Cardiorespiratory function and metabolism of heterothermic bats

Shannon E. Currie

(BSc Honours, University of Western Australia)

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Declaration

I certify that the contents of this thesis have not already been submitted for any other degree and are not currently being submitted for any other degree or qualification.

I verify that any assistance in preparation of this thesis, and all sources used, have been acknowledged.

Shannon Currie

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For my dearest Cody and the bats.

Bat, bat, come under my hat
and I'll give you a piece of bacon,
and when I bake, I'll give you a cake,
if I am not mistaken
-English Nursery Rhyme

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List of Abbreviations

BHR - basal heart rate

BMR - basal metabolic rate

BM - body mass

ECG - electrocardiogram

HR - heart rate

MR - metabolic rate

OP - oxygen pulse

PIT - passive integrated transponder

T_a - ambient temperature

T_b - body temperature

THR - torpor heart rate

TMR - torpor metabolic rate

TNZ - thermoneutral zone

T_{set} - set point temperature

T_{sk} - skin temperature

T_{sub} - subcutaneous temperature

T_{rec} - rectal temperature

RHR - resting heart rate

RMR - resting metabolic rate

$\dot{V}O_2$ - rate of oxygen consumption

VR - ventilation rate

Summary

Bats are fascinating in their ability to maintain coordinated cardiorespiratory function at the extremes of metabolism- during flight and torpor. As the only mammals capable of powered flight, bats have developed relatively large and highly efficient hearts and lungs compared to their non-flying counterparts. In addition to this expensive form of locomotion, bats must cope with high heat loss associated with their high surface area to volume ratios because of their small size. To balance these energetic challenges many bats are capable of entering torpor where metabolic rate (MR), cardiac function and body temperature (T_b) are substantially reduced.

Torpor use is essential to many bats, can occur almost daily in some species and is often expressed throughout the year. There are two forms of torpor exhibited by heterothermic mammals including bats; daily heterothermy, which consists of short term daily torpor <24h or longer multiday hibernation, which comprises longer multiday torpor bouts punctuated by periodic spontaneous arousals. Previous work on torpor physiology in bats has primarily been undertaken on the thermal energetics of northern hemisphere temperate zone insectivorous species that hibernate in thermally stable environments such as caves or houses. On the other hand, many tree-dwelling bats in Australia enter torpor in thermally labile roosts under tree bark or among exposed foliage and can experience large fluctuations in ambient temperature (T_a). Although the cardiorespiratory system is central to the coordination and maintenance of torpor, as its role in the circulation of blood gases and hormones directly impact on thermoregulation, to date there has been limited investigation of cardiac function in heterothermic bats. Therefore, I aimed to determine the patterns of cardiorespiratory function during torpor in two species of Australian bats that use either hibernation, Gould's long-eared bat (*Nyctophilus gouldi* Vespertilionidae), or daily torpor, common blossom bat (*Syconycteris australis* Pteropodidae). I also examined the influence of T_a on the relationships between heart rate (HR), ventilation rate (VR), oxygen consumption

($\dot{V}O_2$) and subcutaneous temperature (T_{sub}), either at a constant T_a or an increasing T_a profile.

My study provides the first quantitative data of HR as a function of temperature for a 'fruit bat' during torpor and the HRs I recorded in a small pteropodid bat as well as a hibernating vespertilionid insectivore were amongst the lowest reported. Qualitatively torpor was similar in both bat species; however the depth, duration and mechanisms reducing HR, MR and T_{sub} in torpor were substantially different between the daily heterotherm and hibernator. In both species there was a strong linear correlation between $\dot{V}O_2$ and HR during steady-state torpor and at rest, however, these relationships differed significantly between the species and thermoregulatory states. At all T_a and T_b tested, the hibernator *N. gouldi* maintained a substantially lower HR during torpor than the daily heterotherm *S. australis* and this was also true for $\dot{V}O_2$. During torpor, *N. gouldi* maintained a very small differential between T_{sub} and T_a and thermoconformed down to T_a around 5°C, while *S. australis* maintained a much higher average T_{sub}-T_a differential in torpor and only thermoconformed to a T_a of 15°C.

Interestingly, arousal from torpor did not differ significantly between the two species either for maximum rewarming rates or the time taken to arouse thus indicating the importance of a swift return to normothermy following torpor, regardless of torpor pattern. When *N. gouldi* were exposed to an increasing T_a to facilitate passive rewarming, HR and $\dot{V}O_2$ remained low over a large T_{sub} range and increased concurrently with increasing T_a (Q_{10} 2.4 and 2.5, respectively). During active arousals, mean HR and $\dot{V}O_2$ were considerably higher than during passive rewarming at corresponding T_{sub}. In addition, partial passive rewarming reduced the energetic cost of arousal from torpor by 53% compared to entirely endogenous arousal at constant low T_a. Therefore, I show that passive rewarming may contribute to minimizing exposure to oxidative stress as well as demands on the cardiovascular system by significantly reducing arousal costs and time taken to actively rewarm.

