline; lipopolysaccharide (LPS)-challenged finches were injected with 1, 10, 100 or 1000 µg kg^{-1} of the bacterial endotoxin LPS.

(Table 1, Fig. 3). On the day of injection (day 1), we found a linear dose-dependent reduction in both seed consumption (P<0.001; Fig. 3A) and body mass gain (P=0.045; Fig. 3C). However, body mass loss during the night did not differ between experimental treatments (P=0.68; Table 1). On the day after injection (day 2), only the birds injected with the highest LPS dose (1000 µg LPS kg^{-1}) showed suppressed body mass gain and food consumption, resulting in a curvilinear relationship between LPS dose and both seed consumption (P=0.0035; Fig. 3B) and body mass gain (P=0.013; Fig. 3D).

Responses to a LPS challenge at night

The direction of the body temperature response to a LPS challenge at night was opposite to that observed during the day (Table 1, Fig. 4). Both LPS-challenged birds and control birds reduced body temperature in a similar way during the first hour after injection. However, from 1 to 3 h after injection, body temperature continued to decrease in control birds, but increased slightly in LPS-challenged birds. This body temperature increase peaked after 3 h, when LPS-challenged birds maintained their body temperature 1.2°C above that of controls (Fig. 4, Table 1). Body temperature in the LPS-challenged birds subsequently decreased, but was nevertheless 0.7°C higher than in control birds throughout the night. There was no difference in body temperature between LPS-injected and control birds by the time of the last measurement (at 07:00 h, when lights were switched on,10 h after injection). Birds injected with LPS in the evening did not lose more body mass overnight than did control birds (P=0.97; Table 1).

Comparison of body temperature during the peak response in the day and at night

LPS-challenged birds (100 µg LPS kg^{-1}) maintained a relatively similar body temperature at the peak response (3 h after injection) regardless of the timing of the challenge (0.4°C lower at night, P=0.058; Table 1, Fig. 5). In contrast, the body temperature in control birds 3 h after injection was 2.1°C lower at night than in the day (P<0.001; Table 1, Fig. 5).

DISCUSSION

By simulating a bacterial infection in male zebra finches we have shown that the body temperature changes during an acute phase response are subject to both pronounced circadian variation (Fig. 5) and dose dependence during the day (Figs 1, 2). Specifically, birds challenged with LPS reduced body temperature relative to control birds during the day when challenged in the morning (hypothermia; Fig. 1), but increased body temperature relative to controls during the night when challenged in the evening (fever; Fig. 4). Furthermore, the body temperature response at night lasted longer and was of a larger magnitude than the response during the day. The duration and magnitude of the LPS-induced hypothermia increased with increasing LPS dose during the day (Fig. 1), and was accompanied by a dose-dependent reduction in both seed consumption and body mass gain (Fig. 3). These results imply that the time frame and strength of physiological and behavioural changes during an acute phase response may depend on both the circadian timing (i.e. day or night) and the magnitude (i.e. LPS dose) of an endotoxin challenge. We do not think this conclusion would have been different had we continued to monitor birds that

