

## RESEARCH ARTICLE

## Heart rate as a predictor of metabolic rate in heterothermic bats

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## ABSTRACT

While heart rate ( $f_H$ ) has been used as an indicator of energy expenditure, quantitative data showing the relationship between these variables are only available for normothermic animals. To determine whether  $f_H$  also predicts oxygen consumption ( $\dot{V}_{O_2}$ ) during torpor, we simultaneously measured  $\dot{V}_{O_2}$ ,  $f_H$  and subcutaneous body temperature ( $T_{sub}$ ) of a hibernator, Gould's long-eared bats (*Nyctophilus gouldi*, 9 g,  $N=18$ ), at ambient temperatures ( $T_a$ ) between 0 and 25°C. At rest,  $f_H$  of normothermic resting bats was negatively correlated with  $T_a$ , with maximum  $f_H$  of 803 beats  $min^{-1}$  ( $T_a=5^\circ C$ ). During torpor, the relationship between  $f_H$  and  $T_a$  was curvilinear, and at low  $T_{sub}$  ( $\sim 6^\circ C$ ),  $f_H$  fell to a minimum average of 8 beats  $min^{-1}$ . The minimum average values for both  $\dot{V}_{O_2}$  and  $f_H$  in torpor reported here were among the lowest recorded for bats. The relationship between  $f_H$  and  $\dot{V}_{O_2}$  was significant for both resting ( $r^2=0.64$ ,  $P<0.001$ ) and torpid bats ( $r^2=0.84$ ,  $P<0.001$ ), with no overlap between the two states. These variables were also significantly correlated ( $r^2=0.44$ ,  $P<0.001$ ) for entire torpor bouts. Moreover, estimates of  $\dot{V}_{O_2}$  from  $f_H$  did not differ significantly from measured values during the different physiological states. Our study is the first to investigate the accuracy of  $f_H$  as a predictor of  $\dot{V}_{O_2}$  during torpor and indicates the reliability of this method as a potential measure of energy expenditure in the field. Nevertheless,  $f_H$  should only be used to predict  $\dot{V}_{O_2}$  within the range of activities for which robust correlations have been established.

**KEY WORDS:** *Nyctophilus gouldi*, Hibernation, Normothermia, Metabolism, Torpor

## INTRODUCTION

Energy is essential for all life processes and therefore its appropriate use and acquisition are crucial for animals. Measurements of energy expenditure of free-ranging animals are of particular interest to ecological and evolutionary physiologists as they provide an understanding of how animals budget their energy. In captivity, energy use in endothermic animals is often quantified as basal metabolic rate (BMR) in resting animals under thermo-neutral conditions, and it is often assumed to be proportional to field metabolic rate (FMR). However, such extrapolations can misrepresent energy demands of individuals in the wild (Nagy, 1987; Koteja, 1991). Ambient temperature ( $T_a$ ), activity, reproductive status, resource availability and predator avoidance are just a few of the challenges faced by animals in their natural habitats that require appropriate adjustment of energy expenditure which are not likely to be represented by extrapolations from BMR. This is particularly evident in a species whose BMR is lower than expected based on size, and whose FMR is much higher than predicted (Geiser and Coburn, 1999).

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Received 24 October 2013; Accepted 8 January 2014

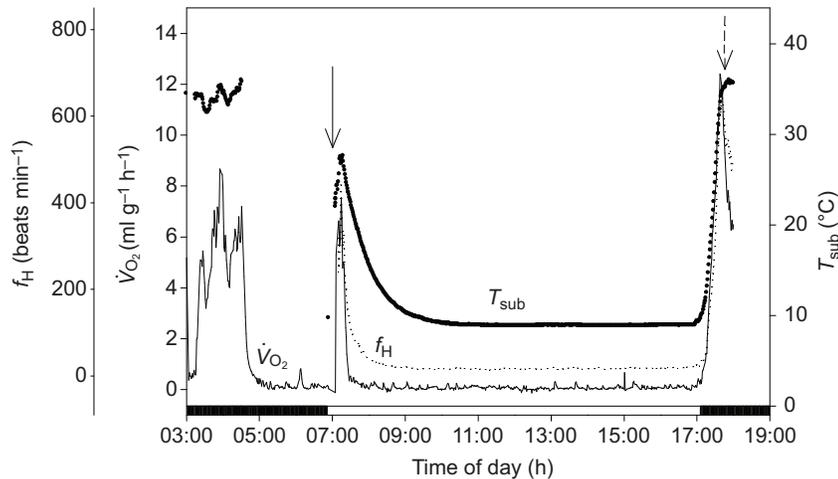
Currently, there are two widely used quantitative approaches for direct measurement of FMR that overcome these problems – the doubly labelled water (DLW) and heart rate ( $f_H$ ) methods (Nagy, 1987; Speakman, 1999; McCarron et al., 2001). The DLW method quantifies  $CO_2$  production over time by measuring the proportional washout of isotopically labelled oxygen and hydrogen from body water. It is generally used as a measure of daily energy expenditure (DEE) in the field and has been the most widely applied approach for measuring FMR. The high metabolic turnover and elusive nature of many small animals means that DLW measurements are often restricted to short time periods, reducing the robustness of the technique. Moreover, free-ranging small animals show enormous differences in energy expenditure between activity and rest, and these cannot be made apparent from average DEE.

