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ORIGINAL PAPER

Body temperatures of hibernating little brown bats reveal pronounced behavioural activity during deep torpor and suggest a fever response during white-nose syndrome

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Abstract Hibernating animals use torpor [reduced body temperature (T_b) and metabolic rate] to reduce energy expenditure during winter. Periodic arousals to normal T_b are energetically expensive, so hibernators trade off arousal benefits against energetic costs. This is especially important for bats with white-nose syndrome (WNS), a fungal disease causing increased arousal frequency. Little brown bats (*Myotis lucifugus*) with WNS show upregulation of endogenous pyrogens and sickness behaviour. Therefore, we hypothesized that WNS should cause a fever response characterized by elevated T_b. Hibernators could also accrue some benefits of arousals with minimal T_b increase, thus avoiding full arousal costs. We compared skin temperature (T_{sk}) of captive *Myotis lucifugus* inoculated with the WNS-causing fungus to T_{sk} of sham-inoculated controls. Infected bats re-warmed to higher T_{sk} during arousals which is consistent with a fever response. Torpid T_{sk} did not differ. During what we term "cold arousals", bats exhibited movement following T_{sk} increases of only 2.2 ± 0.3 °C, compared to >20 °C increases during normal arousals. Cold arousals occurred in both infected and control bats, suggesting they are not a pathophysiological consequence of WNS. Fever responses are energetically costly and could exacerbate energy limitation and premature fat depletion for bats with WNS. Cold arousals could represent an energy-saving mechanism for both healthy and WNS-affected bats when complete arousals are unnecessary or too costly. A few cold arousals were observed mid-hibernation, typically in response to disturbances. Cold arousals may, therefore, represent a voluntary restriction of arousal temperature instead of loss of thermoregulatory control.

Keywords Arousals · *Myotis lucifugus* · Hibernation energetics · WNS · Heterothermy

Abbreviations

T_b	Body temperature
T_{sk}	Skin temperature
T_a	Ambient temperature
WNS	White-nose syndrome
IR	Infrared

Introduction

Many mammals use hibernation during prolonged periods of cold ambient temperature (T_a) and/or food scarcity to dramatically reduce energy expenditure. The hibernation period varies from several weeks to many months, depending on geographic location and winter duration (Geiser 2004; Humphries et al. 2002). Fat or food stores accumulated prior to hibernation must be carefully budgeted, especially in regions with long winters and the energy budget challenges of hibernation are well documented (Thomas

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et al. 1990; Humphries et al. 2002, 2003; Geiser 2004; Boyles et al. 2007; Jonasson and Willis 2012; Czenze et al. 2017). Hibernators undergo multi-day torpor bouts, during which body temperature (T_b) approaches T_a. Torpor bouts are periodically interrupted by comparatively brief increases to typical normothermic metabolic rate and T_b. The function of periodic arousals may include excretion of metabolic wastes, restoration of water balance, changing roost position, re-establishing immune function, and repayment of sleep debt (Park et al. 2000; Daan et al. 1991; Prendergast et al. 2002; Thomas and Geiser 1997). Regardless of the function, re-warming to normothermic T_b, defending normothermic T_b for up to several hours, and then returning to torpor are extremely energetically costly relative to steady-state torpor. While representing only <1–7% of the time budget during winter (Thomas et al. 1990; Jonasson and Willis 2012), arousals can account for more than 90% of energetic costs of hibernation (Thomas et al. 1990).

Many species of bats hibernate during winter when cold T_a of temperate latitudes and lack of insect prey preclude continued activity. Although regions with milder winters may allow for occasional winter foraging (Hope and Jones 2012), in harsher regions bats must rely entirely on stored fat to survive the winter. In extreme cases hibernation can last up to 9 months, as observed for some little brown bats (*Myotis lucifugus*) in central Manitoba, Canada (Norquay and Willis 2014). Prior to hibernation, bats deposit large fat stores required to survive hibernation (Kunz et al. 1998; McGuire et al. 2009, 2016a), although the amount of fat stored varies by species, location, and typical winter severity. For example, *M. lucifugus* in Vermont USA gain an average of 2.2 g (31% body mass increase) prior to hibernation (Kunz et al. 1998), while, further north in central Manitoba, *M. lucifugus* gain approximately 4.5 g (59% body mass increase) (Matheson et al. 2010; Jonasson and Willis 2011). Despite the capacity to deposit large amounts of fat, bats must carefully budget energy stores if they are to survive until spring when conditions once again permit foraging (Czenze et al. 2017; Willis 2017).

The energetics of hibernation in bats have become particularly important in the context of white-nose syndrome (WNS), a recently emerged fungal disease (Warnecke et al. 2012, 2013; Verant et al. 2014). WNS is caused by the fungus *Pseudogymnoascus destructans* (Lorch et al. 2011; Warnecke et al. 2012) and has devastated bat populations throughout eastern North America (Langwig et al. 2012; Frick et al. 2015). Along with cutaneous fungal infection, skin lesions, and premature cessation of hibernation, WNS also causes an increase in arousal frequency, especially in the late stages of hibernation (Warnecke et al. 2012; Reeder et al. 2012; Grieneisen et al. 2015). Bats with WNS, therefore, deplete their energy stores much more quickly than healthy animals.