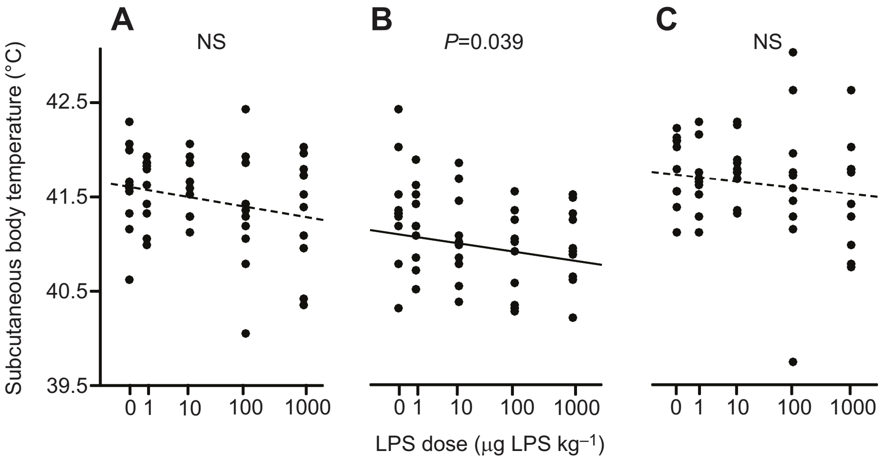

Fig. 2. Body temperature response as a function of strength of the LPS challenge at different stages in the acute phase response. Male zebra finches were injected with either saline (control, 0 µg kg^{-1} LPS) or different doses of LPS (1, 10, 100 or 1000 µg kg^{-1}). Body temperature is shown (A) at the time of the initial body temperature response in LPS-challenged birds (0.7±0.33 h post-injection, P=0.11), (B) during the maximum response to LPS (3.0±0.33 h post-injection, y=41.27−0.13x, P=0.039) and (C) during the late part of the response at the end of the day (12.0±0.33 h post-injection, P=0.26). Note that the x-axis labels are plotted on a logarithmic scale. N=10 for all treatment groups. NS, not significant.

Table 2. The effects of LPS challenge on body temperature in different species of birds when challenged at different circadian phases

Species	Body mass (g)	Circadian phase	LPS dose (μg kg^{-1})	Direction of change	Reference
Pekin duck (*Anas platyrhynchos domestica*)	2900	Day	1–100	+	Maloney and Gray, 1998
	2800	Day	100	+	Marais et al., 2011a,c
Domestic chicken (*Gallus gallus domesticus*)	760	Day	100–5000	+	Leshchinsky and Klasing, 2001
Pigeon (*Columba livia*)	578	Day	10	+	Nomoto, 1996
	578	Night	10	+	Nomoto, 1996
Japanese quail (*Coturnix japonica*)	55	Day	500–2500	+	Koutsos and Klasing, 2001
Song sparrow (*Melospiza melodia morphna*)	32	Night	2100	+	Adelman et al., 2010a,b
	32	Day	2100	+	Adelman et al., 2010b
House sparrow (*Passer domesticus*)	28	Day	1000, 5000	–	King and Swanson, 2013
	28	Night	1000	+	Coon et al., 2011
	28	Day	1000	–	Coon et al., 2011
Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelli*)	26	Day	1000	–	Owen-Ashley et al., 2006
Great tit (*Parus major*)	19	Night	100	+	Nord et al., 2013
Zebra finch (*Taeniopygia guttata*)	14	Day	1000	–	Burness et al., 2010
	14	Day	100	–	This study
	14	Night	100	+	This study

The table gives an overview of studies that first challenged the immune system of birds with the non-pathogenic bacterial endotoxin LPS and subsequently measured the change in the subjects' body temperature during the acute phase response.

Circadian phase denotes whether birds were challenged and measured during the photophase (day) or during the scotophase (night). The direction of change denotes whether body temperature increased (+) or decreased (–) after the LPS challenge.

were challenged during the day through the night and vice versa. We base this statement on data showing that body temperature in the experimental groups converged on that of control finches at the end of the sampling period during both daytime and night-time sessions, which suggests that there were no further differences between groups from this point onwards (Figs 1, 4).

A trade-off between foraging and immune function?

