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Cold-hearted bats: Uncoupling of heart rate and metabolism during torpor at subzero temperatures

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Summary Statement

Thermoregulation during torpor at low temperatures is energetically expensive and results in a considerable but disproportionate increase in heart rate and metabolism.

Abstract

Many hibernating animals thermoregulate during torpor and defend their body temperature (T_b) below 10°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 subzero temperatures during winter. Through simultaneous measurement of heart rate (HR) 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 T_a 0.7 ± 0.4°C, with a corresponding T_b of 1.8 ± 1.2°C. Below T_{crit} animals began to thermoregulate, indicated by a considerable but disproportionate increase in both HR and $\dot{V}O_2$. The maximum increase in HR was only 4-fold greater than the average thermoconforming minimum, compared to a 46-fold increase in $\dot{V}O_2$. The differential response of HR 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 HR 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 HR in an unstudied physiological state that may occur frequently in the wild and can be extremely costly for heterothermic animals.

Introduction

Extremely low ambient temperatures (T_a) creates 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 (HR) and T_b, is therefore critical for many small mammals to save energy during inclement conditions (Ruf & Geiser 2015). During torpor, the hypothalamic control for 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 a minimum T_b.

Details regarding thermoregulation during torpor at subzero temperatures are scant and restricted to medium-sized northern species such as ground squirrels (Geiser & Kenagy 1988; Buck & Barnes 2000; Richter *et al.* 2015). However, small hibernators, such as temperate insectivorous bats often use torpor year round (Eisentraut 1956; Davis & Reite 1967; Masing & Lutsar 2007; Wojciechowski, Jefimow & Tegowska 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, Tøien, Buck & Barnes 2009; Richter *et al.* 2015). At extremely low T_a, defending T_b above T_{crit} likely requires 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, Zimmer & Harris 2001). In addition, to minimise metabolic costs during torpor, adequate perfusion is only retained in vital tissues and organs due to circulatory adjustments (Carey, Andrews &

Martin 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 & Blinks 1959; van Veen, van der Heyden & van Rijen 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 & 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 interrelations between HR 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 subzero T_a (Law & Chidel 2007; Turbill 2008; Clement & Castleberry 2012; Stawski & 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 & Davis 1966; Stawski & Geiser 2011).

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 HR would be substantially reduced during steady-state torpor, with corresponding 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 HR, but maintain a steady T_b.

Materials and methods

Six non-reproductive *Chalinolobus gouldii* (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, during the austral autumn (April) 2015. Individuals were kept in outdoor aviaries at UNE for three months and measurements were conducted from May - July 2015 (austral late Autumn/Winter). Food (*Tenebrio molitor* larvae dusted with a supplement of Wombaroo^{TM} 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 & 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 litres) 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) 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, 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). $\dot{V}O_2$ values were calculated per minute using standardised gas volumes and equation 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, Körtner and Geiser (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. Electrocardiograms were recorded using LabChart v7.3 software and analysed to determine HR by calculating instantaneous HR per second.

T_a above zero °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, either in response to changing T_a or the stimulus of lights off in the evening.

T_a below zero °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 & Davis 1966; S.E. Currie personal observ.) 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 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 HR. We measured T_b within 1 min of removing the bat from the respirometry chamber and since 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 RMR at the same T_a. During steady-state torpor, HR 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 that, or below that, of bats with a T_b within 2°C of T_a, at the same T_a. The Q_{10} for rates of $\dot{V}O_2$ or HR of thermoconforming torpid bats was calculated using the following equation;

$$Q_{10} = \left(\frac{R1}{R2}\right)^{10/(Ta1-Ta2)} \quad (1)$$

where R is the $\dot{V}O_2$ or HR 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) by the corresponding average HR (bpm). The percentage contribution of HR to increases in oxygen transport in thermoregulating bats was calculated following Bartholomew and Tucker (1963);

$$\%\mathrm{HR} = \frac{HR_2-HR_1}{HR_1} \div \left(\frac{HR_2-HR_1}{HR_1} + \frac{OP_2-OP_1}{OP_1}\right) \quad (2)$$

using HR and OP at T_a below 1°C (T_a 1 = 0.5°C; T_a 2 = -2.1°C).

