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RESEARCH ARTICLE

Cold-hearted bats: uncoupling of heart rate and metabolism during torpor at sub-zero temperatures

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ABSTRACT

Many hibernating animals thermoregulate during torpor and defend their body temperature (T_b) near 0°C by an increase in metabolic rate. Above a critical temperature (T_{crit}), animals usually thermoconform. We investigated the physiological responses above and below T_{crit} for a small tree-dwelling bat (*Chalinolobus gouldii*, ∼14 g) that is often exposed to sub-zero temperatures during winter. Through simultaneous measurement of heart rate (f_H) and oxygen consumption ($\dot{V}_{O_2}$), we show that the relationship between oxygen transport and cardiac function is substantially altered in thermoregulating torpid bats between 1 and −2°C, compared with thermoconforming torpid bats at mild ambient temperatures (T_a 5–20°C). T_{crit} for this species was at a T_a of 0.7±0.4°C, with a corresponding T_b of 1.8±1.2°C. Below T_{crit}, animals began to thermoregulate, as indicated by a considerable but disproportionate increase in both f_H and $\dot{V}_{O_2}$. The maximum increase in f_H was only 4-fold greater than the average thermoconforming minimum, compared with a 46-fold increase in $\dot{V}_{O_2}$. The differential response of f_H and $\dot{V}_{O_2}$ to low T_a was reflected in a 15-fold increase in oxygen delivery per heart beat (cardiac oxygen pulse). During torpor at low T_a, thermoregulating bats maintained a relatively slow f_H and compensated for increased metabolic demands by significantly increasing stroke volume and tissue oxygen extraction. Our study provides new information on the relationship between metabolism and f_H in an unstudied physiological state that may occur frequently in the wild and can be extremely costly for heterothermic animals.

KEY WORDS: Oxygen consumption, Thermoregulation, Hibernation, Endotherm, Oxygen pulse, Thermoregulation, Thermogenic capacity

INTRODUCTION

Extremely low ambient temperatures (T_a) create an energetic hurdle for many animals, particularly small endotherms that must markedly increase heat production to maintain a constant high body temperature (T_b). Torpor, a controlled reduction of metabolic rate (MR), heart rate (f_H) and T_b, is therefore critical for many small mammals to save energy during inclement conditions (Ruf and Geiser, 2015). During torpor, the hypothalamic control of temperature regulation is adjusted to a new, lower minimum T_b that is regulated at or just above a critical external temperature (T_{crit}). Depending on ambient conditions, animals will thermoconform down to this T_b/T_a (Heller, 1979; Geiser, 2011). However, when T_a falls below T_{crit}, animals can either rewarm entirely to a normothermic T_b or remain torpid and increase metabolic heat production to thermoregulate and defend minimum T_b.

Details regarding thermoregulation during torpor at sub-zero temperatures are scant and restricted to medium-sized northern mammals such as ground squirrels (Geiser and Kenagy, 1988; Buck and Barnes, 2000; Richter et al., 2015). However, small hibernators, such as temperate insectivorous bats, often use torpor year round (Eisentraut, 1956; Davis and Reite, 1967; Masing and Lutsar, 2007; Wojciechowski et al., 2007). Thermoregulation during torpor, while still conserving substantial amounts of energy, can be expensive when used over long periods and against a large T_b–T_a differential (Karpovich et al., 2009; Richter et al., 2015). At extremely low T_a, defending T_b above T_{crit} is likely to require an alteration of a number of physiological components essential to thermoregulation. The cardiovascular system is vital for maintaining physiological processes during torpor as the heart regulates the supply of blood gases, nutrients and hormones to the body. While there are data on the thermal energetics of torpor at low T_a, detailed information on cardiac function and its relationship to metabolism are entirely lacking.

During torpor at low T_b, the heart must facilitate adequate blood supply under conditions of reduced blood flow and slow ventilation/low oxygen intake (Milsom et al., 2001). In addition, to minimise metabolic costs during torpor, adequate perfusion is only retained in vital tissues and organs as a result of circulatory adjustments (Carey et al., 2003). The hearts of hibernating animals are adapted to function at low T_b and are capable of withstanding temperatures well below the critical point for fibrillation and death in non-hibernating species (Lyman and Blinks, 1959; van Veen et al., 2008). It is widely known that during torpor in thermoconforming hibernators, the heart continues to beat in a co-ordinated rhythm and is resistant to detrimental arrhythmia (Johansson, 1996; van der Heyden and Opthof, 2005). However, when animals are forced to thermoregulate during torpor, the cardiovascular system must adjust to coincide with changes in metabolism.

We investigated the thermal physiology, in particular the interrelationship between f_H and MR below and above T_{crit}, for torpid *Chalinolobus gouldii*, a common tree-dwelling bat whose distribution extends across Australia (Churchill, 2008). While temperate zone northern hemisphere bats tend to hibernate in thermally stable caves or mines during winter, many southern hemisphere bats hibernate in tree hollows or under bark. These roosts are thermally labile and often experience sub-zero T_a (Law and Chidel, 2007; Turbill, 2008; Clement and Castleberry, 2013; Stawski and Currie, 2016). Information concerning cardiac function and its relationship to metabolism and thermal energetics in this species, or at such low temperatures in any bat, is entirely lacking. Moreover, explicit investigations of T_{crit} and thermogenic capacity in hibernating bats are extremely limited (Reite and Davis, 1966; Stawski and Geiser, 2011).