We found a dose-dependent reduction in seed consumption and body mass gain during the day following injection with LPS in the morning (Fig. 3). Birds challenged with the highest LPS dose were still affected on the day after the challenge (i.e. 24–36 h after injection) when this group still consumed fewer seeds and gained less body mass (Fig. 3). Decreased food intake during infection has been suggested to be beneficial, because by decreasing energy intake the host may restrict access to micronutrients necessary for pathogen proliferation and hence limit the infection (Murray and Murray, 1977; Hart, 1988). In addition, both energy requirements and predation risk (if activity related; cf. Martin et al., 2000) for the host should decrease if foraging activity is reduced (Hart, 1988). Thus, the decreased food intake (and concomitant reduction in body mass gain) in our study was probably not an undesired consequence of the LPS challenge, but rather a behavioural adaptation to reduce the negative effects induced by a real pathogen. The dose dependence of this response indicates a potential trade-off between foraging and immune function, such that hosts attempt to

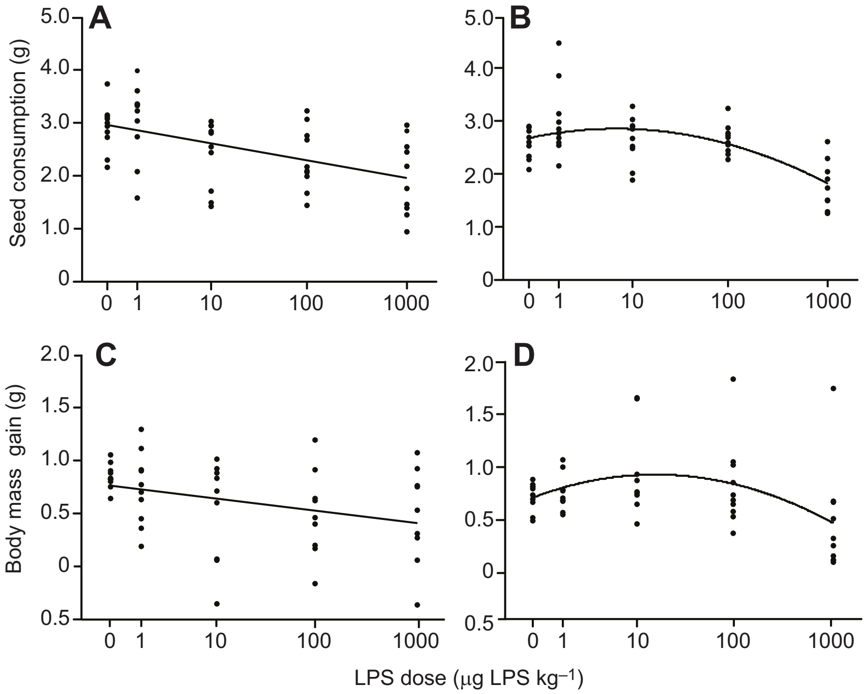

Fig. 3. Seed consumption and body mass gain as a function of LPS dose. Male zebra finches were injected with either saline (control, 0 μg kg^{-1} LPS) or different doses of LPS (1, 10, 100 or 1000 μg kg^{-1}) and the effects were monitored over 2 days. (A) Seed consumption during the day of the experimental injection, when birds demonstrated a linear dose-dependent decrease in consumption (y=2.96+0.33x, P<0.0001). (B) Seed consumption during the day after the LPS challenge, when consumption was curvilinearly related to the strength of the LPS challenge (y=2.69+0.41x+0.23x^2, P=0.0035). (C) Body mass gain during the day of the experimental injection, when birds showed a linear dose-dependent decrease in body mass gain (y=0.75+0.12x, P=0.045). (D) Body mass gain during the day after injection, when body mass gain showed a curvilinear dose dependence (y=0.68+0.37x+0.15x^2, P=0.013). Note that the x-axis labels are plotted on a logarithmic scale. N=10 for all treatment groups.