The cause of increased arousal rate in bats with WNS is not yet understood but two non-exclusive hypotheses, water loss and immune response, have some support. Infected little brown bats (*Myotis lucifugus*) exhibit hypotonic dehydration presumably due to fluid loss across wing tissue damaged by fungal invasion (Cryan et al. 2013; Warnecke et al. 2013). Water loss has been implicated in arousal patterns of healthy hibernators, presumably triggering arousals for restoration of water balance (Thomas et al. 1990; Thomas and Geiser 1997). Therefore, increased cutaneous fluid loss during infection could explain why bats with WNS arouse more often than normal (Willis et al. 2011). However, in addition to showing higher rates of water loss, little brown bats with WNS upregulate a range of pro-inflammatory cytokines associated with inflammation and regulation of immune responses to infection (Rapin et al. 2014; Field et al. 2015; Lilley et al. 2017; Davy et al. 2017). Some of these cytokines trigger so-called 'sickness behaviour' in vertebrates (i.e. reduced behavioural activity and self-isolation) (Hart 1988, Dantzer 1998 a, b) and are often referred to as pyrogens because of their role in the fever response (Evans et al. 2015). Interestingly, bats with WNS show the classic symptoms of sickness behaviour during arousals (Langwig et al. 2012; Wilcox et al. 2014; Bohn et al. 2016) but, to date, a fever response to infection with *P. destructans* has not been demonstrated despite the potential of energetically expensive fever to disrupt hibernation energetics.

Although traditionally it has been assumed that torpor is associated with immobility and reduced responsiveness to stimuli (International Union of Physiological Sciences Thermal Commission 2003), many mammalian species can be active with relatively low T_b (e.g. Willis and Brigham 2003; Warnecke et al. 2008), and it is possible that hibernating bats could be active behaviourally without increasing T_b to normothermic levels, albeit with reduced performance (Rojas et al. 2012). Recent evidence from free-living *Myotis myotis* indicates that hibernating bats may exhibit limited activity without increasing T_b to normothermic levels (Bartonička et al. 2017). If torpid bats can respond to disturbance, assess their physiological and environmental conditions, and accomplish any of the other necessary functions of arousal without fully re-warming to normothermic T_b, they could save substantial amounts of energy and increase the likelihood of over-winter survival.

We conducted a controlled study of captive hibernating little brown bats with continuous monitoring of skin temperature (T_{sk}) using data loggers and activity using near-infrared (IR) video. We first tested two predictions of what we term the sickness behaviour/fever hypothesis (Bohn et al. 2016), namely that bats with WNS exhibit higher skin temperature during (1) torpor and (2) arousal compared to un-infected controls. We also evaluated four predictions

of the hypothesis that hibernating bats can reduce winter energy expenditure by not fully re-warming to normothermic T_b during some of their over-winter arousals: (1) both infected and control bats would be capable of using cold arousals; (2) cold arousals would be more common in infected bats because infected bats are known to deplete energy stores at a faster rate than uninfected bats; (3) cold arousals would be more common late in hibernation when energy stores are depleted; and (4) cold arousals do not reflect a loss of thermoregulatory control (i.e. an inability to re-warm because of depleted fat reserves), but rather, bats may undergo a cold arousal and subsequently return to using normal arousals.

Materials and methods

Data for this study were collected between November 2013 and April 2014 during two experiments on WNS and hibernation energetics (e.g. Cheng et al. 2016; McGuire et al. in prep). All methods were approved by the University of Winnipeg Animal Care Committee and conducted under Manitoba Conservation Wildlife Scientific Permit WB15396. Adult male little brown bats, negative for *P. destructans* (confirmed by quantitative PCR (qPCR); Muller et al. 2013; McGuire et al. 2016b), were collected from two WNS-negative caves in central Manitoba, Canada. At the time of the study, these caves were at least 1000 km from the nearest confirmed WNS-positive site in Northern Ontario. Only adult males were used in this experiment to eliminate the possibility of age- and/or sex-related differences in arousal patterns (Jonasson and Willis 2012; Czenze et al. 2017) and to avoid impacts of removing females from the population. Bats were placed in cloth bags and transported to the University of Winnipeg in a temperature-controlled cooler. The first collection ($n=54$) was made from a site north of Grand Rapids, Manitoba, Canada (53.4°N 99.5°W) on 7 November 2013. We inoculated with *P. destructans* following the methods of Warnecke et al. (2012) and randomly assigned bats to three cages for hibernation (see below). A second group of adult male little brown bats ($n=17$, also confirmed negative for *P. destructans* using qPCR) was collected on 29 November 2013 from a cave approximately 75 km east of The Pas, Manitoba, Canada (53.8°N 101.2°W) and approximately 121 km northwest of the first collection site. These bats were sham-inoculated as controls (i.e. no exposure to the fungus) following the same procedures as for the inoculated bats. At the end of the experiments, we confirmed the effectiveness of our inoculation using qPCR (Muller et al. 2013) and histological examination (Cheng et al. 2016), and similarly confirmed that our controls were negative for *P. destructans*. The observations we report here were not part of a planned study, but rather collected incidentally during other ongoing projects (Cheng et al. 2016; McGuire et al. in prep). Therefore, our inoculated and control bats were not collected at the same time from the same site. However, all handling and experimental methods were identical among treatments. Little brown bats in our study region travel long-distances among hibernacula (Norquay et al. 2013) and population genetic structure is relatively weak between the sites we studied (Davy et al. 2015). Therefore, we have no reason to suspect that capture site would have influenced any of the effects we observed.