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Given that *C. gouldii* regularly experience low T_a, we hypothesised that the T_{crit} for this species would be close to 0°C. As is the case for other hibernators, we predicted that f_H would be substantially reduced during steady-state torpor, with correspondingly low T_b and MR, until T_a reached T_{crit}. However, below T_{crit}, we hypothesised that bats would increase MR with a proportionate increase in f_H, but maintain a steady T_b.

MATERIALS AND METHODS

Six non-reproductive *Chalinolobus gouldii* (Gray 1841) (3 females and 3 males; capture mass 14.3±2.6 g) were collected from a residential roof near the University of New England (UNE) in Armidale, NSW, Australia, during the austral autumn (April) 2015. Individuals were kept in outdoor aviaries at UNE for 3 months and measurements were conducted from May to July 2015 (austral late autumn/winter). Food (*Tenebrio molitor* larvae dusted with a supplement of Wombaroo™ Insectivore Mix) and water were provided *ad libitum*. Prior to release at their capture site, bats were fitted with temperature-sensitive radio transmitters as part of another study (Stawski and Currie, 2016).

Respirometry and electrocardiogram measurements

In the late afternoon, bats were placed in air-tight respirometry chambers in a temperature-controlled cabinet where they remained overnight and into the following day(s). All individuals were exposed to T_a between −2 and 24°C over an experimental period of 8 weeks. Animals were not given access to food or water during respirometry measurements to ensure they were post-absorptive. Bats were weighed to the nearest 0.1 g before and after measurements. The T_a in the chamber was recorded to the nearest 0.1°C using a calibrated thermocouple placed 5 mm within the chamber and read using a digital thermometer.

Respirometry chambers (0.53 l) were made from modified polycarbonate enclosures with clear lids, lined with a small patch of hessian cloth (burlap) from which the bats could roost. Air flow through the chambers was controlled by rotameters and measured with mass flowmeters (Omega FMA-5606, Stamford, CT, USA) that were calibrated prior to the start of experimentation. Flow rate (200 ml min^{-1}) was adjusted to ensure that 99% equilibrium was reached within 12 min. Oxygen concentration was measured using a FC-1B Oxygen Analyser (Sable Systems International Inc., Las Vegas, NV, USA; resolution 0.001%). Measurements were taken from the chamber every minute for 15 min and then switched to outside air for reference readings (3 min) using solenoid valves. Digital outputs of the oxygen analyser, flowmeter and digital thermometer were recorded on a PC using custom-written data-acquisition software (G. Körtner, Centre for Behavioural and Physiological Ecology, Zoology, University of New England, Australia). $\dot{V}_{O_2}$ values were calculated per minute using standardised gas volumes and eqn 3a of Withers (1977), assuming a respiratory quotient of 0.85.

Electrocardiograms (ECG) were recorded from two leads attached to adhesive electrodes on the forearms of each bat following the methods of Currie et al. (2014). Attachment of the electrodes took place once bats were torpid, either following lights on in the morning or during the night following rectal T_b measurements. All bats returned to torpor within 1 h of lead attachment. ECGs were recorded using LabChart v7.3 software and analysed to determine f_H by calculating instantaneous f_H per second.

T_a above 0°C

For measurements of steady-state torpor of thermoconforming bats at mild T_a, bats were placed in respirometry chambers overnight at T_a between 5 and 15°C. Following ECG lead attachment and a return to steady-state torpor, the T_a of the chamber was increased by 2°C and once the new T_a was reached, this was left unchanged for at least 2 h. The T_a was then progressively increased until animals rewarmed, in response to either changing T_a or the stimulus of lights-off in the evening.

T_a below 0°C

Bats were placed in respirometry chambers overnight at a T_a of between 1 and 5°C. Following lights on and/or the attachment of ECG leads, T_a was progressively decreased in 1°C increments when it was below 3°C. Individuals were exposed to each temperature for a minimum of 2 h before the temperature was further reduced. Previous data (Reite and Davis, 1966; S.E.C., personal observation) suggest that swift reductions in T_a (≥3–5°C) often induce arousal from hibernation. Therefore, we took care to ensure T_a was gradually reduced to obtain an accurate representation of thermoregulatory T_{crit} and minimum T_a prior to spontaneous arousal. The minimum temperature reached before animals regularly aroused was −2°C, and therefore no measurements were taken below this temperature. All individuals were exposed to the same range of T_a during experimentation.

T_b measurements

T_b was measured to the nearest 0.1°C using a digital thermometer (Omega, HH-71T) and a calibrated thermocouple probe inserted ∼2 cm into the rectum. Rectal T_b was recorded for animals that had been torpid for at least 2 h, indicated by a low MR and f_H. We measured T_b within 1 min of removing the bat from the respirometry chamber and, as the rate of rewarming from torpor is initially slow, rectal T_b was considered to be within 0.5°C of T_b prior to the disturbance. Following rewarming from torpor, in all experiments bats were returned to aviaries where food was available *ad libitum*.