When *N. gouldi* entered torpor there was a distinct change in breathing pattern from evenly spaced breaths to intermittent breathing following an initial slowing of VR. Ventilation was confined to cycles of short breathing bouts, where VR was a linear function of T_a, interspersed by long apnoeas up to 74min. During torpor, electrical conduction through the heart in *N. gouldi*, slowed in a pattern similar to other heterothermic species. There was a curvilinear relationship between T_{sub} during torpor and cardiac conduction intervals in hibernating bats, however, prolongation of ventricular conduction was minimal. These animals also showed rapid ventricular repolarisation common to all other heterothermic mammals studied, and this was retained in torpor until T_{sub} fell below ~15°C. The retention of a close association between the different aspects of the ventricular conduction cycle is widely considered a cardio-protective mechanism, enabling animals to withstand low T_b without the development of detrimental arrhythmias. I observed only two clear atrial premature beats out of >2500h of electrocardiogram recording. This is certainly likely for hibernating *N. gouldi* and may also be the result of selection pressures related to the need for a swift transition to flight following arousal from torpor in bats.

In conclusion, my study is the first to provide detailed data for HR as a function of T_a in a chiropteran daily heterotherm compared to a hibernator. I have shown that there is a significant difference between cardiac and metabolic physiology between the two patterns of torpor at least for bats. In addition, I present the first data investigating the inter-relations between $\dot{V}O_2$, HR, VR and T_{sub} throughout all phases of torpor and as a function of constant T_a versus increasing T_a. Lastly, I confirm that the hearts of hibernating mammals have a specialised conduction system enabling them to withstand low T_bs indicative of torpor without significant arrhythmogenesis.

Chapter 1

Introduction and Aims

Animals are heterotrophic and must acquire essential macromolecules for growth, maintenance and energy by ingesting other organisms or their products. Ingested food is broken down and transported to cells where the nutrients animals are unable to synthesize by themselves are used for biosynthesis or to fuel energy metabolism through the production of high energy compounds such as adenosine triphosphate (ATP). It is essential for all animals to acquire enough energy to maintain vital processes at the cellular and organismal level throughout all phases of life. The rate at which animals can obtain and convert food to fuel is driven by temperature, both of the animal's body and its surroundings.

TEMPERATURE

Temperature is arguably the most influential factor affecting the functional properties of biological systems. The thermal environment significantly affects body temperature (T_b) of an animal and in turn its physiological processes. In general, biochemical reactions change with increasing temperature such that at low temperatures reaction rates are slow and increase exponentially with increasing temperature. This typically occurs at a rate of between two and three fold over a 10°C increment and is termed the Q_{10} (Withers, 1992; Schmidt-Nielsen, 1997). Within animals, increasing temperature affects the rate of oxygen uptake by tissues, diffusion of oxygen in tissues, speed of electrical conduction of the nervous system and these in turn affect the frequency of contraction and functional capacity of muscles and other tissues and the rate at which animals can acquire and process foods.

Heat transfer between an organism and its environment occurs through a number of avenues. Heat is gained either by radiation, or conduction and convection from a warmer

surface or fluid, while heat loss can occur via evaporation, convection or conduction with a cooler surface and radiation. Animals that obtain their heat primarily from external sources are ectothermic and their body temperature (T_b) is largely a function of the external environment. These animals must be able to deal with fluctuating T_b and tend to balance their heat loss and heat gain mainly through behavioural thermoregulation. Many terrestrial ectotherms are diurnal and use direct solar radiation as a primary means of raising T_b. By making appropriate postural adjustments and selecting thermally suitable microclimates within their habitats these animals are able to regulate T_b within a fairly narrow range over most of the day when they are active, but usually not during rest at night (Stevenson, 1985).

Alternatively, endothermic animals are capable of generating enough metabolic heat internally to maintain a high T_b over a wide range of ambient temperatures (T_a). Although behavioural thermoregulation remains important for endotherms, they are capable of more substantial physiological means of balancing heat than most ectotherms. Typical endotherms have substantially higher metabolic rates (MR) than those of similar sized ectotherms and this is further exacerbated at low T_a where the MR of a typical ectotherm is only a small fraction of that of a similar sized endotherm (Bennett and Ruben, 1979). Having a high and steady T_b regardless of T_a has numerous advantages, enabling endotherms to be active throughout both the day and night and leading to the habitation of a broad array of environments.

ENDOTHERMY- HOMEOTHERMY

Ideally, endothermic animals will maintain a stable optimal T_b for efficiency of bodily functions and a constant rate of biochemical processes (Tattersall et al., 2012). This is certainly the case for homeothermic endotherms that regulate T_b within a narrow range of only a few degrees by proportional adjustments of metabolism at T_as below the thermoneutral zone (TNZ), in which MR is basal (basal metabolic rate- BMR), to compensate for heat loss which is related to the T_b-T_a differential (Withers, 1992). In many homeothermic endotherms even a slight reduction of T_b can have catastrophic effects,

impeding the function of many biological systems, such as the cardiovascular system, and can result in mortality. The T_b of endothermic mammals and birds is essentially determined by their MR and thermal conductance over their body surface, which relate to body size and the insulation properties of fur and feathers. Although homeothermy generally ensures stability of the body systems, the maintenance of a constant high T_b requires significant amounts of fuel.

Considering that in many environments there is a large gradient between optimal endothermic T_b and T_a, the costs of maintaining this balance may prove prohibitively expensive for some animals when faced with unfavourable conditions. This is particularly true for small mammals and birds that require a high intake of energy to produce enough heat for maintenance of high T_b and must compensate for high heat loss due to large surface area to volume ratios. Throughout the year seasonal variations in T_a, food availability, rainfall and access to water have significant impacts on the ability of small animals to cope with the costs of daily living.