We used iButton dataloggers (modified as described by Reeder et al. 2012 to reduce mass) to record T_{sk} (±0.5 °C) from each bat. We calibrated iButtons prior to deployment using a temperature cabinet (Pelt cabinet and Pelt5 temperature controller, Sable Systems International, Las Vegas USA). Cabinet temperature was verified using a NIST-traceable thermocouple thermometer (TC-2000, Sable Systems International, Las Vegas USA). We held iButtons in the cabinet for >7 h at each of five sequential temperatures (5, 10, 20, 30 and 40 °C) and used the values recorded by individual iButtons and the reference temperatures from the thermocouple to determine calibration equations for each iButton individually. Each modified iButton was marked with a unique pattern to enable visual identification of individual bats in near-IR video recordings obtained throughout hibernation (see below). We trimmed a small area of fur in the interscapular region and attached the modified iButton with ostomy cement (OSTOBOND, Montreal Ostomy, Montreal, Quebec, Canada). Intact iButton dataloggers can emit ultrasonic noise which could disturb bats (Willis et al. 2009) but the modifications described by Reeder et al. (2012), especially the plastic coating, eliminate this noise.

All bats (control and inoculated) were housed in 23×38×38 cm nylon mesh cages (Fig. 1; modified from Exo Terra, Rolf C. Hagen Inc., Montreal, Quebec, Canada; 1 cage of 17 control bats and 3 cages of 18 inoculated bats) built into temperature- and humidity-controlled incubators set to 7 °C and 98% relative humidity (Caron Products 6040-1, Marietta, OH, USA). The ceiling of each cage was removed and a camera (see below) was positioned 25 cm above the ceiling to ensure an unobstructed field of view for all bats in a given cage. Walls of each cage between ceiling and camera (i.e. above the nylon mesh) were made from plastic vapour barrier to prevent bats from climbing up to the camera (Fig. 1, Supplementary Videos). We recorded temperature and relative humidity of the chambers at 10-min intervals throughout the experiment (HOBO Microstation Datalogger, H21-002, USA). Temperature and humidity recordings were downloaded and checked daily to ensure microclimate stability in the chambers. A dish at the bottom of each cage provided ad libitum fresh drinking water. We flushed the drinking water every other

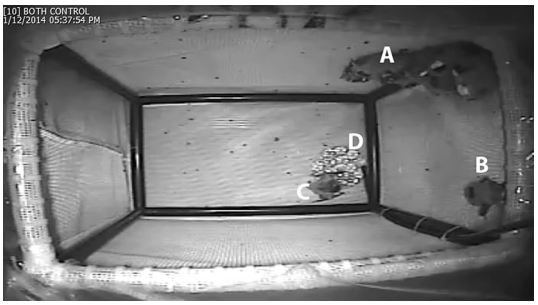

Fig. 1 Screen capture from IR video of the control group hibernation chamber. IR video was motion triggered to record all movements. The camera is mounted above, looking down into the cage. A cluster of hibernating bats (*A*) is visible in an upper corner of the cage as well as a solitary roosting bat in the opposite corner (*B*). Note the modified iButtons attached to the bats to record skin temperature. An active bat is visible on the floor of the cage (*C*) getting a drink of water from the water dish filled with glass beads (*D*). The hose that is used to flush the water dish is attached to the corner post of the cage, running outside of the environmental chamber so that fresh water can be provided without opening the chamber and disturbing the bats

day using supply tubes that ran outside the environmental chamber to avoid opening the door and disturbing the hibernating bats. Motion-activated near-IR cameras (Model HT6501RVFHQ; Speco Technologies, New York, NY, USA) were affixed above each cage in the incubators as described above. Activity by bats during their arousals triggered the camera and we watched videos every day to identify which animals were active and for how long. The iButtons logged an instantaneous measurement of T_{sk} every 15 min throughout hibernation.

After placing bats in the environmental chambers at the beginning of the experiment, they were left to hibernate undisturbed and we monitored their condition based on video recordings. Any bats exhibiting signs of morbidity (roosting alone, near the bottom of the cage and/or with wings outspread) were removed for assessment with as little disturbance to remaining bats as possible. Bats were considered moribund and were humanely euthanized under isoflurane anaesthesia if they were unresponsive during health assessments, which were performed based on a combination of symptoms including arousal on consecutive days, unsteady movements during arousals, roosting alone at the bottom of the cage, or roosting with wings spread ($n=9$ infected, 6 control bats; Cheng et al. 2016; Warnecke et al. 2012). The experiment involving the inoculated bats was terminated after 112 days of hibernation, while a different experimental design allowed the control bats to continue longer (see Cheng et al. 2016). We considered all available data for tests of the sickness behaviour/fever hypothesis, identifying cold arousals, and comparing characteristics of cold arousals and normal arousals. To avoid over-sampling cold arousals from the control group, we standardized the duration of our sampling period to compare the proportion of infected versus control bats exhibiting cold arousals. This meant that we excluded cold arousals observed in control bats after the end date of the inoculation experiment.

Cold arousals were identified using the combination of T_{sk} and video data. To account for small but potentially important differences in torpid and normothermic T_{sk} among bats due to differences in iButton application (e.g. more or less glue, variation in contact with the body surface) we used the change in T_{sk} (ΔT_{sk}) to quantify the magnitude of T_b change during arousals and to characterize types of arousals. We refer to arousals during which activity was noted in videos, but T_b was not elevated to normothermic levels, as "cold arousals". Arousals during which T_b was elevated to near-normothermic T_b are referred to as "normal arousals". Given ambient cooling of external transmitters in this relatively cold environment, $T_{sk}>5$ °C likely reflects a normal pattern of arousal (Jonasson and Willis 2012).