We calculated average resting HR (RHR) 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 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 HR extremely difficult. In addition, animals often moved during the final stages of rewarming, which resulted in detachment of ECG electrodes and cessation of HR recording. Therefore, data for RHR were only available for three bats across five T_a.

All statistical analyses were performed using R v3.1.3 (R Core Team 2014), assuming a significance level of $p<0.05$. Means are presented ± standard deviations 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; Pinheiro *et al.* 2014) were used to assess the relationship between HR or $\dot{V}O_2$ and T_a in thermoregulating torpid bats and resting bats. To determine if 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 individuals as a random factor. For comparisons between the slopes of HR 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, Duursma, Falster & Taskinen 2012) to assess the correlation between $\dot{V}O_2$ and HR 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 for HR against $\dot{V}O_2$ between the two torpid states. For all analyses pseudo-replication was accounted for by using the degrees of freedom as for mixed effect linear modelling 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 is available via Dryad at; doi:10.5061/dryad.jr74d.

Results

Rest

At rest, following arousal from torpor, HR and $\dot{V}O_2$ of normothermic bats increased with decreasing T_a in a qualitatively similar linear pattern (Figs 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 RHR increased from 412 to 698 bpm (Fig 1B; n=3, N=6), however the relationship between HR 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 HR 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 $\dot{V}O_2$ prior (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 HR tracked T_a in a curvilinear manner (n=6, N=54). 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 T_a 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). HR fell to an absolute minimum of 3 bpm at T_a = 1.0°C while the average minimum torpor HR (THR) was 8 ± 2 bpm (n=6, N=7) and only 1.1% of RHR recorded at 1.4°C (698 bpm, n=1, N=1). Even when bats were torpid at mild T_a (19.6°C) HR and $\dot{V}O_2$ were <11% of the corresponding resting rates (THR = 10.3%; $\dot{V}O_2$ = 3.7%). When $\dot{V}O_2$ was plotted against HR 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 HR 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 corresponding $\dot{V}O_2$ equal to 0.03 ml g^{-1} h^{-1} and HR of 7 bpm.

The transport of oxygen per heart beat (oxygen pulse) was qualitatively similar to HR and $\dot{V}O_2$ in bats during steady-state torpor, however 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 T_a of 19.6 and 2.1°C. When compared to the basal metabolic rate (BMR) previously determined for this species (1.44 ml g^{-1} h^{-1}; Hosken & Withers 1997) average Q_{10} was also 3.8. However, the corresponding Q_{10} for HR during thermoconforming torpor was only 2.6.

Thermoregulating torpid bats

Below a T_{crit} of 0.7 ± 0.4°C (n=6, N=7) both HR and $\dot{V}O_2$ substantially increased and bats began thermoregulating (Figs 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}; HR = 22 bpm). Thermoregulating torpid bats exposed to T_a below zero exhibited up to a 46-fold increase in $\dot{V}O_2$ when compared to minimum $\dot{V}O_2$ during torpor at T_a 2°C (n=6, N=15). In contrast, HR 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 HR recorded in thermoregulating torpid bats was 31 bpm at T_a -1.1°C with a corresponding $\dot{V}O_2$ of 0.93 ml g^{-1} h^{-1}.

There was a significant linear correlation between HR 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 T_a greater than 1°C (r^2 = 0.88, $p<0.001$; ANCOVA, $p<0.001$; Fig 2). Both HR and $\dot{V}O_2$ increased linearly with decreasing T_a in thermoregulating individuals (HR, r^2 = 0.71, $p<0.01$; MR, r^2 = 0.61, $p<0.01$; Figs 4 & 5). However, following log transformation, $\dot{V}O_2$ showed a significantly steeper response to declining T_a than HR (ANCOVA; $p<0.05$; Fig

6). This disproportionate increase in HR and $\dot{V}O_2$ was also evident via a significant linear increase in oxygen pulse 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 oxygen pulse 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 HR to changes in oxygen transport was minimal, at only 31% (calculated using Eq 2). Interestingly, the slope of the relationship between $\dot{V}O_2$ and T_a, an indicator of thermal conductance, did not differ during thermoregulating torpor from that of resting bats (ANCOVA; p=0.63; Fig 4).