ENDOTHERMY- HETEROTHERMY

As a mechanism of energy conservation, many small mammals and birds are capable of entering torpor, which is a controlled physiological state during which energy expenditure and other processes are minimised. Torpor is characterised by a significant reduction in MR, heart rate (HR), ventilation rate (VR) and T_b (Lyman et al., 1982). During entrance into torpor the hypothalamic set point temperature (T_{set}) for thermoregulation is adjusted and declines until a minimal T_{set} that is also dependent on T_a, is reached in steady-state torpor (Heller et al., 1977). The down-regulation of T_{set} results in an initial fall in metabolic heat production to basal levels and further reductions in MR are related to temperature effects of low T_b on metabolic processes and possible metabolic inhibition (Geiser, 2004). During torpor, T_b tends to follow T_a in thermoconforming animals. It is not until T_a falls below the minimum T_{set} that animals will increase MR and thermoregulate to maintain T_b at or above this minimum T_{set} (Geiser, 2004). Entering this state not only eliminates the

high costs of keeping warm but also extends the life of limited energy reserves (Carpenter and Hixon, 1988; Koteja et al., 2001).

Historically the use of torpor was thought to be strictly confined to cold climates as a mechanism of energy conservation in animals that are thermally and energetically stressed. However, mounting evidence has shown that in many species torpor can be used throughout the year, largely regardless of thermal conditions, in tropical and subtropical climates. This has led to a broadening of our understanding of the functions of torpor (Geiser and Brigham, 2012). Although for the most part, energy conservation is an essential facet of torpor use, the frequency and depth of torpor bouts may also relate to water conservation, predator avoidance, and improvements in reproductive fitness. Torpor use has been reported during pregnancy in small marsupials *(Dasycercus cristicauda*; Geiser and Masters, 1994; *Sminthopsis macroura*; Geiser et al., 2005b), echidnas (*Tachyglossus aculeatus*; Morrow and Nicol, 2009), and many bats (Geiser et al., 2001; Willis et al., 2006) and in bats may be beneficial to delay parturition to periods when resources may be more abundant. Sperm storage in bats is prolonged in comparison to many other species and this is also likely to be facilitated by torpor use. The temporal displacement of reproductive events in these animals enables females to synchronize fertilization and therefore births with favourable conditions, regardless of the presence of male partners in spring, as well as allowing females to improve the quality of offspring via sperm-competition within the reproductive tract during sperm storage (Geiser and Brigham, 2012).

In arid zone species and animals living in regions with seasonal droughts, torpor has also been suggested as a strategy for hydroregulation, reducing evaporative water loss by upwards of 50% (Cooper et al., 2005), even down to unmeasurable levels (Withers et al., 1990). Water balance is clearly important for all terrestrial animals and has even been suggested as a driving factor behind the increased frequency of arousals during the hibernation season that has resulted in the mortality of hundreds of thousands of bats with white-nose syndrome (Willis et al., 2011). Survival rates are comparatively high in

heterothermic animals not only because they are able to survive poor quality/seasonal environments, but the use of torpor also reduces exposure to predation as animals are inactive for significant portion of the day/year (Turbill et al., 2011). Frequent torpor use when conditions are favourable is common in some heterothermic mammals and it is possible that reduced activity is related to predator avoidance (Bieber and Ruf, 2009; Stawski and Geiser, 2010), which is in turn reflected in the longevity of many heterothermic species.

DAILY TORPOR VERSUS HIBERNATION

There are two major forms of torpor expressed by heterothermic mammals; daily short-term torpor in the daily heterotherms, or longer multiday, often seasonal torpor in the hibernators. Daily torpor is a short-term reduction in T_b, MR, HR and respiration usually lasting only a few hours, always less than 24hrs. Daily heterotherms generally reduce T_b to moderate temperatures (>10°C) with a reduction of MR to approximately 30% of BMR (Ruf and Geiser, 2014). On the other hand, hibernation can last for months over which the animal enters multiday torpor bouts of up to a few weeks, punctuated by periodic spontaneous arousals, followed by periods of 'normothermia'. Animals capable of hibernation can maintain a very low T_b in torpor (as low as -2.9°C; Barnes, 1989) with a reduction of metabolism significantly lower than daily heterotherms at 5% of BMR on average and approaching 1% of BMR in small species (Geiser, 2004).

Many heterotherms can simply be categorized into one of these two groups as their torpor patterns are distinct and consistent. Small dasyurids (carnivorous marsupials), for example, are strict daily heterotherms and, because they require the ability to forage regularly, only exhibit short, shallow daily torpor. Within this entire family no hibernation has been recorded (Riek and Geiser, 2014). Conversely, obligate seasonal hibernators such as temperate zone/arctic rodents have no option to forage during winter as food is not available at all and short-term bouts of torpor, apart from those early and late in the hibernation season, usually only occur in the laboratory under mild ambient conditions.