We arbitrarily selected $\Delta T_{sk}>15$ °C as a threshold to differentiate normal and cold arousals. We maintained hibernation chambers at $T_a=7$ °C; therefore, a T_{sk} increase of 15 °C ($\Delta T_{sk}=15$ °C) corresponds with T_{sk} greater than approximately 22 °C. The exact threshold temperature did not influence our findings because there was an obvious distinction between normal and cold arousals and a wide range of ΔT_{sk} threshold values (7–17 °C) led to the same interpretation of our data (see results). When reviewing video files, we were blind to the corresponding T_{sk} data for each individual. Thus, iButtons and video provided two independent methods for observing arousals. For each cold arousal we recorded ΔT_{sk} and the number of days before the end of hibernation for the individual bat in question (i.e. when the experiment was terminated or the bat met intervention criteria for morbidity and was humanely euthanized, see Cheng et al. 2016 and above). We used days before end of hibernation to understand the timing of cold versus normal arousals throughout hibernation because this variable should provide a better indicator of remaining fat stores for individual bats than rate of fat depletion which could vary among individuals.

All statistical analyses were conducted using R (v3.3.2; R Core Team 2016). To test the sickness behaviour/fever hypothesis we identified all normal arousals from each bat (7.7 ± 0.2 arousals per bat, range 3–11) and recorded the date, torpid T_{sk} prior to the arousal, maximum T_{sk} during the arousal, and ΔT_{sk}. We compared inoculated and control bats using linear mixed effect models, including date of arousal as a covariate, and individual as a random effect to account for repeated measurements of individuals. We tested for cage effects by initially comparing the three inoculated groups. There was no difference among the

inoculated groups (see results), so they were combined and we considered them as one treatment for comparison with controls.

We compared the proportion of individuals using cold arousals between inoculated and control groups using function prop.test in R. We used two-sample *t* tests to compare ΔT_{sk} and the timing of cold arousals between inoculated and control bats. In cases where the assumption of normality was violated, we used Mann–Whitney tests. To test whether cold arousals represented a failure of thermoregulation due to fat depletion at the end of hibernation, we also determined if cold arousals were subsequently followed by normal arousals for each individual.

Results

Of the 17 control and 54 inoculated bats, iButtons successfully recorded data for 13 controls and 41 inoculated individuals. We excluded from our analysis one inoculated bat that did not properly enter hibernation after capture, hibernating <50 days.

We did not observe any differences among the three groups of inoculated bats (all $p > 0.1$) so we considered only the overall effect of inoculation in our analyses. Contrary to our first prediction of the sickness behaviour/fever hypothesis, we did not observe higher torpid T_{sk} in inoculated bats compared to controls. Torpid T_{sk} was 0.7 ± 0.1 °C lower in inoculated bats than controls (likelihood ratio = 23.8, $df = 1$, $p < 0.0001$); however, this difference is within the range of measurement error of the iButtons (±0.5 °C per iButton) and, therefore, we conclude that torpid T_{sk} was similar for control and inoculated bats, and certainly not higher in inoculated bats as predicted. However, consistent with one prediction of the sickness behaviour/fever hypothesis, ΔT_{sk} of inoculated bats was 2.3 ± 0.6 °C greater during normal arousals (Fig. 2; 23.1 ± 0.3 °C versus 20.7 ± 0.6 °C; likelihood ratio = 11.5, $df = 1$, $p < 0.0001$). Maximum T_{sk} during arousals was also greater in inoculated bats than controls (1.6 ± 0.7 °C greater in inoculated bats; likelihood ratio = 5.8, $df = 1$, $p = 0.02$).

Activity that triggered video recordings usually corresponded with a substantial increase in T_{sk} (i.e. above our 15 °C threshold, $n = 407$ normal arousals). However, we identified 34 cold arousals during which an arousal was indicated by an active bat in the video recordings but without a corresponding increase to normothermic T_b (Figs. 3, 4). We did not observe any instances when an arousal was indicated based on T_{sk} without corresponding activity recorded in the video. During cold arousals bat movements were sluggish and shaky (Supplementary Video 1). Bats moved slowly and deliberately during cold arousals in a way that was distinct from typical movement observed during normal arousals and the movements of normothermic bats during the active season (Supplementary Video 2).

We observed 17 cold arousals from 8 individual control bats and 17 cold arousals from 10 inoculated bats (Table 1). Groups of inoculated bats were euthanized after 112 days of hibernation (see methods). Therefore, to ensure an equivalent sampling period, we did not include cold arousals that were observed for control bats after 112 hibernation days. Considering only this equivalent time period between the two groups, there was no difference in the proportion of bats exhibiting cold arousals ($\chi^2 = 0.34$, $df = 1$, $p = 0.56$). There was also no difference in ΔT_{sk} (control: 1.9 ± 0.4 °C, inoculated: 2.5 ± 0.4 °C; $t_{31.9} = 0.95$,