Discussion

While thermoregulating in torpor below T_{crit} animals not only require the ability to produce enough heat to maintain the gradient between T_b and T_a, but also to ensure adequate cardiovascular function to support thermogenic needs. Our study is the first to provide simultaneously recorded data 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 HRs than anticipated for animals this size which compare to torpid HRs found in much larger hibernators (Lyman 1951; Augee & Ealey 1968; Swoap, Körtner & Geiser 2017). We also established a new T_{crit} for thermoregulation of around 1°C, substantially lower than previous estimates for this species (Kulzer, Nelson, McKean & Möhres 1970; Hosken & Withers 1997). Below T_{crit} animals began to defend T_b by increasing metabolic heat production and HR, and all animals in our study were able to rewarm from the lowest T_a (-2°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 HR to support elevated heat production. The bats we studied increased oxygen consumption by 46-fold, compared to a maximum increase of only 4-fold in HR. 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 & Menaker 1963; Smith & Katzung 1966; South & Jacobs 1973). In addition, it is possible that excessive cardiac stimulation and HR acceleration at low T_b could induce detrimental arrhythmias. Therefore, changes in circulation and the relationship between HR 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 HR 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 HR and $\dot{V}O_2$ for thermoregulating torpid individuals compared to thermoconforming individuals with a greater $\dot{V}O_2$ at almost every HR recorded for thermoregulating bats.

The novel highlight of our data are the differential responses of the cardiovascular and metabolic systems to thermogenic requirements at low T_a which would otherwise be unknown had we not recorded these variables simultaneously. Following the Fick equation ($\dot{V}O_2$ (ml/min) = HR × SV × (CaO_2 - CvO_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; CaO_2 - CvO_2). Due to the difficulties associated with measurements of stroke volume 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, calculated using Eq 7 of Bishop 1997; using the heart mass reported for *C. gouldii* from Bullen, McKenzie, Bullen & Williams 2009) we calculate that arteriovenous difference must increase to a maximum of 26.23 ml O_2/dl 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 blood and 100% saturated in thermoregulating torpid bats. Previous reports of Hb content of the blood of bats vary, with only one report for

hibernating individuals (Wołk & Bogdanowicz 1987), however values ≥20 g/dl blood have been reported in resting bats and would suggest our results are not outside of expectations for oxygen carrying capacity in these animals (Jurgens, Bartels & Bartels 1981; Arévalo, Pérez-Suárez & López-Luna 1987; Bishop 1997). In the wake 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 & O'Brien, 1963; Milsom *et al.*, 1999; Zosky & Larcombe, 2003; Braulke & Heldmaier, 2010). Although the autonomic nervous system plays an essential role in reducing HR 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 likely 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 Q_{10} values of HR in thermoconforming bats and also in part by the limited response of HR to decreasing T_a in thermoregulating torpid animals. While nervous control is not entirely withdrawn, we interpret our results to suggest that during thermoregulating torpid bats at low T_b excessive cardiac stimulation is likely inhibited to protect against arrhythmia.

Additionally, the change in relationship between HR 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 & 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, Willis, Malan & Wang 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 not only enable individuals 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, Noy & Geiser 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, while still conserving substantial energy compared with normothermic thermoregulation at low T_a (99% less at -1°C), it is more energetically demanding than for thermoconforming torpid individuals (~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 increasingly lower 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 to free-living animals (Geiser & 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 due to 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). Conversely, unlike rodents that hibernate in thermally stable hibernacula, *C. gouldii* roost in thermally labile environments,

such as tree roosts and buildings (Lumsden, Bennett & Silins 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 & 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 & 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 T_a may often fall below freezing (Mormann & Robbins 2007). When exposed to T_a below 0°C during torpor, *L. borealis* showed a similar range of HR as *C. gouldii* in this study, increasing from an average of 12 bpm at 5°C to 25-40 bpm at -2°C (Reite & Davis 1966). Like *C. gouldii*, *L. borealis* also take advantage of mild winter days and passively rewarm (Dunbar & Tomasi 2006), but have been found to remain torpid unless external temperatures reach at least 15°C (Davis & Lidicker 1956; Davis 1970). The physiological similarities between *L. borealis* and *C. gouldii* with regard to response to low T_a during torpor likely reflect similar roosting habits over winter. On the contrary, little brown bats (*Myotis lucifugus*), which overwinter in stable environments, responded to decreasing T_a by increasing HR to ~100 bpm at -2°C (Reite & 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 most likely a response and selection to the long-term environmental temperatures of their habitat and can be close to