However, it remains controversial whether daily torpor and hibernation differ physiologically or simply represent a temporal prolongation of the same process (for review see; Lyman et al., 1982; Ruf and Geiser, 2014). Much of the controversy surrounding the distinction between torpor patterns is the result of (often unintentional) misuse of the terminology attributed to either form of torpor related to the duration of torpor bouts (e.g. 'daily torpor' is often used to describe any torpor bout that last less than one day). This confusion stems from animals that appear to blur the lines between the two patterns and may express short term bouts of torpor in nature or the laboratory, but are capable of hibernation (McKechnie and Mzilikazi, 2011). In many of these animals, such as the eastern pygmy possum (*Cercartetus nanus*), T_b reduction and MR during torpor <24hrs is often indistinguishable from reductions during multiday hibernation and is often much lower than values measured for similar sized daily heterotherms under similar thermal conditions (Song et al., 1997). In addition many bat species that hibernate over winter will use short term bouts of torpor throughout the rest of the year that have been referred to as 'daily torpor' (Kurta and Kunz, 1988). However, the short-term torpor bouts expressed by these animals are not reflective of the daily torpor exhibited by daily heterotherms, but simply a short bout of hibernation (Geiser and Brigham, 2000). The importance of distinguishing the physiological capacities of these groups is pertinent to the general understanding of their evolution and ecology as well as how they may budget energy in the wild, and has direct implications to their appropriate management and conservation.

A recent update of a review of daily torpor and hibernation included a much broader species range with a large geographic distribution incorporating phylogenetic analyses to assess the distinctions between torpor use in 214 species of mammals and birds (Ruf and Geiser, 2014). This analysis showed that minimum T_b alone was not always effective as a means of distinguishing between the two torpor patterns, whereas the minimum MR during torpor (TMR) and torpor bout duration was. Further, a combination of temporal and metabolic variables resulted in a clear distinction between daily heterotherms and

hibernators, even when accounting for phylogeny. To date however, other physiological variables such as the cardiovascular system have not been incorporated into these studies, even though it is likely that its function differs profoundly between the two patterns of torpor.

CARDIOVASCULAR CHANGES DURING TORPOR

Regardless of the delineation of torpor patterns, the cardiovascular system exhibits profound alterations when animals enter torpor. Considering the vital role of the heart for distribution of blood gases and perfusion of essential organs throughout all phases of an animal's life, it is important to understand how this organ functions throughout entrance and arousal from torpor, as well as its role in thermoregulation and metabolic maintenance during steady-state conditions at low T_b.

Animals capable of entering and arousing from states of low T_b indicative of torpor have intrinsically specialised tissues. For example, it has been well documented that there is an inherent distinction between the functional capacity of myocardium of animals capable of torpor and typical homeotherms (Smith and Katzung, 1966; South and Jacobs, 1973; Kamm et al., 1979; Caprette and Senturia, 1984; Burlington and Darvish, 1988; Johansson, 1996; van Veen et al., 2008). Both in vitro and in vivo studies show that heterothermic mammals maintain coordinated function of the heart down to temperatures approaching 0°C; while the hearts of typical homeotherms become arrhythmic and/or fibrillate and cease to function at temperatures between 10°C and 15°C (Dawe and Morrison, 1955; Lyman and Blinks, 1959) and even at ~24°C in humans (Mallet, 2002).

In homeotherms increased excitability of cardiac myocytes with reductions in temperature stimulates the onset of ventricular fibrillation (Nielsen and Owman, 1968) which generally progresses as a breakdown in the cardiac conduction system resulting in either excitation of ventricles via random pathways or organized spinning electrical pathways called 'rotors' (Noujaim et al., 2007). In contrast, the hearts of heterothermic animals are resistant to discontinuity of the cardiac conduction system, not only associated with low temperatures

but also when induced by pharmacological and physical means that result in fibrillation in non-hibernators. The conduction velocity of the hibernator heart is rapid and remains relatively unchanged with reduction in temperature compared to the hearts of non-hibernators (Duker et al., 1983; Federov et al., 2005) and the propagation of papillary action potentials show no plateau period when the heart is cooled (Svensson et al., 1988). This has been suggested to be supported by the up-regulation of gap junction proteins such as connexin43 and connexin45 (Saitongdee et al., 2000; Federov et al., 2005). Furthermore, the optimal temperatures for cardiac contraction are often much lower in hibernators than non-hibernators at around 15-20°C compared with 31°C (South and Jacobs, 1973).

In addition to an improved capacity of cardiac muscle tissue and conductive systems, adequate cardiovascular function for perfusion of essential organs under the conditions of reduced flow and low T_b during torpor requires the maintenance of adequate blood properties such as oxygen carrying capacity and viscosity (Maclean, 1981). At low T_bs (<20°C) blood viscosity in hibernators has been shown to be substantially lower than non-hibernators (Maclean, 1981). This is due to intrinsic differences between the blood serum and plasma of hibernators and non-hibernators which result in reduced agglutination of red cells (Spurrier and Dawe, 1973) and a resistance to increased blood pH at low temperatures. Red blood cells of hibernators are also resistant to deformation and haemolysis (Spurrier and Dawe, 1973).