Analysis and statistics

Bats were considered torpid when $\dot{V}_{O_2}$ fell to less than 75% of the resting MR at the same T_a. During steady-state torpor, f_H and $\dot{V}_{O_2}$ were averaged over the same time period, for at least 30 min, corresponding to minimum torpid $\dot{V}_{O_2}$. Bats were deemed to be thermoconforming when T_b was within 2°C of T_a. When T_b was not known, individuals were considered to be thermoconforming when $\dot{V}_{O_2}$ values fell to, or below, those of bats with a T_b within 2°C of T_a, at the same T_a. The Q_{10} for $\dot{V}_{O_2}$ or f_H of thermoconforming torpid bats was calculated using the following equation:

$$Q_{10} = \left(\frac{R_1}{R_2}\right)^{10/(T_{a,1}-T_{a,2})}, \quad (1)$$

where R is the $\dot{V}_{O_2}$ or f_H at a particular T_a (1 or 2). Animals were considered to be thermoregulating in torpor when minimum $\dot{V}_{O_2}$ was at least double that of minimum average thermoconforming values at the same T_a. Oxygen pulse (OP), the oxygen delivery per heart beat, was calculated for torpid bats by dividing the average $\dot{V}_{O_2}$ (ml min^{-1}) by the corresponding average f_H (beats min^{-1}). The percentage contribution of f_H to increases in oxygen transport in thermoregulating bats was calculated following Bartholomew and Tucker (1963);

$$\%f_H = \frac{f_{H,2}-f_{H,1}}{f_{H,1}} \div \left(\frac{f_{H,2}-f_{H,1}}{f_{H,1}} + \frac{OP_2 - OP_1}{OP_1}\right), \quad (2)$$

using f_H and OP at T_a below 1°C ($T_{a,1}$=0.5°C; $T_{a,2}$=−2.1°C).

We calculated average resting f_H ($f_{H,rest}$) and $\dot{V}_{O_2}$ from the period following arousal for at least 5 min when $\dot{V}_{O_2}$ had fallen to ≤75% of

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maximum $\dot{V}_{O_2}$ at the peak of rewarming. Unfortunately, during arousal at low T_a, shivering associated with rewarming caused artefact on ECG recordings, making calculation of f_H extremely difficult. In addition, animals often moved during the final stages of rewarming, which resulted in detachment of ECG electrodes and cessation of f_H recording. Therefore, data for $f_{H,rest}$ were only available for three bats across five T_a.

All statistical analyses were performed using R v3.1.3 (http://www.R-project.org/), assuming a significance level of $P<0.05$. Means are presented ±s.d. for number of animals (n) and number of observations (N). We used analysis of covariance (ANCOVA) to assess whether minimum $\dot{V}_{O_2}$ was significantly different following disturbance associated with ECG lead attachment. Linear mixed effects models (nlme package; https://CRAN.R-project.org/package=nlme) were used to assess the relationship between f_H or $\dot{V}_{O_2}$ and T_a in thermoregulating torpid bats and resting bats. To determine whether there were any differences in slope between regressions for resting and thermoregulating $\dot{V}_{O_2}$ with respect to T_a, we performed an ANCOVA using nlme with individual as a random factor. For comparison between the slopes of f_H and $\dot{V}_{O_2}$, we log transformed the data prior to ANCOVA using nlme, again with individual as a random factor. Standardised major axis regressions were performed (smatr package; Warton et al., 2012) to assess the correlation between $\dot{V}_{O_2}$ and f_H when animals were either thermoconforming or thermoregulating during torpor. We used ANCOVA in smatr to determine whether there was a significant difference in the slopes of f_H against $\dot{V}_{O_2}$ between the two torpid states. For all analyses, pseudo-replication was accounted for by using the degrees of freedom from mixed effect linear modelling, which were adjusted for repeated measures. We included sex as an interaction term for all linear models and it was found to have no significant effect; therefore, the data were pooled for further analyses.

All procedures were approved by the University of New England Animal Ethics Committee and New South Wales National Parks and Wildlife Service. Data are available from the Dryad digital repository (Currie et al., 2017; doi:10.5061/dryad.jr74d).

RESULTS

Rest

At rest, following arousal from torpor, $\dot{V}_{O_2}$ and f_H of normothermic bats increased with decreasing T_a in a qualitatively similar linear pattern (Fig. 1A,B). Resting $\dot{V}_{O_2}$ ranged from 1.44 to 10.04 ml g^{-1} h^{-1} as T_a fell from 24.1 to −0.4°C (Fig. 1A; n=6, N=81). Over a similar T_a range (19.4–1.7°C), the values we were able to obtain for $f_{H,rest}$ increased from 412 to 698 beats min^{-1} (Fig. 1B; n=3, N=6); however, the relationship between f_H and T_a was not statistically significant when animal was included as a random factor (nlme; r^2=0.96, P=0.07).

Thermoconforming torpid bats

All bats entered torpor at all T_a and reached low $\dot{V}_{O_2}$ and f_H values indicative of steady-state torpor within 3 h of disturbance associated with ECG lead attachment. There was no impact of this disturbance evident, as minimum $\dot{V}_{O_2}$ following ECG lead attachment was not significantly different from the minimum $\dot{V}_{O_2}$ beforehand (P=0.85). Below T_a of 20°C, most individuals remained torpid until the lights went off, except for a few times when individuals hibernated for up to 48 h or rewarmed in response to low T_a. Importantly, all bats were capable of spontaneously rewarming from the lowest T_a they were exposed to in our study.