An extreme example of temporal fluctuations in energy expenditure and body temperature ( $T_b$ ) is torpor, which is characterised by a controlled reduction in metabolism, often up to 1–10% of BMR (Geiser, 2004). Bouts of torpor can last for short periods of time <24 h in daily heterotherms or up to days or weeks in hibernators (Geiser and Ruf, 1995). Although the DLW method provides a valid measure of energy expenditure over time, this approach cannot reveal the pronounced short-term physiological changes, and metabolic savings, associated with heterothermy. For example, a relatively low FMR in insectivorous bats for their size (Nagy et al., 1999) only suggests that these bats may have used torpor during the period of sampling but cannot provide any further information regarding torpor use in the energy budgets of these animals.

The  $f_H$  method, in contrast, relies on the intrinsic relationship between oxygen consumption ( $\dot{V}_{O_2}$ ) and  $f_H$ , and can provide instantaneous and continuous measurements of metabolism over extended periods ( $\sim 1$  year) (McPhee et al., 2003). This permits comparison of energy expenditure across various activities and life stages and provides a more specific understanding of the various components of an animal's cost of living. Several validation studies comparing both the DLW and  $f_H$  method to standard respirometry show that  $f_H$  is as accurate a predictor of metabolic rate as the DLW method in homeothermic birds and mammals (Bevan et al., 1994; McCarron et al., 2001; Butler et al., 2004).

As the relationship between  $f_H$  and  $\dot{V}_{O_2}$  changes with exercise, it has been important to validate the method for a range of activities. Statistically significant relationships between  $f_H$  and  $\dot{V}_{O_2}$  have been demonstrated for normothermic animals whilst walking (Bevan et al., 1994), diving (Bevan and Butler, 1992), flying (Weimerskirch et al., 2000; Ward et al., 2002) and swimming (Nolet et al., 1992; McPhee et al., 2003), with  $f_H$  providing a more precise estimate of  $\dot{V}_{O_2}$  than the DLW method in some cases. Unfortunately, current data are mainly limited to large homeothermic mammals and birds. However, in recent years technological advancements have led to the development of small, lightweight devices for  $f_H$  telemetry, making measurements of  $f_H$  in small animals feasible (Dechmann et al., 2011).

Considering the dramatic changes in  $\dot{V}_{O_2}$  between rest and torpor, and the fact that more than half of all mammalian orders contain



**Fig. 1. Heart rate, oxygen consumption and subcutaneous temperature of *Nyctophilus gouldi* at ambient temperature of 10°C.** Representative heart rate ( $f_H$ ; dotted line), oxygen consumption ( $\dot{V}_{O_2}$ ; solid line) and subcutaneous temperature ( $T_{sub}$ ; filled circles – missing data are where the bat moved out of range of the scanner) data recorded at an ambient temperature ( $T_a$ ) of 10°C. The filled bar on the horizontal axis represents scotophase. The animal entered torpor in the early morning prior to lights on, exhibited a partial arousal associated with electrocardiogram (ECG) lead attachment (indicated by the solid arrow) and then proceeded to re-enter and remain in torpor until spontaneously arousing when the lights went off in the evening (indicated by the dashed arrow).

heterothermic species (Geiser, 2013), knowledge about whether the same relationship between  $\dot{V}_{O_2}$  and  $f_H$  applies is highly desirable. It is known that  $f_H$  decreases with metabolic rate during torpor, but whether it can be used to estimate energy expenditure has not been established. We therefore aimed to determine the accuracy of  $f_H$  as a measure of  $\dot{V}_{O_2}$  in long-eared bats *Nyctophilus gouldi* (Tomes 1858) during normothermia and torpor as a function of  $T_b$  and  $T_a$ . This insectivorous bat hibernates in temperate areas of Australia and spends a large proportion of its life in a state of torpor (Turbill and Geiser, 2008), but there are no data on FMR for the species. Additionally, we investigated the precision of the  $f_H$  method during entire torpor bouts, incorporating the transitional periods of torpor entry and arousal. Detailed knowledge of the relationship between  $\dot{V}_{O_2}$  and  $f_H$  is particularly important for the study of bats because their metabolism changes substantially between activity, rest and especially during torpor, which may be used throughout the year.

## RESULTS

All bats entered torpor overnight or in the early morning and, following a partial arousal associated with electrocardiogram (ECG) lead attachment,  $\dot{V}_{O_2}$  and  $f_H$  fell concurrently and reached steady-state minima when subcutaneous body temperature ( $T_{sub}$ ) had declined to within  $\sim 2^\circ\text{C}$  of minimum  $T_{sub}$  (Fig. 1). Disturbance did not affect minimum  $\dot{V}_{O_2}$  values (paired  $t$ -test;  $t=2.0478$ , d.f.=9,  $P>0.05$ ). At  $T_a$  below  $20^\circ\text{C}$ , bats remained torpid until shortly after lights off when  $\dot{V}_{O_2}$ ,  $f_H$  and  $T_{sub}$  increased, beginning with an increase in  $\dot{V}_{O_2}$  and  $f_H$  associated with evening arousal.