Entry into torpor

As animals enter into torpor heart rate (HR) falls before any discernible drop in T_b and this coincides with a general slowing of ventilation rate (VR) and/or a change to an episodic breathing pattern which occurs in parallel to reductions in MR (Landau and Dawe, 1958; Elvert and Heldmaier, 2005). In concert with the reduction in HR is a considerable drop in blood pressure and a redistribution of blood flow via differential regional vasoconstriction. Peripheral vascular resistance increases due to constriction of vascular beds that restrict circulation to the extremities as well as the increase in viscosity of cold blood (Lyman and

O'Brien, 1960; Swoap and Gutilla, 2009). The observed decrease in peripheral blood flow also contributes to reductions in thermal conductance as animals enter torpor, which may slow the rate of cooling. Perfusion of critical organs such as the heart, brain and lungs is maintained throughout this period while blood is shunted away from nonessential systems such as the digestive tract (Rauch and Beatty, 1975).

The initial slowing of the heart can be extreme and is driven by nervous input rather than simply T_b decline, as shown in the California ground squirrel (*Citellus beechyi*) where HR fell by 50% with only a 1°C decrease in T_b at the beginning of torpor entry (Strumwasser, 1959). In addition, HR is much slower at any given T_b throughout torpor entry in undisturbed animals compared with hypothermic individuals (Lyman, 1958). Hypothermia in endotherms is a state physiologically distinct from torpor as it is the result of an animal's inability to maintain metabolic heat production, and in many cases in the literature is induced by anaesthesia (Geiser et al., 2014). In general during entrance into torpor HR is initially reduced by an extension of the period between beats and this may occur in a regular or arrhythmic pattern (Twente and Twente, 1978). During this phase HR often oscillates between bradycardic and tachycardic rhythms which may or may not be associated with ventilatory episodes and are driven by cyclical activation of the parasympathetic and sympathetic nervous systems (Lyman, 1982a; Milsom et al., 1999). In contrast, in non-hibernating animals during induced hypothermia the HR slows in a regular rhythm during cooling, suggesting that the controlled arrhythmic pattern of HR typical of early stage entry into torpor in large hibernators is not simply an effect of reduced MR or T_b alone (Twente and Twente, 1978; Milsom et al., 2001). During entry into torpor, the addition of atropine, a parasympathetic blockade, results in a rapid increase in HR to almost double that of undisturbed individuals and return to regular HR rhythm indicating the important role of vagal tone to slow the heart, at least in the early stages of entry (Lyman and O'Brien, 1963). Animals atropinized after entry into torpor has already begun are often able to continue into torpor, however atropine administration prior to torpor entrance impedes complete entry into hibernation (Lyman, 1982a). As entrance

into torpor progresses, the role of the vagus on tachy-brady rhythms is proportionately reduced with the fall in T_b until animals reach the extremely slow HRs indicative of steady-state torpor (Milsom et al., 2001). Hence, HR becomes a function of low T_b as animals reach steady-state conditions.

Steady-state torpor

When animals are in steady-state torpor, HR, MR and T_b have reached minimum values and remain low and fairly stable for the duration of the torpor bout. HR falls to ≤10bpm in most hibernators (for review see; Johansson, 1967; Lyman, 1982b) while the few daily heterotherms that have been studied retained a much higher HR of around 70bpm during torpor (Morhardt, 1970; Zosky, 2002; Mertens et al., 2008; Swoap and Gutilla, 2009). Cardiac output is dramatically reduced in torpor yet adequate supply of oxygen is maintained due to the low metabolism of tissues. In hibernating thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) cardiac output fell from an average of 69ml min^{-1} at rest to around 1ml min^{-1} at T_b of 6°C (Popovic, 1964).

There is also a reduction in the number of circulating red blood cells when animals are torpid, which is thought to be the result of considerable storage of erythrocytes in the spleen (Krutzsch and Hughes, 1959; Spurrier and Dawe, 1973). Differential vasoconstriction in torpor maintains an increase in peripheral resistance and relatively high diastolic pressures compared to active animals, around 40mmHg throughout a torpor bout (Lyman, 1982b; Swoap and Gutilla, 2009). This also results in significantly greater perfusion of the heart per unit weight when compared to active animals (Wells, 1971).

The role of the autonomic nervous system in controlling the heart during torpor at low T_b, remains enigmatic as results from different hibernating animals show large variability. As previously mentioned, once animals are in steady-state torpor modulation of HR is to a large extent likely a direct function of low T_b and metabolism. However during torpor homeostatic mechanisms are still active and evidence suggests that differential influences of the parasympathetic and/or sympathetic systems may still directly influence the

cardiovascular system. Some studies suggest that both stems of the autonomic nervous system are active, but their action is proportionately slow at low T_b (Twente and Twente, 1978; Milsom et al., 1993). Support for the role of parasympathetic tone during torpor comes from studies where infusion of atropine or vagotomy resulted in cardio-acceleration and a cessation of cardiorespiratory association (Twente and Twente, 1978; Milsom et al., 1993; Zosky and Larcombe, 2003). However, a reduced ability to elicit a response from the parasympathetic system in other studies has led to the conclusion that sympathetic tone is the primary control of the heart during torpor (Lyman and O'Brien, 1960; Lyman and O'Brien, 1963). In addition the stimulation of adrenergic receptors in hibernators resulted in increased HR and, when the sympathetic system was blocked, peripheral resistance, blood pressure and HR all declined (Lyman, 1982b; Senturia et al., 1986). The length of time spent torpid and depth of a torpor bout is also likely to impact the sensitivity of the system and the activity of control mechanisms. For example, the cardiorespiratory association evident in episodic breathers disappears after multiple days in torpor at low T_b (~5°C) (Milsom et al., 2001). This also indicates that there are likely broad inter-specific differences in cardiorespiratory function and control between animals capable of hibernation and those only able to enter short bouts of daily torpor.