When bats were in steady-state torpor and thermoconforming, $\dot{V}_{O_2}$ (n=6, N=62) and f_H (n=6, N=54) tracked T_a in a curvilinear manner. T_b fell to within 1.0±0.9°C of T_a in thermoconforming individuals (n=6, N=20). The minimum average $\dot{V}_{O_2}$ of thermoconforming bats was 0.02±0.01 ml g^{-1} h^{-1} (n=6, N=9) recorded at a T_a of 2.1±0.3°C, which was <1% of resting values at a similar T_a (1.4±0.5°C, $\dot{V}_{O_2}$=10.23±1.58 ml g^{-1} h^{-1}, n=2, N=2). f_H fell to an absolute minimum of 3 beats min^{-1} at T_a=1.0°C while the average minimum torpor f_H ($f_{H,torpor}$) was 8±2 beats min^{-1} (n=6, N=7) and only 1.1% of $f_{H,rest}$ recorded at 1.4°C (698 beats min^{-1}, n=1, N=1). Even when bats were torpid at mild T_a (19.6°C), f_H and $\dot{V}_{O_2}$ were <11% of the corresponding resting rates ($f_{H,torpor}$=10.3%; $\dot{V}_{O_2}$=3.7%). When $\dot{V}_{O_2}$ was plotted against f_H in thermoconforming individuals, there was a significant positive linear correlation (r^2=0.88, $P<0.001$; Fig. 2). On occasion, animals maintained a low $\dot{V}_{O_2}$ and f_H when T_a fell below 1°C and one animal thermoconformed down to a T_a of −1°C with a T_b of 0.6°C and a corresponding $\dot{V}_{O_2}$ equal to 0.03 ml g^{-1} h^{-1} and f_H of 7 beats min^{-1}.

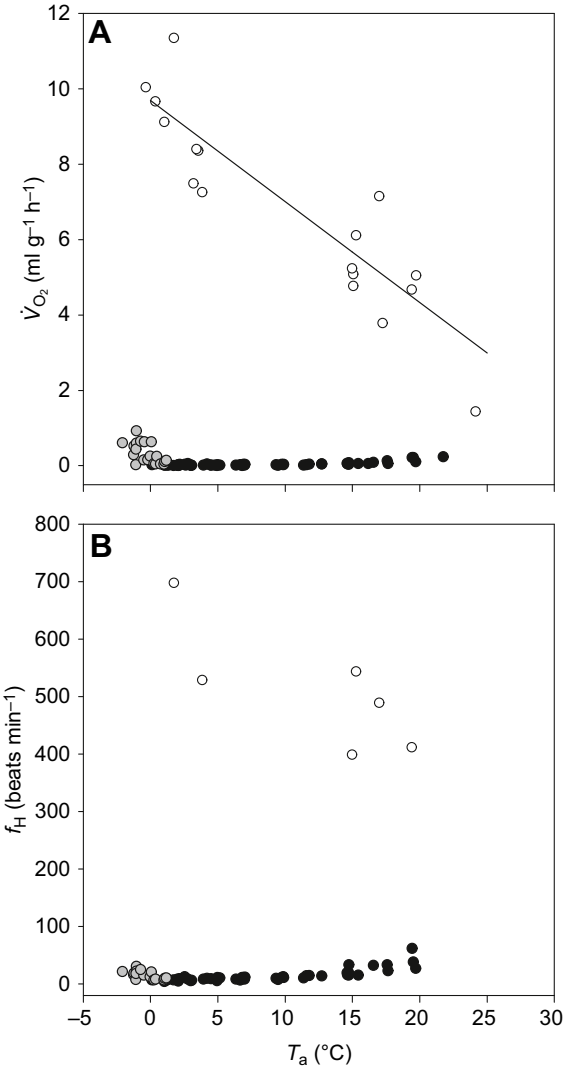

Fig. 1. Oxygen consumption and heart rate for *Chalinolobus gouldii* at rest and during torpor. Oxygen consumption ($\dot{V}_{O_2}$, A) and heart rate (f_H, B) are shown as a function of ambient temperature (T_a) for normothermic individuals at rest [white circles, solid line; $\dot{V}_{O_2}$=9.69–0.27(T_a), r^2=0.91, $P<0.001$; $\dot{V}_{O_2}$ n=6 animals, N=17 observations, f_H n=3, N=6] and during torpor when thermoconforming (black circles, n=6; $\dot{V}_{O_2}$ N=62, f_H N=54) or thermoregulating (grey circles, n=6; $\dot{V}_{O_2}$ N=18, f_H N=15).

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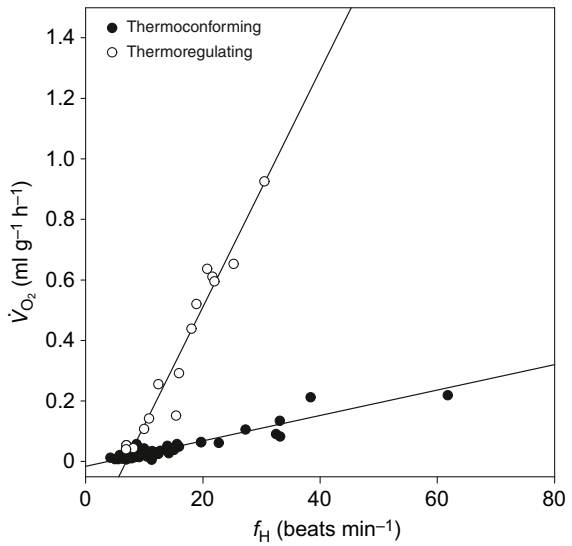

Fig. 2. The relationship between f_H and $\dot{V}_{O_2}$ in torpid *C. gouldii*. Data are given for thermoconforming individuals [$\dot{V}_{O_2}$=0.004(f_H)–0.016, r^2=0.88, P<0.001; n=6, N=54] or thermoregulating individuals [white circles, dashed line; $\dot{V}_{O_2}$=0.039(f_H)–0.272, r^2=0.95, P<0.001; n=6, N=15].