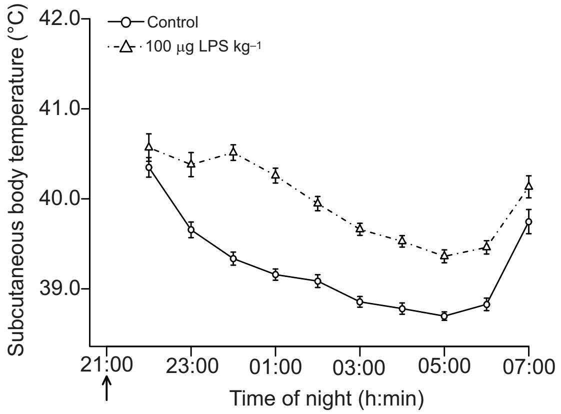

Fig. 4. Body temperature response to LPS challenge as a function of time of night. Mean (±s.e.) body temperature is shown for male zebra finches that were injected with either saline (control, 0 μg kg^{-1}, *N*=12) or 100 μg kg^{-1} of the bacterial endotoxin LPS (*N*=12) at the beginning of the evening. Each data point represents the mean of three consecutive measurements, which were obtained 20 min apart. The arrow indicates the time of injection.

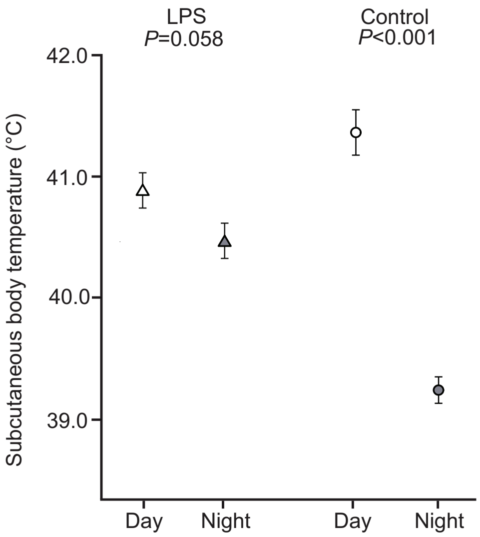

Fig. 5. Maximum body temperature response to LPS challenge during the day and at night. Mean (±s.e.) body temperature is shown for male zebra finches that were injected with either saline (control, 0 μg kg^{-1},*N*=12) or 100 μg kg^{-1} of the bacterial endotoxin LPS (*N*=12), at the time of the maximum body temperature response to the LPS challenge (3.0±0.33 h post-injection). The maximum response was measured both during the day (following morning injection) and during the night (following evening injection).

minimize any negative effects pertaining to reduced food intake by regulating the expression of sickness behaviours to the strength of the infection.

Proximate explanations for the occurrence and lack of fever

Our results do not support the hypothesis that changes in body temperature during an acute phase response occur primarily to create a hostile environment for pathogens (Blatteis, 2003), because LPS-challenged birds did not develop fever during the day, and kept their body temperature within the range of circadian variation in control birds both during the day and at night (Fig. 5). Nor do we think that the primary reason for diurnal hypothermia after LPS administration was to create a hostile environment for pathogens, because the maximum decrease in body temperature in LPS-challenged birds never went below the minimum nocturnal body temperature in control birds. However, hypothermia may have positive effects on other aspects of pathophysiology independent of the thermal environment for the pathogen. For example, Liu et al. (2012) found that rats that developed a ca. 2°C hypothermia after inoculation with severe doses of septic or aseptic endotoxins showed suppressed leakage of endotoxins into the bloodstream and reduced levels of visceral organ dysfunction, both of which probably contributed to lower mortality in hypothermic subjects. It remains to be seen whether the shallow drop in body temperature as observed in this study (≤0.5°C) was great enough to carry any similar anti-pathological benefits.

It is unlikely that hypothermia developed because of energetic constraints on the use of fever (cf. Romanovsky and Székely, 1998), because birds in our study were not constrained by resource availability and should therefore have been able to sustain the potentially increased energy expenditure associated with a fever response as well as the increased heat loss that might accompany febrile body temperatures (cf. Owen-Ashley et al., 2006; Owen-Ashley and Wingfield, 2007; Burness et al., 2010). It is also unlikely that LPS-induced hypothermia in our study was a consequence of septic shock (Romanovsky et al., 1996, 2005), because a comparatively moderate dose of LPS (100 μg kg^{-1}) induced hypothermia during the day but fever at night, and shallow daytime hypothermia also occurred in response to injection with very low doses of LPS (Fig. 1). Taken together, this suggests that regulated hypothermia might be a more frequent response to infection during the day, whereas fever is the more common response at night in small birds. However, our data indicate that neither response occurs primarily to create an environment that is unsuitable for pathogen replication.