Arousal from torpor

During rewarming from torpor an animal must increase T_b, often by more than 25°C, to return to a state of normothermy. Firstly HR and MR increase dramatically driven by increased sympathetic activation and this occurs prior to the rise in T_b. At this time any episodic breathing pattern ceases and VR increases along with HR and MR (Milsom et al., 2001). Sympathetic blockade halts the arousal process by slowing HR and preventing any rise in T_b (Chatfield and Lyman, 1950) and this system is so essential to torpor and the rewarming process that, at least placental animals without an intact sympathetic nervous system are unable to enter torpor (Swoap and Weinshenker, 2008; Braulke and Heldmaier, 2010). Once the arousal process is underway animals can rewarm in a matter of hours or even minutes depending on body mass and T_a (Geiser and Baudinette, 1990).

As arousal progresses blood pressure also increases, generally reaching a peak prior to peak HR and this can occur early in arousal (Chatfield and Lyman, 1950; Kirkebö, 1968). Blood flow remains restricted to the anterior portion of the body in most hibernators during the initial phase of rewarming and perfusion of the brown adipose tissue is dramatically increased in placental mammals as this provides an essential source of heat (Bullard and Funkhouser, 1962; Hayward and Ball, 1966; Rauch and Hayward, 1970). The heart is crucial to this phase not only ensuring adequate blood flow as the oxygen requirements of tissues increase, but also as a source of heat (Burlington et al., 1972; Lyman, 1982c). Regional restrictions of circulation enable the rewarming and sequential reperfusion of primary organs with the gut and skin the last to receive warm blood (Rauch, 1973; Kurtz et al., 2006) and this vasoconstriction is important to ensure a swift return to high T_b (Lyman and O'Brien, 1963). Towards the end of an arousal episode there is an 'overshoot' of HR, peaking at around 200bpm faster than the resting HR of normothermic individuals in a steady-state. Around this time, peripheral resistance falls as vasodilation returns a normothermic blood flow to the entire body and this in turn results in a lowered blood pressure, returning the cardiovascular system to a state typical of normothermy (Lyman and O'Brien, 1960).

PREVIOUS WORK

Traditionally, information regarding torpor variables has been gathered from laboratory experiments often involving captive animals. Unfortunately, torpor use in nature is often underestimated in these cases (Geiser et al., 2000) and many animals that are heterothermic in the wild were originally described as strict homeotherms in laboratory studies. Heterothermic animals are very sensitive to disturbance and while previous work has obviously made a significant contribution to the understanding of cardiac function during torpor, there are some potential short-comings mainly in regard to available technology/techniques. This is particularly true for measurements of cardiovascular physiology where there appears to be a trade-off between signal quality and the invasiveness of procedures or the need for restraint of animals, which likely induces

stress and impedes the quality of results. With the advent of miniaturized transmitters and transponders for recording and transmission of HR and/or electrocardiogram (ECG) signal and T_b we now have the opportunity to measure cardiac function in small animals undisturbed in captivity, as well as free-ranging individuals. New information regarding electrophysiological changes during daily torpor which has been previously difficult, have been reported in undisturbed Djungarian hamsters (*Phodopus sungorus*) and laboratory mice (*Mus musculus*) using implantable ECG transmitters (Mertens et al., 2008; Swoap and Gutilla, 2009). Swoap and Gutilla (2009) were also the first to record blood-pressure during torpor in almost half a century and provide the first evidence of blood-pressure alterations associated with shallow torpor. These studies illustrate the importance of reducing stress and disturbance of animals by measuring them unencumbered.

Historically, studies of cardiac function in hibernators have been reductionist in nature with many studies examining the effect of temperature on isolated hearts or myocardial tissue (eg; Clark, 1920; O'Shea, 1987; Geiser et al., 1989). While this technique of investigation has demonstrated innate differences between hibernators and non-hibernators, the findings may not be entirely indicative of physiological function within the intact animal. Adolph (1951) demonstrated the limitations associated with application of in vitro studies, particularly with regard to cardiac physiology, showing that isolated hearts from the rat (*Rattus norvegicus*) and cat (*Felis catus*) beat comparatively slower than in the intact animal. Furthermore, Smith and Katzung (1966) showed that the physiological state of the animal when tissue samples are taken may impact results, with myocardium from hamsters that were not hibernating demonstrably less tolerant of low temperatures than tissue from hibernating specimens. Another likely problem from the past relates to many previous studies of tissue isolations from torpid animals which involved surgery undertaken without anaesthesia (eg; Krutzsch and Hughes, 1959; Duker et al., 1983). Therefore it is likely that the painful disturbance of surgery would have initiated the arousal process and may have confounded the results.

Moreover the studies that have been undertaken on anaesthetised animals used anaesthetic drugs that have been shown to produce significant physiological side-effects (Cox, 1972; Graf et al., 1995). For instance, when examining cardiac function during arousal from torpor Eagles et al. (1988) used sodium pentobarbital to anaesthetise three species of hibernators prior to investigation. Sodium pentobarbital has been shown to act as a cardio-suppressive local anaesthetic, blocking the actions of the vagus and depressing the sinoatrial node as well as impeding the responsiveness of vascular smooth muscle (Cox, 1972). Therefore, findings made in a state of anaesthesia are often not readily comparable to the precisely controlled conditions of torpor.