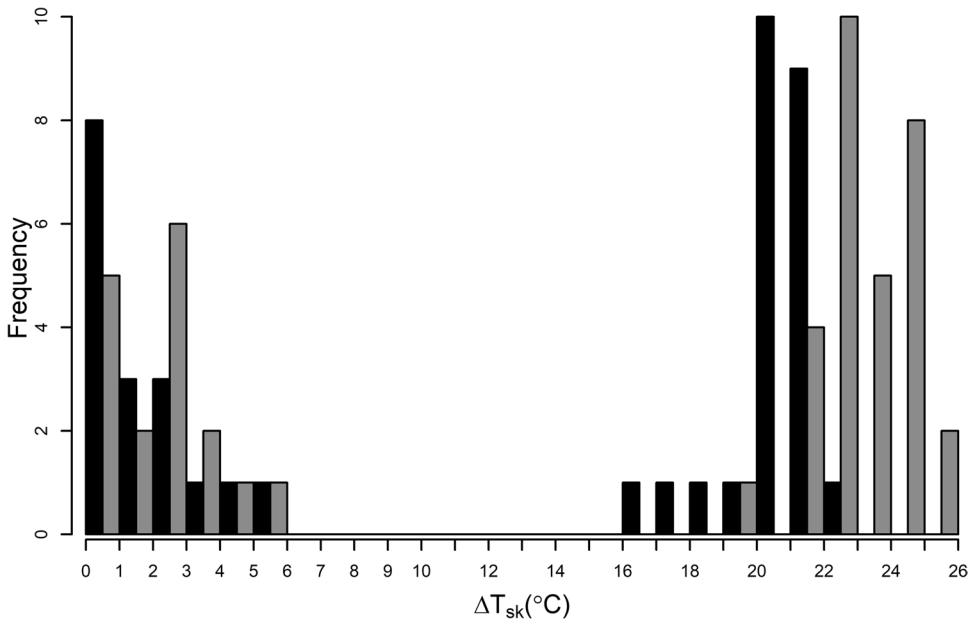

Fig. 2 Distribution of skin temperature increase (ΔT_{sk}) during arousals of inoculated (*black bars*) and control bats (*grey bars*). All cold arousals are represented, as well as three randomly selected representative normal arousals from those bats that exhibited cold arousals. There was no difference in ΔT_{sk} in cold arousals between inoculated and controls, but in normal arousals ΔT_{sk} was greater for inoculated bats than controls

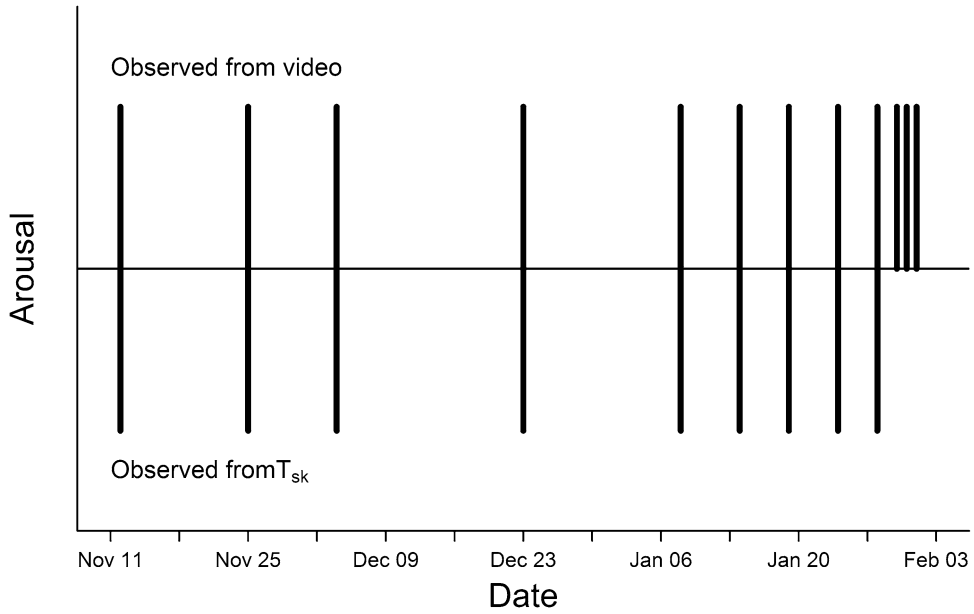

Fig. 3 An example of an arousal profile of an individual bat. The *x*-axis spans the duration of hibernation for this individual. Observed arousals are indicated by *vertical lines* (arbitrary length for visual representation only) on the corresponding date. Arousals observed on video are indicated *above the axis*, and arousals identified from T_{sk} data are indicated *below the axis*. In most cases arousals observed on video correspond with increased T_{sk}, note the final three arousals where this bat was active, as determined from video, but the bat did not arouse to normothermic T_{sk}. This example is from an inoculated bat, hence the arousal frequency increased late in hibernation as commonly observed in bats suffering from WNS

$p=0.35$) or maximum T_{sk} (control: 8.4 ± 0.5 °C, inoculated: 8.4 ± 0.5 °C; $t_{16}=0.05$, $p=0.95$) during cold arousals (Fig. 2). As for behavioural observations described above, cold arousals were clearly distinct from normal arousals in terms of ΔT_{sk}. There was no overlap in ΔT_{sk} of cold arousals ($\Delta T_{sk}=2.2\pm0.3$ °C) and normal arousals ($\Delta T_{sk}=22\pm0.1$ °C) (Fig. 2).