zero, even though subzero 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, Webb & Racey 1991), smoke (Stawski, Matthews, Körtner & Geiser 2015) and disease (Verant *et al.* 2014) have all been shown to increase T_{crit} and therefore metabolic rate during torpor (Geiser 2004) as well as inducing arousal. Increased arousal frequency during winter has already been the cause of devastating losses for 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 the heart is rate limited by low T_b and 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. 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 interests.

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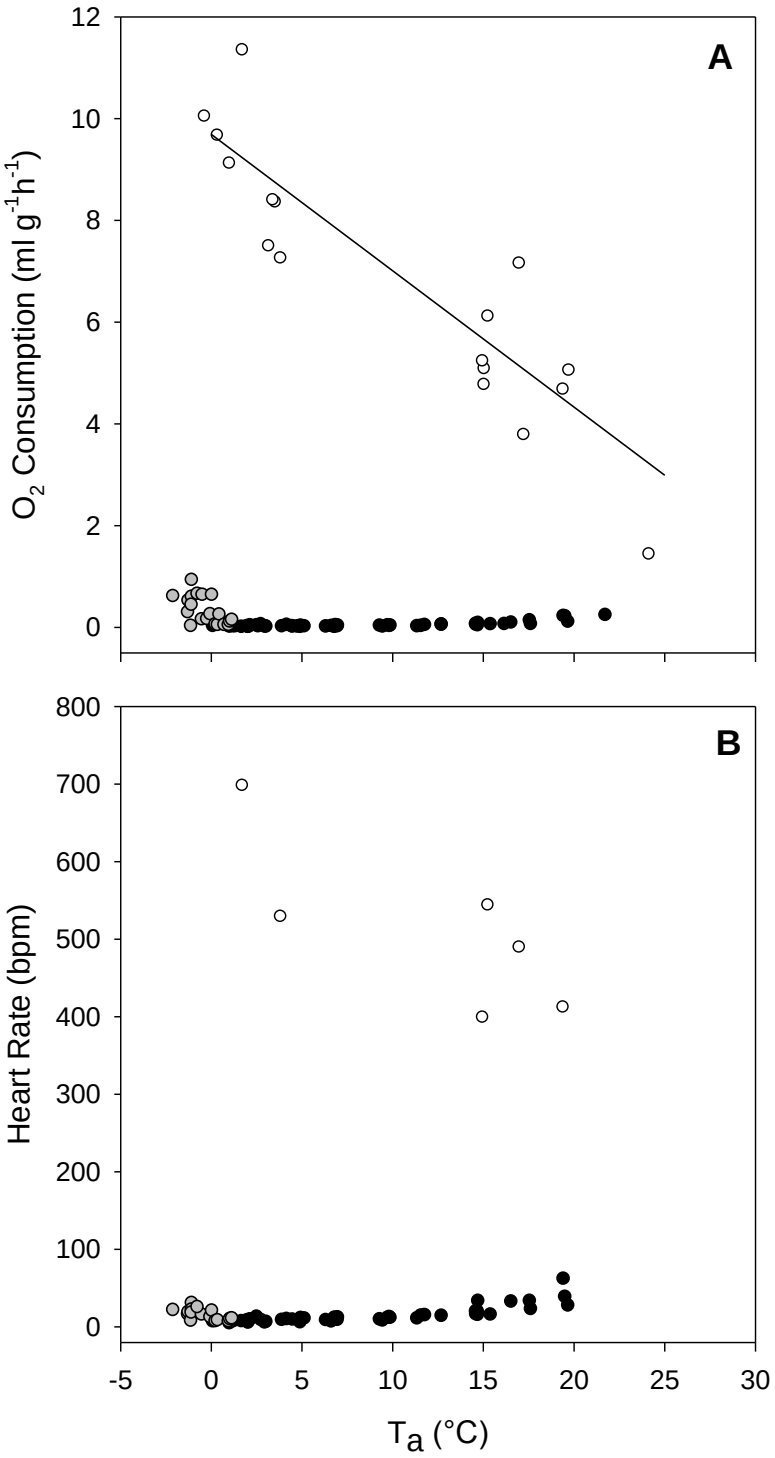