It has been proposed that the immune defence system might work best at a given body temperature or within a range of body temperatures (Nord et al., 2013). The occurrence of daytime hypothermia in our study could then be explained if this optimal body temperature, or body temperature range, is lower than the normal daytime body temperature. Our data do not support the hypothesis of a single optimal temperature for immune function, in which case we would have expected all LPS-challenged birds, irrespective of dose, to maintain this body temperature during the acute phase response (cf. discussion in Nord et al., 2013). However, treatment-wise differences in body temperature were relatively small when integrated over the duration of the response. This might be compatible with the idea that optimum immune function can be realized within a range of body temperatures that are lower than the normal resting body temperature. Further support for this notion is provided by the larger experimental effect at night (Fig. 4), which resulted in a similar body temperature in the immune-challenged birds at the time of the maximum response (3 h post-injection) regardless of the circadian timing of the immune challenge (Fig. 5). Alternatively, the dose dependence of the body temperature response might represent a trade-off between optimal immune function and optimal physiological performance, whereby the change in body temperature is determined by production costs of the response on the one hand and the required time frame for pathogen clearance on the other (Maloney and Gray, 1998). Proper assessment of the dose–response to LPS in zebra finches during the night would provide further insights into the possible existence of such a trade-off.

It is possible that daytime hypothermia was not primarily the result of a thermoregulatory response to LPS. For instance, reduced seed consumption (Fig. 3A,B) might have caused a dose-dependent reduction in the metabolic heat production from digestion (‘heat increment of feeding'; Chappell et al., 1997). This could have been further exacerbated by reduced exercise thermogenesis (Paladino and King, 1984; Prinzinger et al., 1991) if activity was suppressed following LPS injection (Burness et al., 2010; Sköld-Chiriac et al., 2014). In line with this, decreased locomotor activity has previously been put forward as a possible explanation for LPS-induced hypothermia in the California mouse (*Peromyscus californicus*) (Martin et al., 2008). However, body temperature does not necessarily track changes in such processes, because metabolic heat production can partly or completely substitute for shivering thermogenesis at temperatures below thermal neutrality (Paladino and King, 1984; Chappell et al., 1997). If so, any reduction in heat supplied by digestion or activity in our study (which was performed some 8°C below thermal neutrality; Calder, 1964) should have been compensated for by increased shivering to maintain a stable body temperature. This remains speculative in the absence of metabolic data. Thus, it is currently unclear whether dose-dependent daytime hypothermia was merely a consequence of a reduction in metabolic heat production from digestion and activity, or whether it was the result of a direct effect of LPS on the birds' thermoregulatory set point.

Based on data in the literature (Kluger, 1991; Marais et al., 2011c), the increase in body temperature at the maximum response following LPS injection in the evening (Fig. 4) was estimated to raise resting metabolic rate by 12–28%. This is somewhat higher than the 10% increase reported by Burness et al. (2010) for zebra finches injected with a 10 times higher LPS dose than used at night in our study. It is not known whether this 10% increase in resting metabolic rate was associated with fever, because Burness et al. (2010) did not record body temperature during measurements of metabolic rate. While we did not assess the dose–response to LPS at night, the magnitude of fever is proportional to LPS dose in fowl (e.g. Jones et al., 1983; Maloney and Gray, 1998), and great tits (*Parus major*) challenged with LPS in winter maintained similar febrile body temperatures regardless of variation in ambient temperature (Nord et al., 2013). This supports the view that fever in birds might represent a trade-off between optimal immune function and the energy costs of the immune response, and that proper immune function can only be realized within a certain range of body temperatures (see discussion on optimal body temperatures for the immune system, above). Future work should seek to determine whether this is true also for nocturnal fever in zebra finches.