Mean resting  $f_H$  of normothermic bats was a linear function of  $T_a$  ( $r^2=0.82$ ) and increased with decreasing temperature from 228 to 706 beats  $\text{min}^{-1}$  at  $T_a$  between 25 and  $2^\circ\text{C}$  (Fig. 2). The corresponding mean resting  $\dot{V}_{O_2}$  ranged from 1.27 to  $11.18 \text{ ml g}^{-1} \text{ h}^{-1}$  (not shown). Normothermic resting  $T_{sub}$  was not affected by  $T_a$  and the mean was  $34.5 \pm 1.0^\circ\text{C}$  ( $N=8$ ). The maximum  $f_H$  recorded was 803 beats  $\text{min}^{-1}$  at a  $T_a$  of  $5^\circ\text{C}$ . During torpor,  $f_H$  was reduced curvilinearly with  $T_a$  to values as low as 3.5% of resting  $f_H$  at the same  $T_a$  (Fig. 2), while  $\dot{V}_{O_2}$  was reduced to  $\sim 1\%$  resting  $\dot{V}_{O_2}$  (not shown). The maximum average resting  $f_H$  was  $\sim 90$ -fold higher than the minimum average  $f_H$  in torpor ( $T_a$  below  $5^\circ\text{C}$ ).

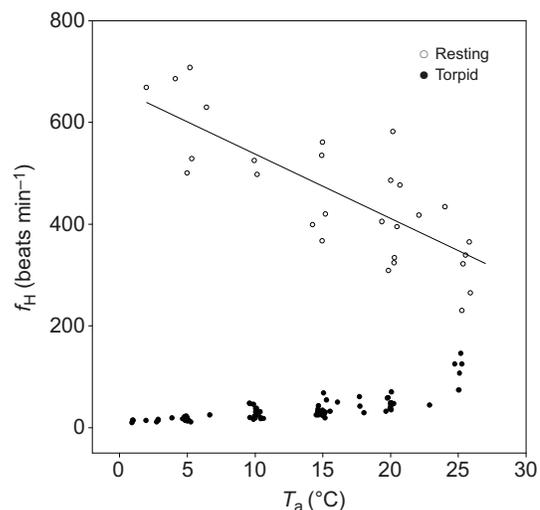
At  $T_a$  between 1 and  $25^\circ\text{C}$ , the mean  $f_H$  of torpid bats over 30 min ranged from 8 to 144 beats  $\text{min}^{-1}$  with corresponding  $\dot{V}_{O_2}$  from 0.02 to  $0.46 \text{ ml g}^{-1} \text{ h}^{-1}$ . Even at  $T_a$  of  $25^\circ\text{C}$ , mean  $f_H$  during torpor was only 35% that of normothermic bats.  $f_H$  during torpor was a curvilinear function of  $T_{sub}$  when plotted on a linear scale, with a  $Q_{10}$  of 2.0 (Fig. 3). Mean  $f_H$  of normothermic bats ranged from 1.3-fold

to 4-fold the values predicted by the extrapolated curve of torpid bats against  $T_{sub}$  (Fig. 3). The minimum  $f_H$  recorded in a torpid bat over 1 min was 5 beats  $\text{min}^{-1}$  at  $T_a=0^\circ\text{C}$ .

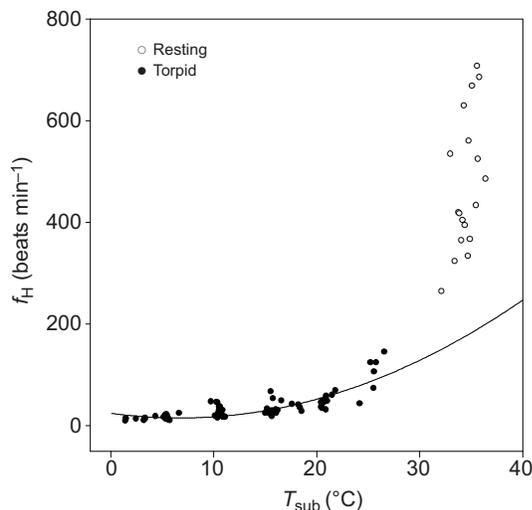
$\log_{10}$  transformation resulted in a linear function for both  $\dot{V}_{O_2}$  and  $f_H$  against  $T_{sub}$  during torpor (Fig. 4). The  $Q_{10}$  of  $\dot{V}_{O_2}$  for torpid bats (2.5) was similar to that for  $f_H$  (2.0), resulting in two near-parallel curves. The slopes of  $\log_{10}$ -transformed  $f_H$  and  $\dot{V}_{O_2}$  against  $T_{sub}$  during torpor did not differ significantly (ANCOVA;  $P>0.05$ ) (Fig. 4). As mean minimum  $f_H$  increased from 15 beats  $\text{min}^{-1}$  at  $T_{sub} 5.5^\circ\text{C}$  to 113 beats  $\text{min}^{-1}$  at  $T_{sub} 26^\circ\text{C}$ , mean minimum  $\dot{V}_{O_2}$  increased from 0.04 to  $0.40 \text{ ml g}^{-1} \text{ h}^{-1}$ . Consequently, the observed increase in  $f_H$  by  $\sim 100$  beats  $\text{min}^{-1}$  resulted in a 10-fold increase in  $\dot{V}_{O_2}$ .