Figure 1. Oxygen consumption (A) and heart rate (B) as a function of ambient temperature (T_a) for *C. gouldii.* Data are presented for normothermic individuals at rest (white circles, solid line; $\dot{V}O_2 = 9.69 - 0.27(T_a)$, $r^2=0.91$, $p<0.001$; $\dot{V}O_2$ n=6, N= 17, HR n=3. N=6), and during torpor when thermoconforming (black circles; n=6, $\dot{V}O_2$ N=62, HR N=54) or thermoregulating (grey circles; n=6, $\dot{V}O_2$ N=18, HR N=15).

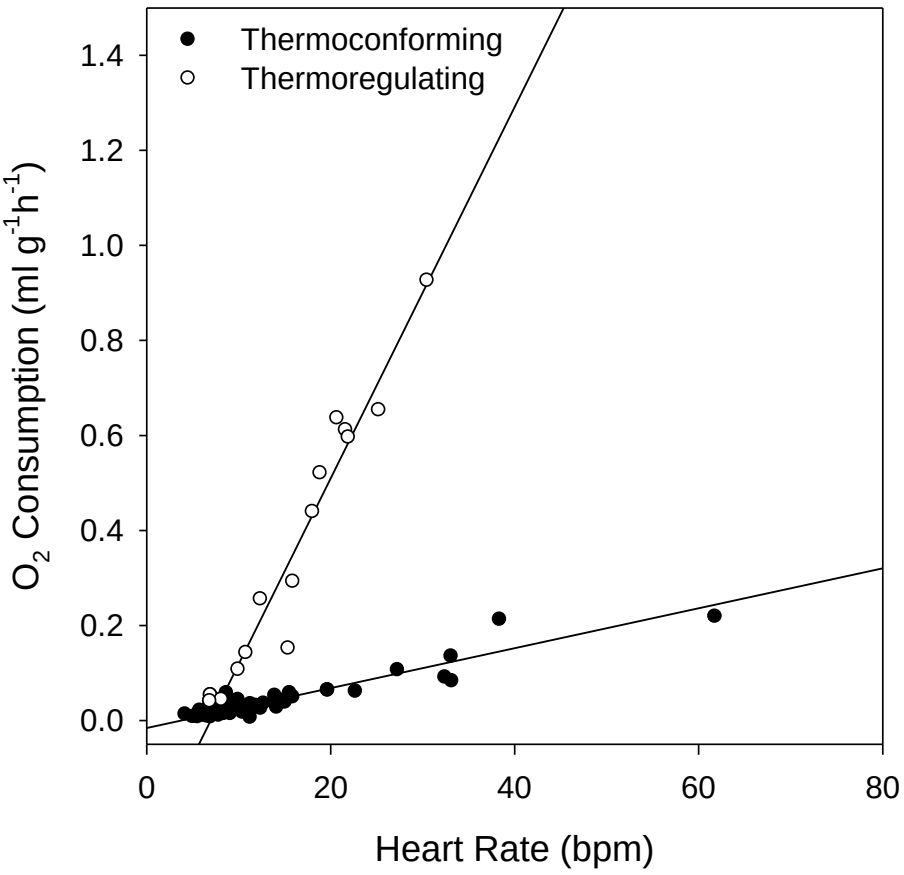

Figure 2. The relationship between heart rate (HR) and oxygen consumption in torpid *C. gouldii.* Data for thermoconforming individuals (black circles, solid line; $\dot{V}O_2$=0.004(HR) - 0.016, r^2=0.88, p<0.001; n=6, N=54) or thermoregulating individuals (white circles, dashed line; $\dot{V}O_2$=0.039(HR) - 0.272, r^2=0.95, p<0.001; n=6, N=15).