We examined the timing of cold arousals to determine whether this strategy was only employed late in hibernation as an emergency measure when fat reserves were depleted. The timing of cold arousals was highly skewed (Fig. 5), with most cold arousals happening within 2 weeks of the end of hibernation (control median day of occurrence = 4 days before end of hibernation, inoculated median day of occurrence = 5 days). Despite the prevalence of cold arousals in later hibernation, however, cold arousals happened as early as 40 days before the end of hibernation. We observed five cold arousals ≥20 days before the end of hibernation, typically in response to disturbance. The interval between a cold arousal and the previous normal arousal (3.1 ± 0.4 days) was much shorter than the interval between successive normal arousals (9.8 ± 0.4 days; $t_{84.3}=11.7$, $p<0.0001$). There was no difference in the timing of cold arousals between inoculated (range 0–40 days before the end of hibernation) and control bats (0–26 days before the end of hibernation) (Mann–Whitney $W=132$, $p=0.68$). Furthermore, while many cold arousals occurred late in hibernation, we observed seven bats that underwent a cold arousal and subsequently expressed a normal arousal.

Discussion

We found support for the sickness behaviour/fever hypothesis. Infected bats increased T_{sk} during normal arousals by 2.3 °C more than controls, which is consistent with a cytokine-mediated fever response and sickness behaviour. Little brown bats with WNS have been shown to upregulate genes associated with the innate immune system including a range of pro-inflammatory cytokines such as interleukins (e.g. IL-1β, IL-6) and tumour necrosis factor (TNFα) (Rapin et al. 2014; Field et al. 2015; Lilley et al. 2017). These cytokines are associated with inflammation and regulation of innate and acquired immune responses to infection. They are also known to trigger sickness behaviour (Hart 1988; Dantzer 1998a, b) and febrile or fever response, leading to elevated T_b in vertebrates (Evans et al. 2015). Little brown bats with WNS show classic symptoms of sickness behaviour during their arousals, including reduced clustering and increased solitary roosting, and reduced behavioural activity (Langwig et al. 2012; Wilcox et al. 2014; Bohn et al. 2016), but our T_{sk} data provide the first evidence of a fever response to infection with *P. destructans* in any WNS-susceptible bat species.

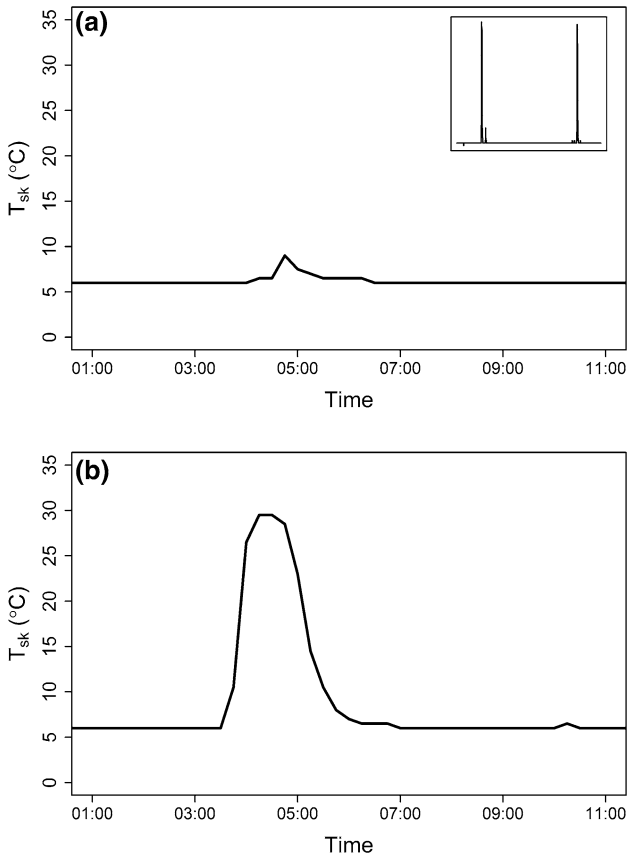

Fig. 4 Representative arousal T_{sk} profiles for an inoculated bat. The bat went through a normal arousal on day 92 of hibernation (detail not shown), then **a** a cold arousal following a disturbance on day 93 and **b** a normal arousal on day 100 of hibernation. The full sequence of the three arousals is presented in the *inset* of panel (**a**). In the normal arousal in (**b**) T_{sk} reached 29 °C, while in the cold arousal only reached 9 °C. The warm arousal on day 100 of hibernation, following a cold arousal on day 93 indicated that cold arousals do not indicate a loss of thermoregulatory control

One question about cytokine upregulation in response to infection with *P. destructans*, and corresponding sickness behaviour/fever, is whether these effects occur during torpor or are restricted to arousals (Wilcox et al. 2014; Field et al. 2015; Bohn et al. 2016; Lilley et al. 2017). This is an important question because, if these responses occur during torpor bouts, and interfere with torpid thermoregulation, they could exert strong influence on arousal frequency for bats with WNS. In golden-mantled ground squirrels (*Spermophilus lateralis*) immune challenge during torpor led to increased T_b during the subsequent arousal but did not appear to trigger arousals or cause a response during torpor itself (Prendergast et al. 2002). Our T_{sk} data are consistent with this pattern in that we did not observe evidence of effects (i.e. higher T_{sk}) during torpor bouts. If anything, torpid T_{sk} was lower for infected bats than controls. We cannot rule out the possibility of a cytokine-mediated response to *P. destructans* during torpor, but the absence of any change in torpid T_{sk}, combined with the pronounced change during arousals, suggests that effects during arousal are more biologically significant. Transcriptome analyses, like those of Field et al. (2015) and Davy et al. (2017) have the potential to help address this question if bats can be sampled at specific time points during torpor, re-warming and arousal to quantify changes in gene expression occurring throughout the phases of torpor and re-warming.