Between-species variation in fever responses among birds

We found pronounced circadian variation in the direction of the change in body temperature during an acute phase response in zebra finches (Figs 1, 3). A review of the avian literature suggests that such circadian variation in the body temperature response is more common in small passerine birds (mean body mass: 22±3 g, range: 14–32 g), whereas larger, non-passerine, birds (mean body mass: 1188±539 g, range: 55–2900 g) develop fever regardless of the circadian timing of the immune challenge (Table 2; although it should be noted that these comparisons suffer from relatively few consistent measurements from both circadian phases in the same species). To the best of our knowledge, diurnal fever in response to LPS has only been observed once in the Passeriformes, in the 32 g song sparrow (*Melospiza melodia*) (Adelman et al., 2010b). It is possible that the slightly higher active-phase body temperature in the Passeriformes (41.6°C) compared with other bird orders in Table 2 (41.2°C) (Prinzinger et al., 1991) may preclude any further rise in body temperature associated with fever during the day (Mackowiak and Boulant, 1996; Gray et al., 2013). However, the consistency of the body temperature change for the larger species in Table 2 (Galliformes, Anseriformes, Columbiformes) suggests that body size may be a more important determinant of the response to LPS than phylogenetic relatedness. For example, exercise hyperthermia scales negatively with body size, such that the activity-induced rise in body temperature is larger in small birds independent of phylogeny (Prinzinger et al., 1991). Moreover, small birds have a comparatively high metabolic intensity (Hulbert et al., 2007) and a limited capacity for fasting (Hohtola, 2012), such that some foraging must occur during the day even during an acute phase response (e.g. Fig. 2). Adding fever to flight-induced peaks in body temperature associated with foraging during the day may increase the risk of overheating, which comes at a high somatic cost (Speakman and Król, 2010). By comparison, larger birds are tolerant of prolonged fasting periods (Sartori et al., 1995; Criscuolo et al., 2000) and might be able to minimize any work-related increase in diurnal body temperature by avoiding excessive activity during the acute phase response. In many (small) bird species, body temperature during the nocturnal roosting period is typically less variable and regulated to a lower set point than during the day (McKechnie and Lovegrove, 2002). Hence, at night, even small birds may be able to use fever to clear an infection without a concomitant increase in the risk of overheating. Further studies on circadian variation in the body temperature response to endotoxin in large passerines and small non-passerines would shed light on the relative importance of phylogeny and body size, respectively, in explaining interspecific variation in fever expression.

Conclusions

We found distinct circadian variation in the body temperature response to LPS injection manifested by the (dose-dependent) use of hypothermia during the day and fever during the night. Thus, the occurrence of diurnal hypothermia in response to an endotoxin challenge does not seem to be a sign of sepsis as has previously been suggested for small mammals (above), but may instead be a normal part of the birds' response to infection. We suggest that this might promote survival by optimizing the body's response according to individual demands at the time of infection: the use of hypothermia might be beneficial in terms of minimizing the risk of overheating, or in avoiding excessive metabolic costs, during the day when body temperature is high and variable, whereas the use of fever might be more beneficial to counteract infection at night when body temperature is lower and less variable. To fully appreciate this (for us, unexpected) circadian rhythmicity in the body temperature response to infection, we need to better understand the proximate mechanisms of fever (Gray et al., 2013) and the extent to which these might vary between distant and related species across a range of body sizes. Based on the results from this study in conjunction with those from others (see above), we propose that changes in body temperature regulation in small birds during infection occur as either: (i) an active response to either maintain body temperature within an optimal range or exceed a body temperature threshold that is required for optimal immune function, or (ii) a passive change in body temperature resulting from a combination of increased metabolic heat production during immune system activation (manifested primarily during the night) and decreased metabolic heat production because of physiological and/or behavioural adjustments during the immune response (manifested primarily during the day). Alternatively, it is possible that both these mechanisms together shape the body temperature response seen in our study of zebra finches, because a passive change in body temperature might explain the daytime response to LPS injection in the morning, and a body temperature threshold for proper immune function might explain the night-time response to LPS injection in the evening.