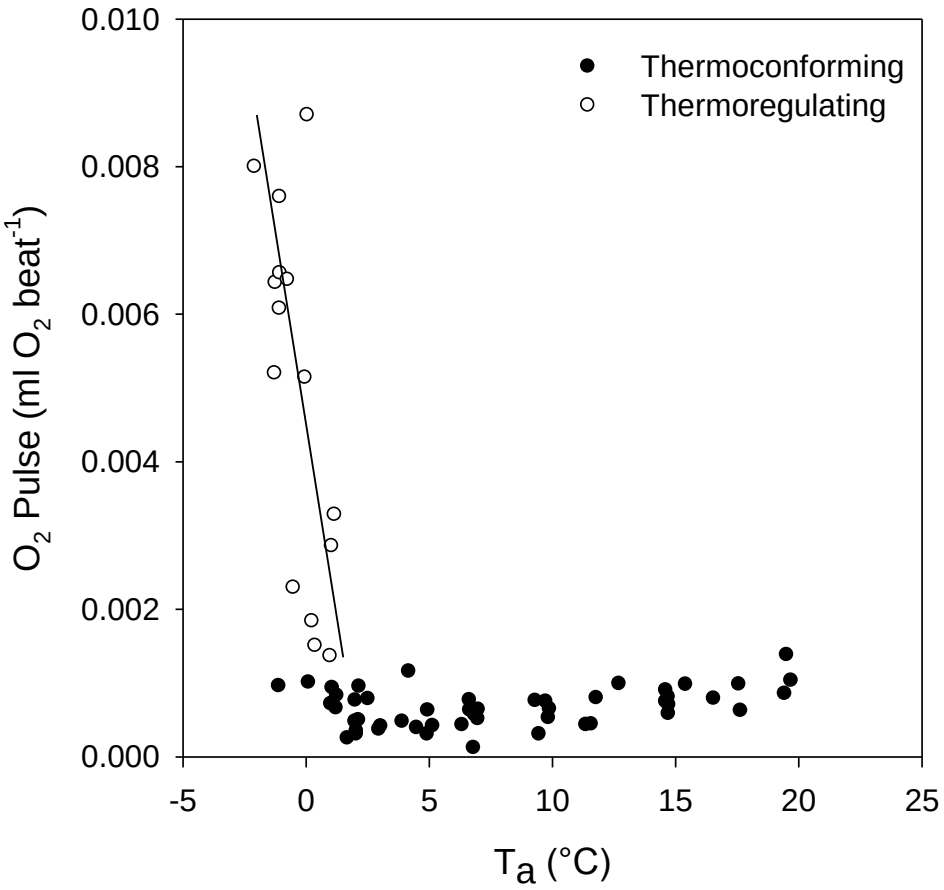

Figure 3. Oxygen pulse (OP) as a function of ambient temperature (T_a) in *C. gouldii* during torpor when thermoconforming (black circles; n=6, N=54) or thermoregulating (white circles; n=6, n=15). The relationship with T_a changes from curvilinear (thermoconforming) to linear when animals begin thermoregulating (solid line; OP = 0.0045-0.0021 (T_a), $r^2 = 0.87$, $p<0.001$)