$\dot{V}_{O_2}$  and  $f_H$  were strongly correlated at rest and during torpor (Fig. 5). However, extrapolation of the line derived from bats during torpor fell below values for normothermic bats and the slopes differed enormously (ANCOVA;  $P<0.01$ ) (Fig. 5). There was no overlap in recorded averages, with none of the normothermic points falling on the torpor regression and vice versa. The relationship between  $\dot{V}_{O_2}$  and  $f_H$  in resting normothermic bats is described by the equation:

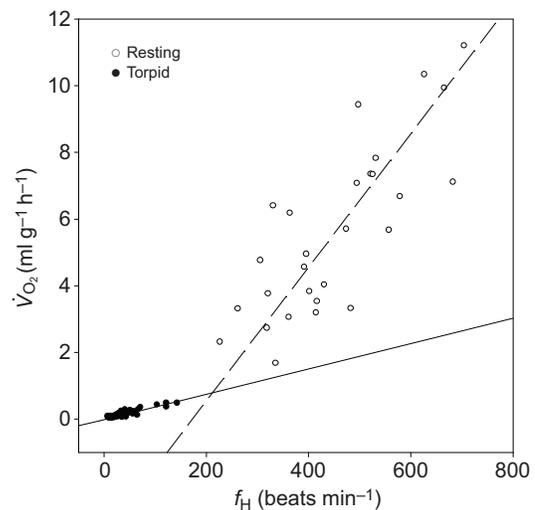
$$\dot{V}_{O_2} = 0.02f_H - 3.458 \quad (1)$$



**Fig. 2.  $f_H$  of normothermic resting and torpid *N. gouldi* as a function of  $T_a$ .** Each point represents an individual measurement taken from 18 individuals in total.  $f_H$  in resting normothermic bats increased linearly with decreasing  $T_a$ :  $f_H=664.8-12.68T_a$ ,  $r^2=0.82$ ,  $P<0.01$ .



**Fig. 3.**  $f_H$  of normothermic resting and torpid *N. Gouldi* as a function of  $T_{sub}$ .  $f_H$  increased in a curvilinear pattern with increasing  $T_{sub}$ ; however, the extrapolated curve for  $f_H$  data in torpid bats fell below values obtained for normothermic resting individuals.



**Fig. 5.**  $\dot{V}_{O_2}$  as a function of  $f_H$  in normothermic resting and torpid *N. Gouldi* at  $T_a$  between 1 and 25°C. Dashed line represents the regression equation for resting individuals ( $\dot{V}_{O_2}=0.02f_H-3.458$ ,  $r^2=0.46$ ,  $P<0.001$ ,  $n=34$ ); solid line represents the regression equation for torpid individuals ( $\dot{V}_{O_2}=0.004f_H-0.013$ ,  $r^2=0.84$ ,  $P<0.001$ ,  $n=74$ ).

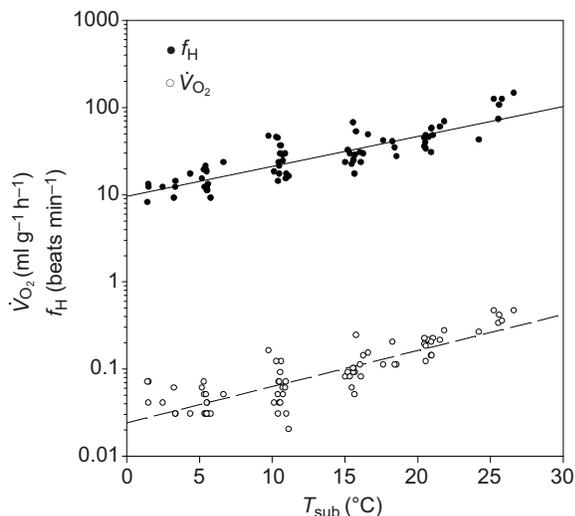
( $r^2=0.64$ ,  $P<0.001$ ) where  $f_H$  is in  $\text{beats min}^{-1}$  and  $\dot{V}_{O_2}$  is measured in  $\text{ml g}^{-1} \text{h}^{-1}$ . For each individual, estimates of resting  $\dot{V}_{O_2}$  did not differ significantly from direct measurements at the same  $f_H$  (paired  $t$ -test;  $t=0.33$ , d.f.=9,  $P=0.749$ ). This was calculated by sequentially removing data from one individual from Eqn 1 and recalculating the regression using data from the remaining bats. During torpor, the relationship between  $\dot{V}_{O_2}$  and  $f_H$  was described by the equation:

$$\dot{V}_{O_2} = 0.004f_H - 0.013 \quad (2)$$

( $r^2=0.84$ ,  $P<0.001$ ). There was also no significant difference between values estimated from modified versions of Eqn 2 and those measured directly at the same  $f_H$  (paired  $t$ -test;  $t=-1.6199$ , d.f.=17,  $P=0.124$ ).