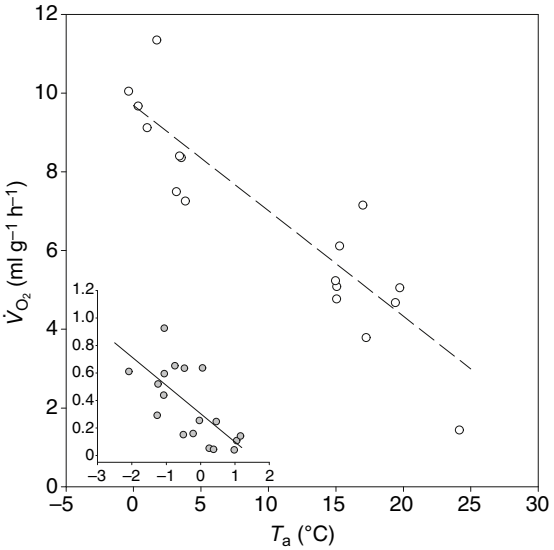

Fig. 4. $\dot{V}_{O_2}$ as a function of T_a in thermoregulating *C. gouldii*. Data are for bats that are normothermic at rest [$\dot{V}_{O_2}$=9.69–0.27(T_a), r^2=0.91, P<0.001; n=6, N=17] or thermoregulating during torpor [inset; $\dot{V}_{O_2}$=0.31–0.21(T_a), r^2=0.62, P<0.01; n=6, N=18]. There was no significant difference between the slopes of $\dot{V}_{O_2}$ and T_a in resting versus thermoregulating torpid individuals (ANCOVA; P=0.63).

The transport of oxygen per heart beat (OP) was qualitatively similar to f_H and $\dot{V}_{O_2}$ in bats during steady-state torpor; however, it declined only slightly with decreasing T_a down to 2°C (n=6, N=53; Fig. 3). The Q_{10} for $\dot{V}_{O_2}$ was 3.8 in thermoconforming bats, determined between a T_a of 19.6 and 2.1°C. When compared with the basal MR previously determined for this species (1.44 ml g^{-1} h^{-1}; Hosken and Withers, 1997), average Q_{10} was also 3.8. However, the corresponding Q_{10} for f_H during torpor in thermoconforming bats was only 2.6.

Thermoregulating torpid bats

Below a T_{crit} of 0.7±0.4°C (n=6, N=7), both f_H and $\dot{V}_{O_2}$ substantially increased and bats began thermoregulating (Fig. 1A,B). Average T_b at T_{crit} was 1.8±1.2°C (n=5, N=5), and as T_a fell to −2°C, bats defended T_b at 2.3±1.6°C (n=2, N=2). The majority of animals rewarmed spontaneously when T_a fell below −1.3°C; however, one animal remained torpid down to a T_a of −2.1°C ($\dot{V}_{O_2}$=0.61 ml g^{-1} h^{-1}; f_H=22 beats min^{-1}). Thermoregulating torpid bats exposed to a T_a below zero exhibited up to a 46-fold increase in $\dot{V}_{O_2}$ when compared with the minimum $\dot{V}_{O_2}$ during torpor at a T_a of 2°C (n=6, N=15). In contrast, f_H in torpid thermoregulating bats only increased on average 2-fold (range 1- to 4-fold) over the same T_a range (n=6, N=15). The maximum f_H recorded in thermoregulating torpid bats was 31 beats min^{-1} at T_a −1.1°C with a corresponding $\dot{V}_{O_2}$ of 0.93 ml g^{-1} h^{-1}.

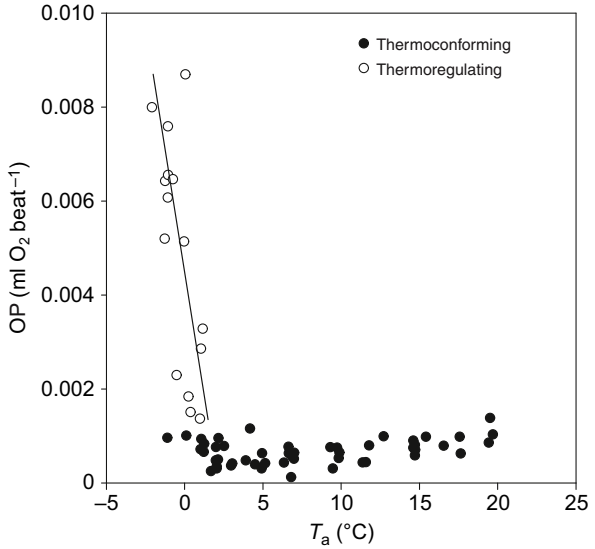

Fig. 3. Oxygen pulse (OP) as a function of T_a in torpid *C. gouldii*. Data are given for thermoconforming (n=6, N=54) or thermoregulating (n=6, N=15) individuals. The relationship with T_a changes from curvilinear (thermoconforming) to linear when animals begin thermoregulating [solid line; OP=0.0045–0.0021(T_a), r^2=0.87, P<0.001].