Even if a cytokine-mediated immune/febrile response does not contribute to the increase in arousal frequency for bats with WNS, it could still help explain premature depletion of energy stores. Fever is extremely energetically costly for endotherms. For example, the relationship between metabolic rate and the $T_b - T_a$ differential for little brown bats quantified by Studier (1981) estimates that an increase in T_b of 2 °C at a T_a of 7 °C would increase arousal costs by approximately 13.5%. Some estimates of metabolic costs of fever for other endothermic species suggest increases that could be more than twice this high. Higher energetic costs resulting from fever could have two significant effects for bats with WNS. Most obviously they would increase the cost of each arousal for bats that are already re-warming too frequently. It could also make it more difficult for bats to re-establish homeostasis during arousals if heightened metabolic costs increase buildup of metabolic wastes or increase pulmonary or cutaneous water loss. More work is needed to understand the energetic implications of the apparent febrile response of little brown bats

Table 1 Comparison of cold arousals in control and inoculated bats

	Control	Inoculated	Significance
Number of cold arousals	17	17	
Number of bats that exhibited cold arousal	8 of 13 bats	10 of 40 bats	See text
Timing of cold arousals (days before end of hibernation)	Median = 4 Range 0–26	Median = 5 Range 1–40	$W = 132$, $p = 0.68$ (Mann–Whitney)
ΔT_{sk}	1.9 ± 0.4 °C Range 0–5.5	2.5 ± 0.4 °C Range 0–6	$t_{31.9} = 0.95$, $p = 0.35$

The same total number of cold arousals were observed in each group, but there was no difference in the proportion of bats undergoing cold arousals when considering equivalent time periods (see text). There was no difference in the timing or increase in skin temperature during cold arousals between the two groups

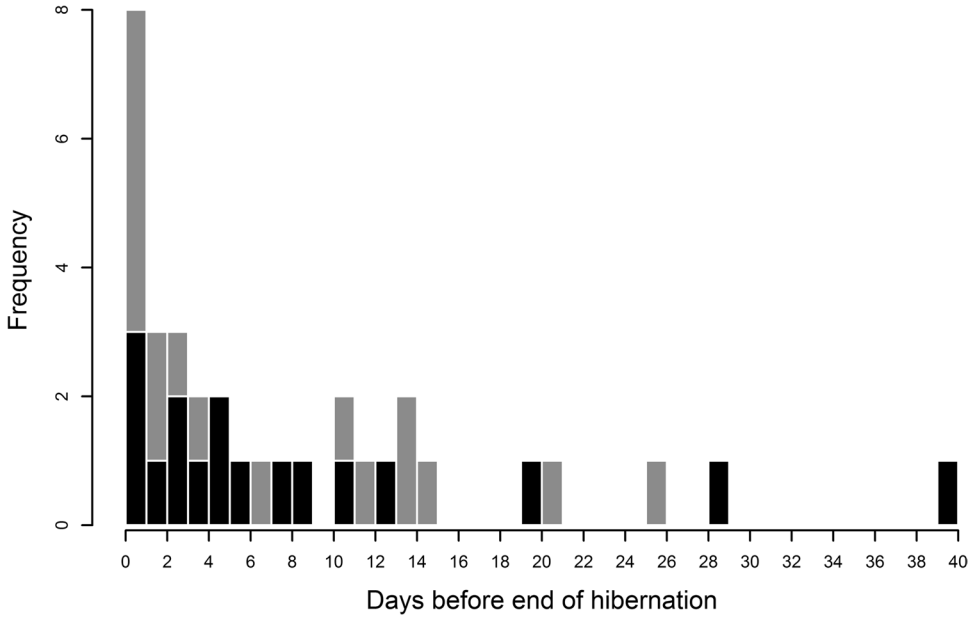

Fig. 5 Distribution of the timing (days before the end of hibernation) of cold arousals. Inoculated bats are indicated in *black bars*, and control bats in *grey bars*. Most cold arousals occurred within 2 weeks of the end of hibernation; however, there were five instances in which a cold arousal occurred ≥20 days before the end of hibernation

to *P. destructans*, but our results suggest it could play an important role in WNS pathophysiology.

In addition to evidence of fever, we also found strong support for our hypothesis that little brown bats can reduce energy expenditure during winter via behavioural activity at low T_b. We observed cold arousals from multiple bats, which accounted for 9% of all arousals we observed. During normal arousals, T_{sk} increased by ≥17 °C compared to ≤6 °C for cold arousals. Cold arousals were equally likely to be exhibited by bats infected with *P. destructans* as controls, despite the pronounced energetic constraints faced by bats with WNS. Cold arousals were dramatically more common late in hibernation when bats' energy stores would have been depleted, but some cold arousals also happened early in hibernation. Moreover, some individuals expressed cold arousals but then later exhibited normal arousals, which suggests that cold arousals do not simply reflect a loss of thermoregulatory control (i.e. the inability to re-warm because of depleted fat reserves). We expected the use of cold arousals to be influenced by the disease status of animals in our study, particularly because WNS leads to dramatic depletion of energy stores, but cold arousals were employed by both inoculated and control bats (Table 1; Fig. 5). There was no difference in ΔT_{sk} or the timing of cold arousals between inoculated and control bats. Moreover, although WNS is known to cause sickness behaviour during normal arousals, and reduced clustering during torpor (e.g. Wilcox et al. 2014; Bohn et al. 2016), observers watching the videos for this study did not observe obvious differences in behaviour between infected and control bats during cold arousals. These findings lead us to conclude that cold arousals are not necessarily associated with, or a symptom of, WNS but rather reflect a physiological adaptation available to hibernating bats more generally. Cold arousals represent an alternative strategy that may be effective under some circumstances and not others.