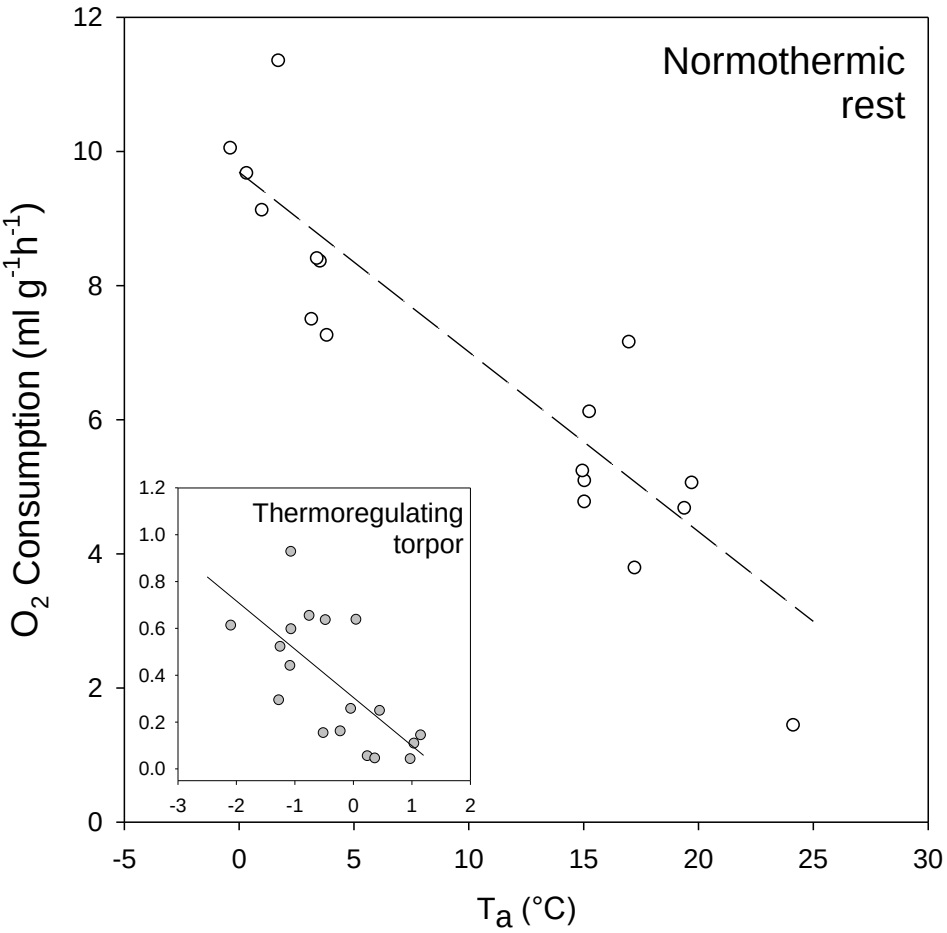

Figure 4. Oxygen consumption as a function of ambient temperature (T_a).

Thermoregulating *C. gouldii* at rest (white circles, dashed line; $\dot{V}O_2 = 9.69 - 0.27(T_a)$, $r^2=0.91$, $p<0.001$; n=6, N=17), or during torpor (inlaid plot: grey circles, solid line; $\dot{V}O_2 = 0.31 - 0.21(T_a)$, $r^2=0.62$, $p<0.01$; 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; $p<0.05$).