There was a significant linear correlation between f_H and $\dot{V}_{O_2}$ in thermoregulating bats (r^2=0.95, P<0.001) and this was significantly steeper than the relationship for thermoconforming bats at a T_a greater than 1°C (r^2=0.88, P<0.001; ANCOVA, P<0.001; Fig. 2). Both $\dot{V}_{O_2}$ and f_H increased linearly with decreasing T_a in thermoregulating individuals (f_H, r^2=0.71, P<0.01; MR, r^2=0.61, P<0.01; Figs 4 and 5). However, following log transformation, $\dot{V}_{O_2}$ showed a significantly steeper response to declining T_a than f_H (ANCOVA; P<0.05; Fig. 6). This disproportionate increase in f_H and $\dot{V}_{O_2}$ was also evident via a significant linear increase in OP as T_a fell below ∼1°C (r^2=0.89, P<0.001; Fig. 3).

There was an almost 15-fold increase in average OP from 2°C to −2°C (5.5×10^{-4} ml O_2 beat^{-1} to 80×10^{-4} ml O_2 beat^{-1}, respectively) and the contribution of f_H to changes in oxygen transport was minimal, at only 31% (calculated using Eqn 2). Interestingly, the slope of the relationship between $\dot{V}_{O_2}$ and T_a, an indicator of thermal conductance, did not differ between thermoregulating torpid and resting bats (ANCOVA; P=0.63; Fig. 4).

DISCUSSION

While thermoregulating in torpor below T_{crit}, animals must not only produce enough heat to maintain the gradient between T_b and T_a but also ensure adequate cardiovascular function to support thermogenic needs. Our study is the first to provide simultaneously recorded data

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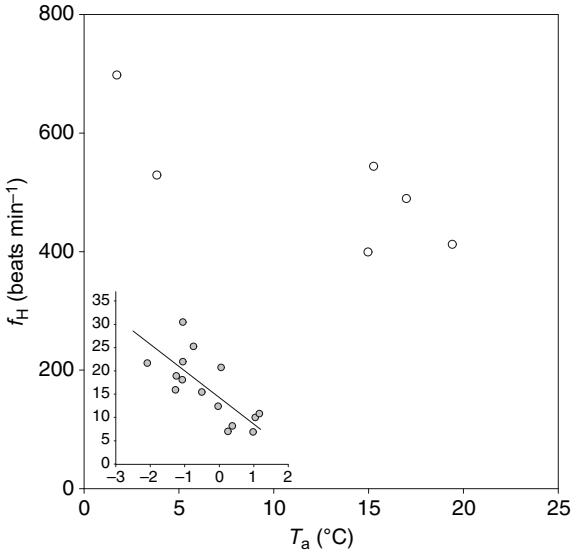

Fig. 5. f_H as a function of T_a in thermoregulating *C. gouldii*. Data are for bats that are normothermic at rest (n=3, N=6) or thermoregulating during torpor [inset; f_H=14.27−5.72(T_a), r^2=0.71, P<0.01; n=6, N=15].

of metabolism and cardiac function in normothermic and torpid bats exposed to T_a at or below 0°C. Confirming our hypothesis, we found that torpid *C. gouldii* are capable of coping with low T_b while maintaining coordinated cardiac function. While our sample size is limited, we report decidedly lower minimum f_H values than anticipated for animals this size, which compare to torpid f_H values found in much larger hibernators (Lyman, 1951; Augee and Ealey, 1968; Swoap et al., 2017). We also established a new T_{crit} for thermoregulation of around 1°C, substantially lower than previous estimates for this species (Kulzer et al., 1970; Hosken and Withers, 1997). Below T_{crit}, animals began to defend T_b by increasing metabolic heat production and f_H, and all animals in our study were able to rewarm from the lowest T_a (−2°C).

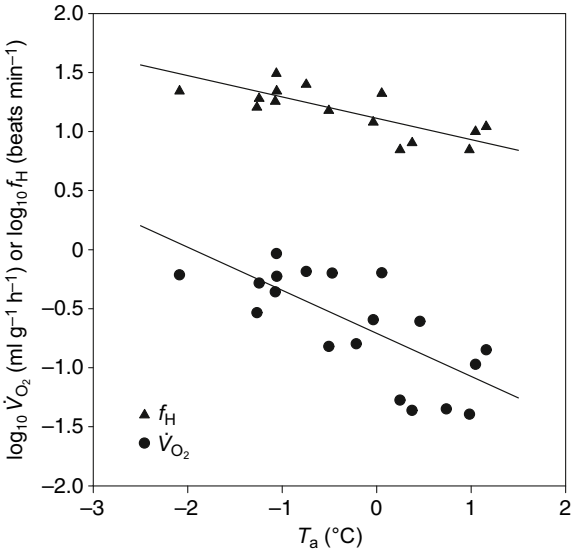

Fig. 6. f_H and $\dot{V}_{O_2}$ as a function of T_a in thermoregulating *C. gouldii* during torpor. $\log_{10}$-transformed f_H [$\log_{10} f_H$=1.11−0.18(T_a), r^2=0.82, P<0.001; n=6, N=15] and $\dot{V}_{O_2}$ [$\log_{10}\dot{V}_{O_2}$=−0.71−0.37(T_a), r^2=0.71, P<0.001; n=6, N=18] increased disproportionately with decreasing T_a. $\dot{V}_{O_2}$ increased at a greater rate than f_H below 1°C.