MATERIALS AND METHODS

Four weeks before the experiment started, we implanted a temperature-sensitive PIT tag (11.5×2.1 mm, 0.06 g; LifeChip BioThermo, Destron Fearing, South St Paul, MN, USA) subcutaneously in the neck of 50 adult male zebra finches. This route of implantation is minimally invasive, and temperatures measured in the neck can be used to accurately predict variation in deep body temperature (Nord et al., 2013). Subsequent to implantation, we measured body mass (to the nearest 0.1 g) and randomly divided birds into four groups (N=12–13 per group), which contained 2–3 individuals from each LPS treatment (see below). One day before the start of the experiment, we transferred all birds from their regular communal cages to individual experimental cages (32×48×32 cm), with *ad libitum* access to commercial seed mixture and water. Cages were placed so that birds were able to hear and see each other, but were visually separated from the investigators. Birds were kept under constant artificial light (14 h:10 h light: dark, lights on between 07:00 h and 21:00 h) and ambient temperature conditions (22±2°C) throughout the experiment. There was no difference in body mass ($F_{4,44}$=0.95, P=0.44) or subcutaneous body temperature ($F_{4,45}$=0.14, P=0.97) between treatments in any of the four groups of birds at the start of the experiment.

We assessed the relationship between subcutaneous temperature (as measured by the PIT tags) and deep body temperature (as measured by a factory-calibrated thermocouple thermometer) on the morning of experimental manipulation ($T_{\mathrm{b,deep}}$=0.94$T_{\mathrm{b,subcut}}$+3.23, R^2=0.63, ΔT_{b}=0.49+0.04°C; see supplementary material Fig. S1 for details). We then measured the birds' body mass and injected them in the pectoral muscle with 50 µl phosphate-buffered saline (PBS, control) or a dose of 1, 10, 100 or 1000 µg LPS kg^{-1} (based on the mean body mass at the time of PIT tag implantation) derived from *E. coli* (Sigma, cat. no. L2880) diluted in 50 µl PBS. Sample size for each treatment group was N=10. Implant calibration and injection were commenced between 08:30 h and 08:45 h and were completed within 20 min. Birds were then immediately transferred back to their experimental cages, and were provided with water *ad libitum* and 25.0 g of seeds (equivalent to about 10 times the amount the birds consumed during a day). Starting 20 min after the mean time of injection, we measured subcutaneous body temperature every 20 min for 12.5 h using a handheld racket antenna (diameter 17.5 cm; Destron Fearing) connected to an FS2001F ISO reader (Destron Fearing) through the cage floor. Both the antenna and the observer were outside the birds' field of view at all times. When lights were switched off in the evening (i.e. at 21:00 h, 12.5 h after injection), we measured the birds' body mass and their seed consumption (by weighing the remaining seeds in the food cup together with any food spill in the cages), after which birds were left undisturbed during the night. Food was not provided again until birds had been weighed the following morning (see below). At 07:30 h the next morning (i.e. 30 min after the lights were switched on, and 22.5 h after injection), we weighed all birds and transferred them to larger individual cages (60×33×57 cm), with *ad libitum* access to water and 25.0 g of seeds. As soon as lights were switched off in the evening of the second day (i.e. 36 h after injection), birds and remaining seeds (including food spill) were weighed, after which the birds were transferred back to their regular, communal cages.