Unfortunately, due to the difficultly of obtaining ECGs without disturbance and/or interference by the animal (i.e. by removing recording apparatus), many studies in the past have resorted to restraining animals. For example, bats have been taped by their wings to wooden boards prior to insertions of subcutaneous electrodes (Reite and Davis, 1966; Davis and Reite, 1967) and electrodes have been secured to animals using bent wire, fish hook or safety pin clips often without anaesthetic and connected to long wires (Nardone, 1955; Bartholomew and Hudson, 1962). Although these studies have provided valuable evidence of ECG changes associated with heterothermy, it is likely that physical restraint and use of encumbering and invasive recording equipment resulted in discomfort and stress, confounding data and misrepresenting the animal's typical torpid state.

An important and interesting phenomenon cited by many studies of torpor is the presence of electrocardiographic arrhythmias. During torpor HR is generally irregular and sinus arrhythmias have been reported in a number of investigations. Dawe and Morrison (1955) refer to the rhythmic changes in HR of hibernating Franklin's ground squirrel (*Spermophilus franklinii*) as "unusual", "pairing of beats or beats in bursts". It is more likely these tachy-brady patterns express cardiorespiratory association of intermittent breathers. Atrioventricular (AV) dissociation and different stages of heart block have been reported during steady-state torpor and arousal (Chatfield and Lyman, 1950; Eagles et al., 1988). However to date, there has been little investigation to characterise the frequency and

implications of these arrhythmias. Moreover, the limitations of equipment sampling rates and signal amplification make detection of the slow, low voltage atrial depolarisation (P wave, Figure 1) indicative of hibernation very difficult and may have resulted in the descriptions of AV dissociation or complete AV block described in the past (eg; Buchanan, 1911a; Buchanan, 1911b). More importantly, the presence of such irregularities is interesting in itself considering the aforementioned ability of the hearts of hibernators to withstand fibrillation.

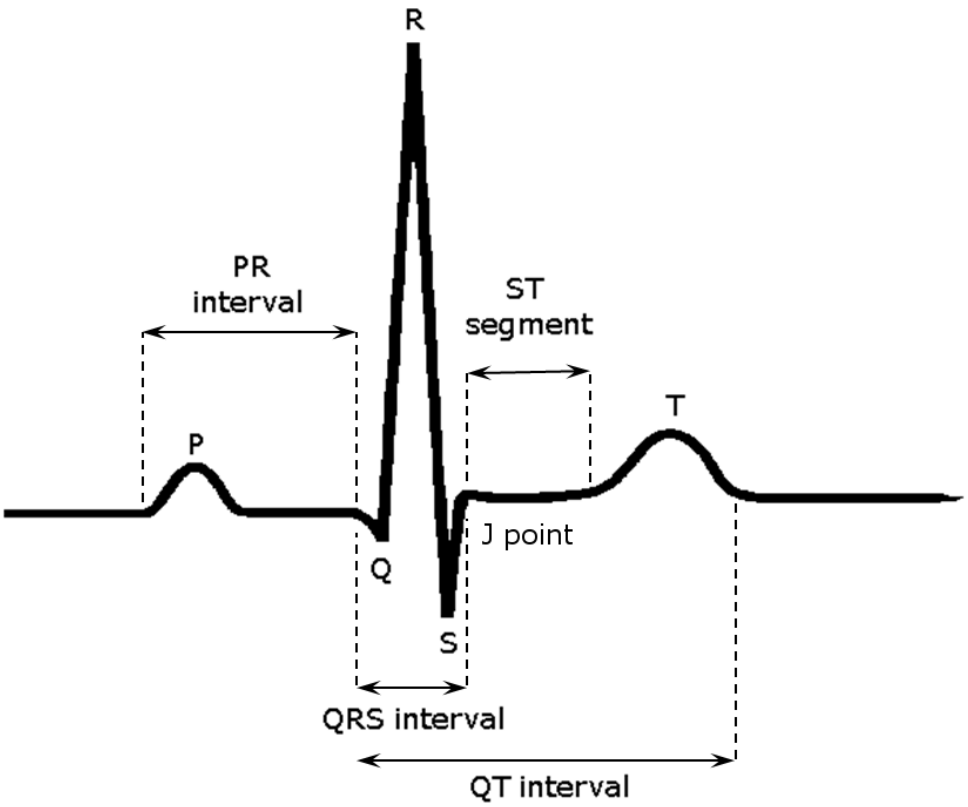

Figure 1. Schematic of a typical mammalian ECG signal showing the waveforms, which represent; atrial depolarisation (P wave), ventricular depolarisation (QRS complex), and ventricular repolarisation (T wave). As well as the relevant intervals and segments representing; AV node conduction (PR interval), ventricular depolarisation and repolarisation time (QT interval), time between depolarisation and repolarisation of the ventricles (ST segment), and the junction between QRS and ST (J point).

BATS

The Chiroptera are the second largest mammalian order with over 1100 species and these animals inhabit a vast array of habitats (Neuweiler, 2000; Kunz and Fenton, 2006).