When animals were exposed to T_a below 1°C, there was a disproportionate response of the cardiovascular and metabolic systems as $\dot{V}_{O_2}$ increased at a greater rate than f_H to support elevated heat production. The bats we studied increased oxygen consumption by 46-fold, compared with a maximum increase of only 4-fold in f_H. During this phase, T_b remained low and therefore the heart was cold. Although hibernators' hearts are capable of withstanding very low T_b, muscle contractility and rate are still limited by temperature (Michael and Menaker, 1963; Smith and Katzung, 1966; South and Jacobs, 1973). In addition, it is possible that excessive cardiac stimulation and f_H acceleration at low T_b could induce detrimental arrhythmias. Therefore, changes in circulation and the relationship between f_H and $\dot{V}_{O_2}$ must be altered to maintain adequate supply under increasingly demanding conditions as T_a falls. In particular, the increased needs of the tissues must be met by an increase in oxygen delivery per heart beat or via increased oxygen extraction rates. Our results are the first to demonstrate a substantial upward shift in oxygen pulse during this phase of almost 15-fold, with f_H only contributing to 31% of the elevated oxygen supply to tissues. Accordingly, we are also the first to illustrate a significant difference in relationship between f_H and $\dot{V}_{O_2}$ for thermoregulating torpid individuals compared with thermoconforming individuals with a greater $\dot{V}_{O_2}$ at almost every f_H recorded for thermoregulating bats.

The novel finding of our data is the differential response of the cardiovascular and metabolic systems to thermogenic requirements at low T_a, which would be unknown had we not recorded these variables simultaneously. Following the Fick equation [$\dot{V}_{O_2}$ (ml min^{-1})=f_H×SV×(Ca_{O_2}−Cv_{O_2})], the significant change in oxygen delivery per beat that we recorded for thermoregulating *C. gouldii* must be the result of substantial increases in stroke volume (SV) and/or oxygen uptake by the tissues (arteriovenous difference in oxygen content; Ca_{O_2}−Cv_{O_2}). Because of the difficulties associated with the measurement of SV and blood characteristics, data for these variables in hibernating animals remain scarce with virtually none available for bats in any physiological state. Under the assumption that SV increases to its maximum capacity (~0.03 ml min^{-1}, calculated using eqn 7 of Bishop, 1997; using the heart mass reported for *C. gouldii* from Bullen et al., 2009), we calculate that arteriovenous difference must increase to a maximum of 26.23 ml O_2 dl^{-1} blood at −2°C. This value is approaching maximal tissue oxygen extraction capacity, and is similar to oxygen extraction rates thought to occur during extreme exercise and flight (Bishop, 1997). To achieve such high levels of oxygen extraction, haemoglobin (Hb) content of the blood must be at least 21 g dl^{-1} blood and 100% saturated in thermoregulating torpid bats. Previous reports of Hb content of bat blood vary, with only one report for hibernating individuals (Wołk and Bogdanowicz, 1987); however, values ≥20 g dl^{-1} blood have been reported in resting bats and would suggest our results are not outside the expectations for oxygen carrying capacity in these animals (Jürgens et al., 1981; Arévalo et al., 1987; Bishop, 1997). In the light of our findings for oxygen pulse, and in line with these calculations, our results suggest that both SV and tissue oxygen extraction approach maximal capacity when bats are thermoregulating during torpor at sub-zero T_a.

There remains some debate as to the control mechanisms behind the cardiac rhythms we see during torpor (Lyman and O'Brien, 1963; Milsom et al., 1999; Zosky and Larcombe, 2003; Braulke and Heldmaier, 2010). Although the autonomic nervous system plays an essential role in reducing f_H at the onset of torpor, at low T_b nervous input is reduced, partially related to the dampening effects of temperature on nerve function but also probably as a result of

withdrawal of control (Milsom et al., 2001). While we did not directly measure neurotransmitters or experimentally test for nerve response at the heart, our results suggest that once thermoconforming bats are in steady-state torpor and the heart is cold, temperature becomes the driving factor behind the patterns that we observe. This is supported by the Q_{10} values of f_H in thermoconforming bats and also in part by the limited response of f_H to decreasing T_a in thermoregulating torpid animals. While nervous control is not entirely withdrawn, we interpret our results to suggest that during thermoregulation in torpid bats at low T_b, excessive cardiac stimulation is probably inhibited to protect against arrhythmia.

Additionally, the change in relationship between f_H and $\dot{V}_{O_2}$ could also be the result of an alteration in blood supply across the body. During torpor, circulation to the periphery is restricted with perfusion of only vital organs such as the heart, lungs and brain retained (Lyman and O'Brien, 1963). This restriction of blood flow results in dramatic changes in blood pressure, enabling animals to maintain sufficient supply to tissues at low T_b when blood viscosity is increased (Lyman et al., 1982). It is possible that when thermoregulating during torpor, animals increase blood supply to essential thermogenic organs, such as brown adipose tissue or muscle, to defend T_b against increasing differentials with T_a. This may enable individuals not only to remain torpid for longer but also to rewarm swiftly should the external temperature drop below a level they are capable of withstanding. It would be interesting to investigate how blood flow during this phase is altered, and whether the increased peripheral resistance and restriction of circulation is partially withdrawn. Our results show that in thermoregulating torpid bats, thermal conductance is similar to that in resting bats, as reported for other heterotherms (Henshaw, 1968; Geiser, 2004), and this may be the result of such changes in blood flow. We have previously suggested that this may be the case in bats during passive rewarming (Currie et al., 2015), and it may be that changes in blood flow influence the relationship between metabolism and heart rate when animals are thermoregulating during torpor.