Two months after the daytime trials, we measured the body temperature response to LPS injection in the evening using a subsample (N=24) of the birds from the first part of the experiment. Previous exposure to LPS does not affect the body temperature response during a second LPS injection when injections are more than 2 weeks apart (at least in Pekin ducks; Marais et al., 2011b). This was true also in our study (previous LPS dose: P=0.61). One day prior to the evening injection, birds were placed individually in the experimental cages described above, with *ad libitum* access to commercial seed mixture and water. On the evening of experimental manipulation, birds were randomly assigned to experimental treatments, weighed and subsequently injected in the pectoral muscle with either 50 µl PBS (control treatment, N=12) or 100 µg LPS kg^{-1} diluted in 50 µl PBS (N=12). We chose to use a single LPS dose only because this dose gave the highest response in the daytime tests. Injections started at 20:45 h and were completed within 20 min. Starting 20 min after the mean time of injection, we then measured subcutaneous body temperature every 20 min for 10 h (i.e. until the lights were switched on in the morning). All body temperature measurements were performed in the dark without handling the birds (as detailed above). We weighed birds again after the last temperature measurement in the morning and then transferred them back to their regular cages. It is important to note that birds did not eat during the night (when cage rooms were completely dark), so body mass loss should reflect the energy consumption during the night.

Data analysis

All statistical tests were performed using SAS for Windows. We analysed the daytime body temperature response to different LPS doses using a linear mixed model (PROC MIXED) with a first order autoregressive covariance structure (AR1), with body temperature as the dependent variable, LPS dose (log-transformed in all analyses) and day of injection (i.e. 'group') as factors, time and (time)^2 (to account for potential non-linearity in the body temperature response) as covariates, and a random intercept for bird identity as random factor. The original model also contained the two-way interactions LPS dose×time and LPS dose×(time)^2. We then performed separate regressions of body temperature as a function of LPS dose and (LPS dose)^2: (i) at the beginning of the day ('initial'=the mean of the first three body temperature measurements of each individual immediately after the injection; time: 0.67±0.33 h post-injection), (ii) at the maximum response ('maximum'=the mean of the three body temperature measurements of each individual at the maximum response; time: 3.00±0.33 h post-injection) and (iii) at the end of the day ('late'=the mean of the last three body temperature measurements of each individual during the day; time: 12.00±0.33 h post-injection). Daytime food consumption following morning injection was tested separately for each day, using seed consumption as the dependent variable, group as a factor and initial body mass, LPS dose and (LPS dose)^2 as explanatory variables. Body mass gain during the 2 days after injection and body mass loss during the night following morning injection were tested in a similar way, viz. each day and night was tested separately using body mass gain during the day – or body mass loss during the night (when the birds were not feeding) – as the dependent variable, group as factor, and body mass at the beginning of the trial, LPS dose and (LPS dose)^2 as explanatory variables. We analysed the body temperature response to the LPS challenge at night in a linear mixed model (PROC MIXED with AR1 covariance structure) with body temperature as the dependent variable, treatment and the previous LPS dose (from the daytime trial) as factors, time and (time)^2 as covariates, and bird identity as a random intercept. The original model also contained the two-way interactions treatment×time and treatment×(time)^2. Differences in body mass loss during the night following evening injection were compared in a linear model with treatment as a factor and body mass at the beginning of the night as a covariate. Finally, to assess whether the body temperature attained during the peak response (defined above) in LPS-challenged birds was different during the day and at night, we compared within-treatment differences in body temperature using independent t-tests (control birds and birds injected with 100 µg LPS kg^{-1} only). In multivariate tests, final models were derived using stepwise backward elimination of non-significant variables (P>0.05) until only significant variables remained. All values are presented as means±s.e. and all significances are two-tailed.

Ethical note

The experimental design follows Swedish legislation and was approved by the Malmö/Lund Animal Ethics Committee before the start of the experiment.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.S.-C. and A.N. conceived and designed the study with input from D.H. and J.-Å.N. The practical work was performed by S.S.-C., A.N. and M.T., and S.S.-C. and A.N. analysed the data. All authors participated in writing and preparation of the final manuscript.

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Supplementary material

Supplementary material available online at
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