## DISCUSSION

Our study is the first to provide continuous quantitative data on  $f_H$ , metabolic rate and  $T_{sub}$  simultaneously and as a function of  $T_a$  for a



**Fig. 4.** Mean  $f_H$  and  $\dot{V}_{O_2}$  of torpid bats plotted against  $T_{sub}$ . Note the logarithmic scale. Linear regressions are shown for  $\log_{10}f_H=0.03x+0.98$ ,  $r^2=0.81$  (solid line) and  $\log_{10}\dot{V}_{O_2}=0.04x-1.62$ ,  $r^2=0.72$  (dashed line).

microbat. We demonstrate a strong positive correlation between metabolism and cardiac function at rest and during torpor. The data suggest that  $f_H$  can be used to reliably quantify the energy expenditure of bats, at least during torpor and rest, in the wild.

Bats showed a strong proclivity to enter torpor in captivity and, despite disturbance associated with  $f_H$  measurements, exhibited similar temporal patterns of torpor use to those described for the same species in previous studies (Geiser and Brigham, 2000). In addition, our results showed that the relationship between  $\dot{V}_{O_2}$ ,  $f_H$  and  $T_{sub}$  as bats entered torpor progressed in a pattern qualitatively similar to other hibernators and daily heterotherms (Lyman, 1958; Swoap and Gutilla, 2009). The minimum mean values for both  $\dot{V}_{O_2}$  and  $f_H$  in torpor reported here were amongst the lowest recorded for bats. At  $T_a$  of 9–11°C, minimum  $\dot{V}_{O_2}$  of torpid bats was not significantly different from previous data for this species (Geiser and Brigham, 2000) (two-tailed  $t$ -test,  $t=0.778$ , d.f.=21,  $P>0.05$ ). Minimum mean  $f_H$  (8  $\text{beats min}^{-1}$ ) in particular was well below values reported for unrestrained northern hemisphere bats of a similar mass (40  $\text{beats min}^{-1}$ ) (Kulzer, 1967). Moreover, the absolute minimum of 5  $\text{beats min}^{-1}$  was similar to that measured in much larger hibernators such as woodchucks (*Marmota monax*, 3–5 kg) (Lyman, 1958) and dormice (*Glis glis*, ~150 g) (Elvert and Heldmaier, 2005).

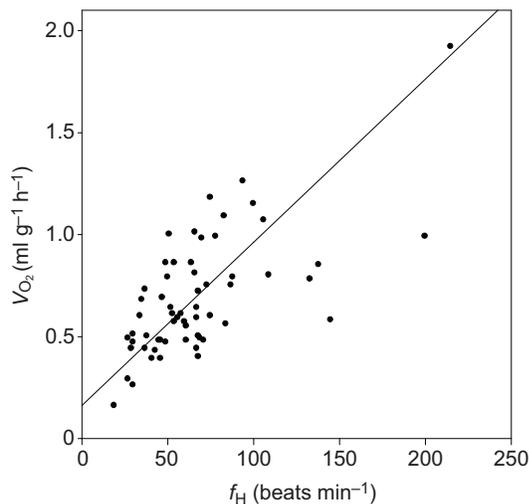
Interestingly, reported  $f_H$  values of ~40  $\text{beats min}^{-1}$  in other studies were similar to those of thermo-regulating individuals (bats that maintained a  $T_{sub}-T_a$  differential  $>2^\circ\text{C}$  when in torpor) at the same  $T_a$  in our study (not shown). This suggests that bats in previous investigations were not thermo-conforming and were not in steady-state torpor. It also indicates the need for simultaneous measurements of other physiological variables such as  $T_b$  to enhance the reliability of  $f_H$  data in torpor.

The maintenance of higher  $T_b-T_a$  differentials in thermo-regulating torpid bats will reduce the energy savings associated with torpor when compared with animals that are thermo-conforming at the same  $T_a$  because a higher differential results in a higher heat loss that needs to be compensated for. However small this difference may be, extended periods of time spent thermo-regulating during torpor will increase energy demands. In free-living bats, increased

energy expenditure associated with disturbance, including that caused by pathogens, has been suggested to deplete energy stores required for survival of the hibernation season, increasing mortality (Speakman et al., 1991; Thomas, 1995; Warnecke et al., 2013). Therefore, precise and detailed measurements of  $f_H$  for bats in different physiological states are required if the  $f_H$  method is to be used to quantify energy expenditure in the wild. To investigate this in free-ranging animals, measurements of temperature are required, both of the individual and of their surroundings, to enable better interpretation of the data and provide information regarding  $T_b-T_a$  differentials. It also has the potential to provide instant information remotely regarding natural disturbances of bats during torpor (i.e. perceived predation risks, etc.) and how often thermo-regulatory heat production is used in free-living animals.