When thermoregulation is activated during torpor, although it still conserves substantial energy compared with normothermic thermoregulation at low T_a (99% less at −1°C), it is more energetically demanding than thermoconforming during torpor (~26-fold greater at −1°C than 2°C). When arctic ground squirrels (*Urocitellus parryii*) and golden-mantled ground squirrels (*Callospermophilus lateralis* and *Callospermophilus saturatus*) were forced to thermoregulate during hibernation at decreasing T_a (down to −30°C), the frequency of spontaneous arousals increased and the amount of body mass lost during the hibernation season almost doubled compared with free-living animals (Geiser and Kenagy, 1988; Richter et al., 2015). Smaller hibernators are unlikely to be able to cope with such large T_b–T_a differentials during torpor because of their larger surface area to volume ratios and comparatively higher costs of thermoregulation. We show that small bats rewarmed before T_a fell below −3°C, a T_b–T_a differential of only a few degrees. It is likely that more frequent arousals and a rapid depletion of fat stores occurs in small hibernating mammals exposed to very low temperatures for long periods.

However, it has been suggested that the comparative costs of arousals are reduced when animals are exposed to decreasing T_a, as the costs of maintaining T_b during torpor increase (Karpovich et al., 2009; Richter et al., 2015). However, unlike rodents that hibernate in thermally stable hibernacula, *C. gouldii* roost in thermally labile environments, such as tree roosts and buildings (Lumsden et al., 2002). The individuals we studied were originally removed from the roof of a building and were radio-tracked to tree roosts for at least 17 days following their release (Stawski and Currie, 2016). In these circumstances, bats may be able to passively rewarm, thus mitigating much of the cost of arousal from low T_a/T_b. Indeed, we found evidence of passive rewarming in these same individuals when radio-tracked following their release, with 83% of all recorded arousals during winter involving passive rewarming (Stawski and Currie, 2016). Although poorly insulated roosts mean that animals may experience temperatures below their torpor T_{crit}, the ability to passively rewarm may outweigh some costs of thermoregulation during torpor.

Red bats (*Lasiurus borealis*) are also a tree-dwelling species and continue to remain in thermally labile roosts even though the T_a may often fall below freezing (Mormann and Robbins, 2007). When exposed to T_a below 0°C during torpor, *L. borealis* showed a similar range of f_H to that of *C. gouldii* in this study, increasing from an average of 12 beats min^{-1} at 5°C to 25–40 beats min^{-1} at −2°C (Reite and Davis, 1966). Like *C. gouldii*, *L. borealis* also take advantage of mild winter days and passively rewarm (Dunbar and Tomasi, 2006), but have been found to remain torpid unless external temperatures reach at least 15°C (Davis and Lidicker, 1956; Davis, 1970). The physiological similarities between *L. borealis* and *C. gouldii* with regard to their response to low T_a during torpor probably reflect similar roosting habits over winter. In contrast, little brown bats (*Myotis lucifugus*), which overwinter in stable environments, responded to decreasing T_a by increasing f_H to ~100 beats min^{-1} at −2°C (Reite and Davis, 1966). However, these data may be more indicative of animals initiating or attempting the arousal process, as Reite and Davis (1966) note that after 4 h, animals kept at −5°C became hypothermic and died. This suggests that bats which hibernate in unstable microclimates are more likely to remain torpid as T_a falls below zero, while bats that overwinter in thermally stable places are more likely to arouse as they can change roost location to select warmer areas within the cave to avoid excessively low temperatures. Our results show that T_{crit} is probably a response of selection related to the long-term environmental temperatures of their habitat and can be close to zero, even though sub-zero temperatures may only be experienced for a few days throughout the year.

Conclusion

Our data provide fundamental information regarding the costs of thermoregulation while in torpor, which has implications for understanding energy budgets of heterothermic animals that regularly experience low T_a in the wild. Disturbances such as sound (Speakman et al., 1991), smoke (Stawski et al., 2015) and disease (Verant et al., 2014) have all been shown to increase T_b and therefore metabolic rate during torpor (Geiser, 2004), as well as inducing arousal. Increased arousal frequency during winter has been the cause of devastating losses of bats infected with white nose syndrome (Willis, 2017), and this could possibly be the result of increased metabolic rate during torpor. As animals are increasingly susceptible to disturbances associated with anthropogenic activity and climate change, it is crucial that we understand the costs associated with thermoregulation during torpor as our data show that it is distinctly different from torpor when bats are thermoconforming. Our results suggest that while f_H is limited by low T_b and the heart continues to beat relatively slowly, other aspects of the cardiovascular system must be increased to near-maximal levels to supply sufficient oxygen during this demanding phase. An increased incidence of thermoregulation during torpor in the wild could have dramatic implications for survival in many

heterothermic species, and this is an area of great interest that is virtually unstudied in nature.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: S.E.C., C.S.; Methodology: S.E.C., C.S., F.G.; Validation: S.E.C.; Formal analysis: S.E.C., C.S.; Investigation: S.E.C.; Resources: C.S., F.G.; Data curation: S.E.C.; Writing - original draft: S.E.C.; Writing - review & editing: S.E.C., C.S., F.G.; Visualization: S.E.C.; Supervision: F.G.; Project administration: S.E.C., F.G.; Funding acquisition: S.E.C., F.G.

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Data availability
Data are available from the Dryad digital repository: http://datadryad.org/resource/doi:10.5061/dryad.jr74d.

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