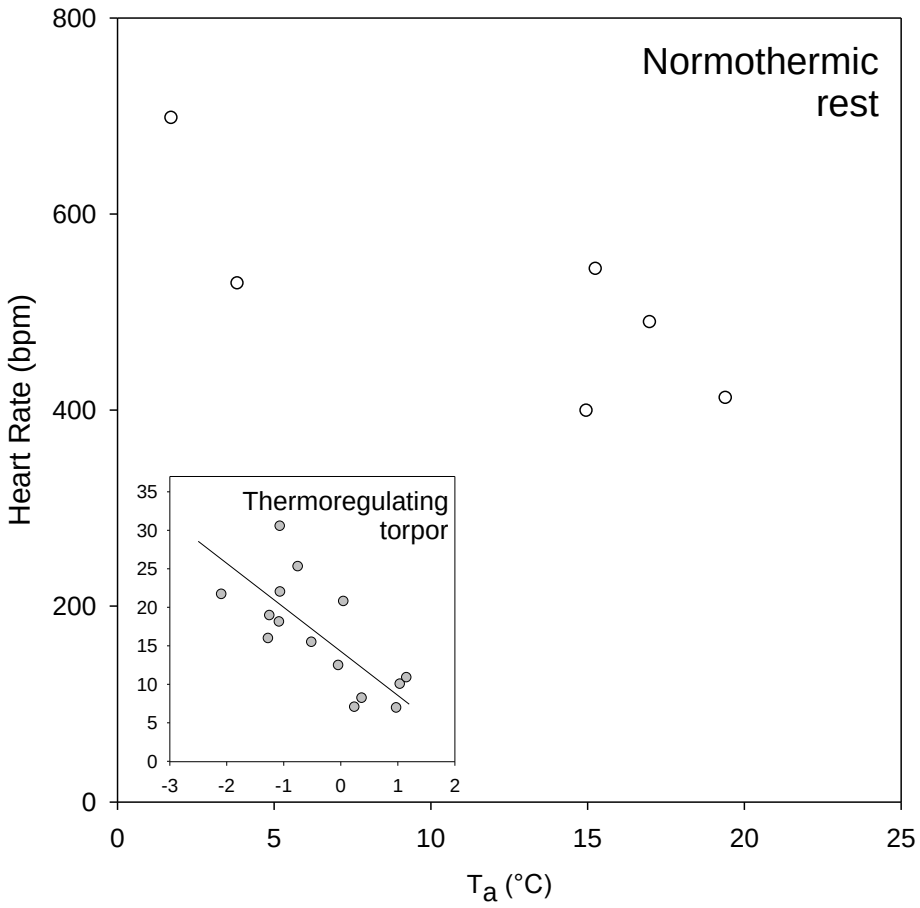

Figure 5. Heart rate as a function of ambient temperature (T_a) in thermoregulating *C. gouldii*. While normothermic at rest (white circles; n=3, N=6), or while thermoregulating during torpor (inlaid plot: grey circles, solid line; HR = 14.27-5.72(T_a), r^2=0.71, p<0.01; n=6, N=15).

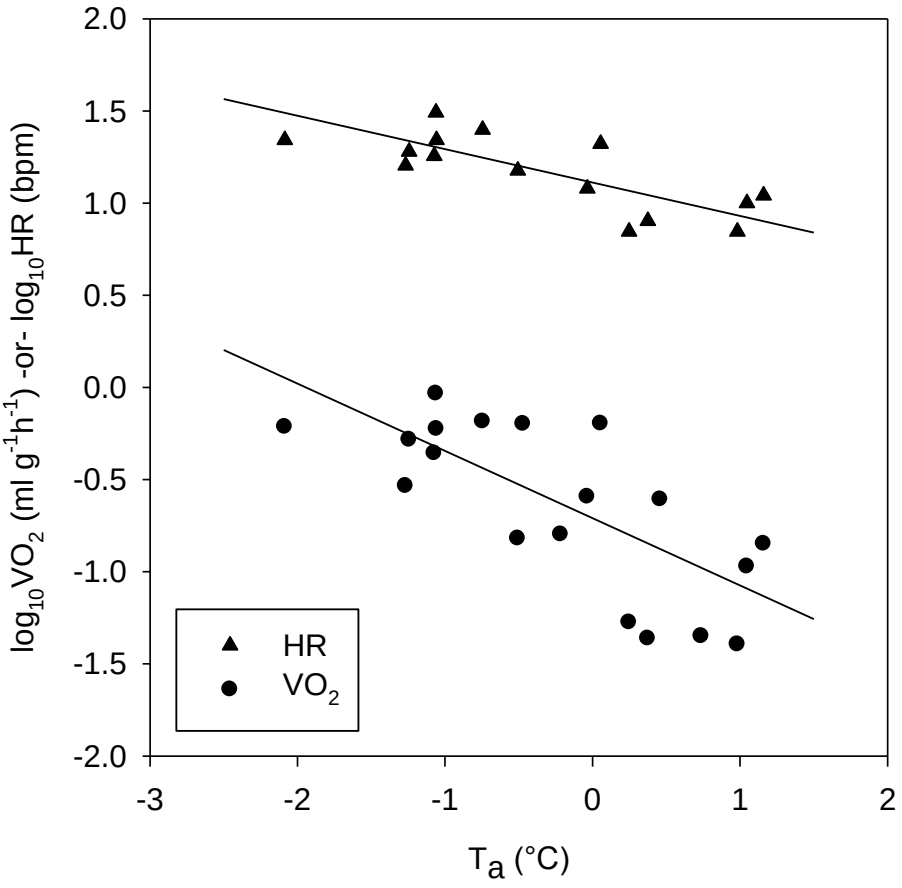

Figure 6. HR and $\dot{V}O_2$ as a function of T_a for thermoregulating bats during torpor. Log_{10} transformed HR (triangles, line; $log_{10}HR = 1.11 - 0.18(T_a)$, r2 = 0.82, $p<0.001$; n=6, N=15) and $\dot{V}O_2$ (circles, solid line; $log_{10}\dot{V}O2 = -0.71 - 0.37(T_a)$, r2=0.71, $p<0.001$; n=6, N=18) for thermoregulating torpid bats increased disproportionately with decreasing T_a. $\dot{V}O2$ increased at a greater rate than HR below 1°C.