We show that during steady-state torpor and at rest, the relationships between  $f_H$  and  $\dot{V}_{O_2}$  are strongly linear. However, this same relationship may not be maintained during more dynamic periods of torpor entry and arousal. During entry into torpor, peripheral blood flow is restricted and  $T_b$  declines in association with the change in  $T_b$  set point (Lyman et al., 1982). Although there is little change in blood pressure associated with the increased viscosity of cold blood,  $f_H$  and metabolism are actively suppressed as demonstrated by a high  $Q_{10}$  (Milsom et al., 1999; Geiser, 2004). As animals arouse from torpor, there is an enormous increase in  $\dot{V}_{O_2}$  and  $f_H$  required for increasing  $T_b$ . Associated with this is a decrease in blood viscosity and reperfusion of the peripheries and organs, which could alter the relationship between  $f_H$  and  $\dot{V}_{O_2}$ . We therefore investigated whether a strong linear correlation remains between  $\dot{V}_{O_2}$  and  $f_H$  when averaged across a complete torpor bout (i.e. from peak values after partial arousal before torpor to peak values following final arousal; Fig. 1). Our results show that the relationship between  $\dot{V}_{O_2}$  and  $f_H$  was still significant with the inclusion of torpor entry and arousal ( $r^2=0.44$ ,  $P<0.001$ ), regardless of time spent in torpor or whether  $T_a$  remained constant throughout a torpor bout (Fig. 6). This signifies the precision of  $f_H$  as a predictor of  $\dot{V}_{O_2}$ , not only during steady-state conditions but also throughout the transition between physiological states.

Nevertheless,  $f_H$  should only be used to predict  $\dot{V}_{O_2}$  within the range of activities for which robust correlations have been established (Nolet et al., 1992). This is of particular importance when studying



**Fig. 6.**  $\dot{V}_{O_2}$  as a function of  $f_H$  averaged for an entire torpor bout for *N. Gouldi* at  $T_a$  between 1 and 25°C. The torpor bout includes entry after partial arousal and final arousal; each point represents one bout. The regression equation was:  $\dot{V}_{O_2}=0.008f_H+0.163$ ,  $r^2=0.44$ ,  $P<0.001$ .

heterothermic animals, which, as we have demonstrated, display a distinct difference between resting and torpor regressions, with no overlap between the two states. At rest, both  $f_H$  and  $\dot{V}_{O_2}$  were related to  $T_a$  in a linear fashion but this became curvilinear when animals were in torpor. Not surprisingly, none of the values for torpor fell near the line derived from  $f_H$  against  $\dot{V}_{O_2}$  in normothermic resting bats. Our results support the findings of a previous study of metabolic rate reductions in hibernators (Song et al., 1997) and demonstrate that torpor is not just an extrapolated reduction of  $f_H$  and  $\dot{V}_{O_2}$  as a function of temperature differentials. Moreover, extrapolation from the regression of torpid bats underestimated resting  $\dot{V}_{O_2}$  by as much as 75%, emphasising the importance of determining correlations for different physiological states.

Essential to any study of energy expenditure in bats is an understanding of the physiological mechanisms and costs associated with flight. Flight is the most energetically expensive form of locomotion (Schmidt-Nielsen, 1972) and energy expenditure in small (5 g) flying bats has been shown to be >16 times higher than that at rest (Voigt and Lewanzik, 2012). Strong correlations between  $f_H$  and  $\dot{V}_{O_2}$  during flight have been reported for geese flying in a wind tunnel and this relationship differed significantly from that of walking geese, with no overlap between exercises (Ward et al., 2002). This illustrates that extrapolations from resting values may be grossly inaccurate for flying bats. In phyllostomid bats,  $f_H$  doubled at the onset of flight, while oxygen consumption increased 4-fold and both  $f_H$  and  $\dot{V}_{O_2}$  returned to resting levels within 30 s of landing (Thomas and Suthers, 1972), further indicating a need for calibration over fine time scales. As flight is essential for survival of all bats and constitutes the highest energetic demand on individuals, determination of correlations between  $f_H$  and  $\dot{V}_{O_2}$  during flight for bat species is essential before this method can be used to quantify energy expenditure in the field.

The DLW method can only provide average energy expenditure over time, with relatively low values in bats suggestive of torpor use (Nagy et al., 1999). A study on two small insectivorous bat species showed that a 5-fold range of average energy metabolism measurements could be generated using this method, when bats employed torpor to differing degrees (Speakman and Racey, 1988). Here, we show that regardless of torpor bout length there is a strong correlation between  $f_H$  and  $\dot{V}_{O_2}$  across a complete torpor bout and at rest, consistent with the potential for the  $f_H$  method to reliably measure field energy expenditure. Although it has been suggested in the past that the  $f_H$  method becomes prohibitively expensive when applied to animals smaller than 1 kg (Butler et al., 2004), miniaturised heart rate transmitters are becoming more readily available and can be used on animals as small as 10 g (Dechmann et al., 2011). Our study highlights the need for validation of this method for small heterothermic animals as torpor plays an important role in energy budgets for these animals and extrapolations from resting values are grossly inaccurate. This may also warrant the development of a torpor ‘cut-off method’ for  $f_H$  similar to those used for  $T_b$  or metabolic rate in most studies of heterothermy in free-living mammals and birds.