Low T_b affects cardiac function and aerobic metabolism, and reduces muscle power output, transmission of neural signals and movement speeds (Rojas et al. 2012). At $T_b = 8$ °C bats exhibit less coordinated movements, powered flight occurs at $T_b > 28$ °C, and maximum movement speeds and fully coordinated movements only occur at normothermic T_b (Choi et al. 1998; Willis and Brigham 2003). Evidence of cold arousals was recently presented for *Myotis myotis*, but movement was only quantified by still images captured at 5-min intervals and, therefore, it is unclear how coordinated these movements may have been (Bartonička et al. 2017). Although the cold arousals we observed were clearly associated with un-coordinated behaviour (see Supplementary Videos), this pattern of behaviour could be adequate for allowing bats to deal with some stimuli. For example, if a torpid bat was simply bumped out of position or dislodged from roosting by an active, normothermic individual and no other potential threat (e.g. a predator) is present, it could be highly beneficial to avoid spending the energy required to re-warm. It may also be possible for bats to perform other behaviours during cold arousals that help restore homeostasis during normothermic arousals (e.g. drinking). We observed bats adjacent to the water dishes in the cages during cold arousals in positions consistent with drinking but higher resolution video observations will be needed to confirm that bats can drink during cold arousals.

Many observations of cold arousals occurred at the end of hibernation, when energy stores are expected to be nearly depleted, and were not subsequently followed by normal arousals. Thus, we initially questioned whether cold

arousals represented a loss of thermoregulatory control in cases of severe disease or fat depletion. This was not the case. We observed cold arousals in both inoculated and control bats, and seven bats that experienced a cold arousal subsequently continued to hibernate and arouse normally. We conclude, therefore, that cold arousals do not reflect a loss of thermoregulatory control, as bats are still able to re-warm completely after experiencing cold arousals. Instead, cold arousals appear to be a voluntary strategy in some cases. Despite the prevalence of cold arousals late in hibernation, there were five examples of cold arousals that occurred ≥20 days before the end of hibernation. Of these five examples of early cold arousals, four seemed to be the result of a disturbance unrelated to the natural arousal pattern of the bat. In each case, the bat in question had previously aroused with a normal arousal 1 or 2 days prior to the disturbance. Disturbances included a bat being knocked off the wall by another bat, or the bat in question being removed from the chamber by a researcher for inspection and then being returned to the cage wall. These cases suggest that there may be two distinct functions of cold arousals. Cold arousals that occur at the end of hibernation (late/spontaneous cold arousals) may serve as an emergency energy-saving mechanism. Cold arousals occurring earlier during hibernation (early cold arousals) may suggest a response to a disturbance as opposed to energy depletion (similar to the observations of Bartonička et al. 2017). Emergency cold arousals may allow a bat to obtain a drink of water or assess whether conditions outside the hibernaculum might be favourable for foraging or early spring emergence, without incurring the full cost of arousal. For example, bats could crawl nearer to the entrance of the hibernaculum and/or assess changes in ambient conditions such as barometric pressure which may influence emergence from hibernation (Czenze and Willis 2015). Earlier in hibernation, cold arousals may be a mechanism that allows bats to respond to disturbances in cases when a normal arousal is not otherwise required (e.g. disturbances following shortly after a normal arousal).

Bats can survive occasional disturbances during hibernation because disturbances affect survival rates non-linearly (Boyles and Brack 2009; Speakman et al. 1991; Thomas 1995). Boyles and Brack (2009) proposed that there is a threshold number of disturbances that hibernating bats can tolerate, determined by the duration of hibernation and the temporal pattern of disturbance. Infrequent disturbances below the threshold should not impact survival because most bats store more energy in fall than they require for hibernation. Disturbances exceeding this threshold, however, or disturbances that are closely spaced in time (e.g. on consecutive days), could dramatically reduce survival (Boyles and Brack 2009). Our findings and those of Bartonička et al. (2017) suggest that bats may be even more flexible in terms of their potential to tolerate disturbance if they can exploit cold arousals to perform some arousal-related activities without incurring the full energetic cost of becoming normothermic.

Previous studies have found similar variation in torpor and arousal patterns that suggest bats can voluntarily adjust the magnitude of T_b change during arousals. Jonasson and Willis (2012) and Czenze et al. (2017), for example, observed numerous instances of free-ranging bats arousing during hibernation but then quickly re-entering shallow torpor in the midst of an arousal, a pattern they referred to as "heterothermic arousals". They suggested that heterothermic arousals represent an energy-saving strategy. Combined with our results, here, the dichotomous view of active, normothermic, and inactive, torpid periods of hibernation is likely overly simplistic. Instead, bat activity and thermoregulation may be more accurately represented as a continuum. Cold arousals (our study; Bartonička et al. 2017) and heterothermic arousals (Jonasson and Willis 2012; Czenze et al. 2017) appear to represent physiological strategies that allow hibernators to flexibly budget energy stores. To our knowledge, cold arousals and heterothermic arousals have not been observed for other long-term hibernators during winter and they suggest that bats may exhibit unique adaptations for extreme energy savings during hibernation (Willis 2017).

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Compliance with Ethical Standards

Ethics approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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