## MATERIALS AND METHODS

We used open-flow respirometry, ECGs and temperature-sensitive passive integrated transponders to measure the relationship between metabolic rate and  $f_H$  of long-eared bats (*N. Gouldi*) during torpor at a range of  $T_a$  (0–25°C). Measurements were conducted on a total of  $N=9$  female and  $N=9$  male *N. Gouldi* (mass at capture:  $10.5\pm 1.5$  g) from May to July 2011 and March to July 2012 (autumn/winter). Bats were captured in mist nests at Imbota Nature Reserve and Newholme Field Station near Armidale, NSW, Australia

(30°35'S, 151°44'E). Both field sites are temperate open woodland areas at ~1000 m elevation. Captured animals were transferred to the University of New England and kept in captivity for a maximum period of 7 months. Bats were kept in large outdoor flight cages with a maximum of eight animals per cage, and provided with mealworms and water *ad libitum*. Twice weekly, mealworms were dusted with a supplement of Wombaroo Insectivore Rearing Mix. Additional food was supplied in the form of moths and other flying insects, and these were attracted into cages by a UV light. Bats remained within 1 g of their body mass at the time of capture while in captivity.

This study was conducted under a scientific licence provided by the NSW Parks and Wildlife Authority (SL100084) and with Animal Ethics approval from the University of New England (AEC11-016).

### Transponder implantation

$T_{\text{sub}}$  was measured using temperature-sensitive transponders (IPTT-300 Bio Medic Data Systems Implantable Programmable Temperature Transponder, Seaford, DE, USA; 0.13 g, 14×2 mm) implanted interscapularly. For small mammals,  $T_{\text{sub}}$  is closely related to  $T_{\text{b}}$ , particularly during torpor when  $T_{\text{b}}-T_{\text{a}}$  differentials are often 1°C or less (Wacker et al., 2012). Transponders were calibrated over a range of 5 to 40°C to the nearest 0.1°C against a precision reference thermometer in a water bath prior to use.

Bats were given a minimum of 3 days to acclimate to captivity and ensure stable body mass before transponder implantation. Transponders were implanted under general isoflurane/oxygen anaesthesia. The skin was sterilised with 70% alcohol before a small (~3 mm) incision was made in the skin just below the shoulder blades for transponder insertion. The insertion site was closed with a single suture (chromic gut, Ethicon, Somerville, MA, USA) and the entire process was complete within 15 min. Bats were given 24 h to recover in a warm room before being returned to outdoor flight cages.

### Respirometry

Bats were placed in respirometry chambers in the early evening and metabolic rate, measured as oxygen consumption ( $\dot{V}_{\text{O}_2}$ ), was monitored overnight and throughout the following day(s) to allow animals to undergo their usual daily thermal cycle. Bats were weighed ( $\pm 0.1$  g) immediately prior to measurement and were removed from the chamber following arousal from torpor on subsequent days and reweighed. A linear rate of mass loss was assumed over each day to calculate mass-specific values.

Respirometry chambers were made from modified polycarbonate enclosures with clear lids (0.26, 0.40 or 0.53 l), lined with a small patch of hessian from which the bats could roost. Chambers were placed inside a temperature-controlled cabinet. Chamber size was randomised between measurements and the values obtained were not affected by chamber volume (ANOVA;  $P > 0.05$ ). The  $T_{\text{a}}$  ( $\pm 0.1^\circ\text{C}$ ) was recorded using a calibrated thermocouple placed 5 mm within the chamber and read using a digital thermometer. Air flow (165–230 ml min<sup>-1</sup>) was adjusted based on chamber size to ensure that 99% equilibrium was reached within <11 min, controlled with rotameters and measured with mass flowmeters (Omega FMA-5606; Stamford, CT, USA).

Oxygen concentration was measured in a constant temperature room to minimise drift using either Sable Systems FC-1B Oxygen Analyser or FOX Field Oxygen Analyser (Version 1.01, FXO301-01R). 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. Outputs of the digital thermocouple thermometer, flowmeter and oxygen analyser were recorded using custom-written data-acquisition software (G.K.) onto a personal computer. The  $\dot{V}_{\text{O}_2}$  was calculated using standardised gas volumes and eqn 3a of Withers (Withers, 1977). A respiratory quotient of 0.85 was assumed throughout.

$T_{\text{sub}}$  was read from each animal with a DAS-7006/7R/S Handheld Reader (Bio Medic Data Systems), which was connected to a personal computer and programmed to take readings every minute, concurrent with respirometry measurements. In addition,  $T_{\text{b}}$  was measured to the nearest 0.1°C by inserting a fine calibrated thermocouple probe 1.5–2 cm rectally. Rectal  $T_{\text{b}}$  was taken within 30 s of removal from respirometry chambers and compared with simultaneous readings of  $T_{\text{sub}}$ , with  $T_{\text{sub}} \leq 1.5^\circ\text{C}$  of  $T_{\text{b}}$  when animals were in torpor. Transponder function varied and on occasion transponders temporarily stopped working when the  $T_{\text{sub}}$  of animals in torpor fell below

7°C. In these cases,  $T_{\text{sub}}$  was estimated to be 0.5°C above  $T_{\text{a}}$ , as this was the average differential for animals with similar  $\dot{V}_{\text{O}_2}$  whose transponders continued to work at low  $T_{\text{a}}$ .

### ECGs

Measurements of  $f_{\text{H}}$  were recorded using established methods (Zosky, 2001). Individuals were placed in respirometry chambers in the evening and left until the following morning; when animals were torpid and  $\dot{V}_{\text{O}_2}$  reached steady-state values, ECG wires (lead I arrangement) were attached to adhesive electrodes on the bat's forearm just after lights on. This resulted in a partial arousal from torpor in most cases; however,  $\dot{V}_{\text{O}_2}$  soon returned to similar or lower values than prior to the disturbance and did not differ significantly (paired *t*-test;  $t=2.0478$ , d.f.=9,  $P > 0.05$ ). The data were therefore considered representative of steady-state torpor.

Electrodes were fashioned from Kendall Care Resting ECG Electrodes (Tyco Healthcare Group) cut into strips of appropriate length and width to fit the forearm of the bat. Lead wires were made from modified KittyCAT Paediatric Monitoring Electrodes (Tyco Healthcare Group) fitted with customised clips at one end. ECGs were measured using either a FE132 BioAmp or ML135 Dual BioAmp (ADInstruments) connected to a Powerlab 4/35 Data Acquisition System and recorded with LabChart Pro v7.3 software. ECGs were analysed to calculate instantaneous  $f_{\text{H}}$ , which was averaged per second using LabChart Pro v7.3 and exported to Microsoft Excel for further analysis.

### Statistical analyses

For the purpose of our study, only data for normothermic resting after arousal from torpor and thermo-conforming animals in steady-state torpor were used for regression analyses (bats that maintained a high  $T_{\text{sub}}-T_{\text{a}}$  differential when in torpor were considered to be thermo-regulating, and were excluded from analyses). Mean minimum values of  $\dot{V}_{\text{O}_2}$ ,  $f_{\text{H}}$  and  $T_{\text{sub}}$  during torpor were taken from times when all variables were lowest for at least 30 min. At  $T_{\text{a}}$  below 10°C, periods of apnoea were generally longer than 30 min (S.E.C., unpublished), and in such cases the sampling time was extended to 45 min to include representative periods of breathing to be able to estimate metabolic rate. Mean  $\dot{V}_{\text{O}_2}$  and  $f_{\text{H}}$  during torpor were calculated for thermo-conforming animals that entered torpor for <24 h exposed to constant  $T_{\text{a}}$ . Means were taken from peak values following partial arousals in the morning, to the peak following arousal from torpor in the afternoon or following lights out (see, for example, Fig. 1). On occasion, torpor lasted for >24 h or bats were exposed to more than one  $T_{\text{a}}$  during a torpor bout. Animals were exposed to a maximum of three different  $T_{\text{a}}$  values for  $\geq 1.5$  h each. The mean under these conditions fell within the range of values for animals exposed to only one temperature, and therefore all data were pooled for analysis.

The  $Q_{10}$  for  $\dot{V}_{\text{O}_2}$  and  $f_{\text{H}}$  of thermo-conforming torpid bats was calculated using the following equation:  $Q_{10} = (\text{value}_1/\text{value}_2)^{10/(T_{b1} - T_{b2})}$ . Values for resting  $\dot{V}_{\text{O}_2}$ ,  $f_{\text{H}}$  and  $T_{\text{sub}}$  were taken from the period following arousal. Because of impedance of the ECG associated with bat movement and/or individuals' intolerance of the electrodes, resting values could only be averaged over a 5 min period. Furthermore, following arousal from torpor, bats often moved out of range of the transponder scanner, which was ~5 cm, and therefore  $T_{\text{sub}}$  was occasionally unavailable. This resulted in more  $f_{\text{H}}$  values of resting normothermic bats against  $T_{\text{a}}$  than  $T_{\text{sub}}$ .

Statistical analyses were performed using R v2.15.2. Standardised major axis regressions were performed using the smatr package (Warton et al., 2012) and we accounted for pseudo-replication by using the degrees of freedom as for mixed effect linear modelling adjusted for repeated measures. Two sample *t*-tests were used to compare mean  $\dot{V}_{\text{O}_2}$  before and after disturbance associated with ECG lead attachment, and predicted  $\dot{V}_{\text{O}_2}$  values with measured values. Analysis of covariance (ANCOVA) was used to compare slopes of regression equations. Means are reported  $\pm$ s.d. for the number of measurements (*n*) and number of individuals (*N*).

### Acknowledgements

The authors thank Daniella Rojas, Artiom Bondarenko and Chris Wacker for their tireless help with animal care and experimentation, and Kodie Noy, Danielle Sisson and Kathryn Lambert for their help in the field.

**Competing interests**

The authors declare no competing financial interests.

**Author contributions**

All authors contributed to writing the paper and devising the study; S.E.C. collected and cared for the bats, carried out the experimental protocol and analysed the data. G.K. and F.G. were also involved in data analysis and initial design of the experimental protocol.

**Funding**

Financial support for this study was received from the Australian Research Council [DP130101506 to F.G].

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