They are highly specialized mammals as the only group capable of powered flight and this characteristic has likely facilitated their almost global distribution. Bats range in size by greater than 2 orders of magnitude with body mass from less than 3g to greater than 1.5kg and they show a suite of morphological and physiological modifications, which relate directly to the ecological and energetic demands of flight. The ability of bats to maintain coordinated cardiovascular function at the extremes of metabolism, from flight to torpor, over a T_b range of 42°C+ (Bondarenco et al., 2014), makes the study of cardiorespiratory function in these animals very interesting.

Cardiovascular system

Specialisations associated with flight are not limited to the external morphology of bats (most conspicuously the forelimbs) but are also evident in modifications of the essential organs associated with cardiac and pulmonary function (Canals et al., 2011). The energetic costs of flight are extremely high and flying bats have MRs at least double those of active terrestrial mammals of a similar mass (Thomas, 1975). As such it is essential that the cardiovascular and respiratory systems function optimally to support such high aerobic scope and maximum levels of oxygen consumption.

Bats have the largest relative heart and lung sizes of all mammals and these are directly correlated with body mass (Canals et al., 2005). The relative large mass of bat hearts is generally related to increased muscle mass of the right ventricle and size of the right atrium. This is associated with increased venous return and need for efficient pulmonary circulation during flight (Neuweiler, 2000). This is further enhanced by exceptionally smooth walls of the inflow and outflow tracts of the right ventricle, suggested to reduce friction and aid swift venous return and pulmonary delivery (Rowlatt, 1967). Cardiac myocytes of bats are also specialized for rapid heart rates, with small muscle fibres tightly packed and supplied with a very dense capillary network (Poupa and Lindstrom, 1983). In addition these cells are very rich in mitochondria and lipid droplets for rapid generation of large amounts of ATP and therefore energy, to support rapid contraction at high rates necessary for flight (Navaratnam et al., 1986; Ayettey et al., 1990).

It has been suggested that small bats compensate for the high energetic cost of flight with a greater alteration of cardiovascular size relative to respiratory mass. This is because while relative heart mass scales with body mass ($BM^{-0.21}$), lung volume remains isometric with body mass in small bats ($BM^{-0.9}$) (Canals et al., 2005). However, the microanatomy of the lungs of bats shows a more extensive surface area of blood-gas barrier tissue relative to birds and terrestrial mammals (Maina and King, 1984). This correlates to a higher capacity for pulmonary diffusion (Maina and King, 1984) and is represented in turn by significantly higher blood oxygen transport in bats than non-flying mammals of a similar size in addition to relatively larger lungs (Jurgens et al., 1981).

Furthermore, to ensure optimal ventilatory efficiency, bats synchronise respiratory rate to coincide with wing beat frequency during flight and this is in part because a considerable proportion of bats navigate their environments and hunt using echolocation. This has also been shown to correlate with echolocation pulses in the greater spear-nosed bat (*Phyllostomus hastatus*) where one or two ultrasonic pulses correlate with either upstroke or down stroke and expiration (Suthers et al., 1972). Consequently, the cost of echolocation during flight is greatly reduced by this synchronization (Voigt and Lewanzik, 2012).

Ecology of bats

Bats are an extremely diverse and often gregarious order with social groups of up to several million individuals. Sociality in bats is complex and highly variable within and among species and can even change throughout the year (Willis and Brigham, 2004; Kerth, 2008).

In many species, groups comprise primarily females and this is particularly evident during the reproductive season when maternity colonies are formed, often representing peak group sizes and the primary social period for many species (Kunz and Lumsden, 2003). Recent studies have demonstrated the complexity of maintained social interactions in a fission-fusion population of wild bats. Over four years individual Bechstein's bats (*Myotis*

bechsteinii) maintained social relationships despite regular and variable splitting and merging of groups, indicating the longevity of social interactions in these animals (Kerth et al., 2011).

Group size is often driven by roost preference/availability and in turn energy constraints of individuals. Roost limitation such as availability of suitable tree-hollows, may promote aggregation of individuals as is the case for many vespertilionids; while solitary bats are often found to roost in open foliage which is not limited (Kunz and Lumsden, 2003; Kerth, 2008). Energy conservation through torpor use as well as social thermoregulation also drives roosting preferences in bats (Willis and Brigham, 2007; Pretzlaff et al., 2010).

The majority of bat species are small with almost half weighing less than 10g (Stawski et al., 2014). Due to the large surface area of their wing membranes, bats have an even larger surface area to volume ratio than other small mammals of a similar size (Neuweiler, 2000) and an overall higher thermal conductance than predicted from body mass (Bradley and Deavers, 1980). As such heat loss in most small bats can be excessive. However, the highly diverse foraging ecology of bats and its relation to flight aerodynamics may be a limiting factor to the size of many species. For example, for insectivorous echolocating bats an increase in body size may reduce maneuverability required for prey detection and capture (Barclay and Brigham, 1991). In addition, fat storage is limited in these small bats (Calder, 1996) making regular foraging necessary and therefore the costs of daily living extremely high.

Heterothermy in bats

Of all mammals, bats are the one taxon especially likely to exhibit torpor and, although heterothermy has only been documented in 12 of 18 bat families, it is likely that the majority of small bats are capable of torpor use in one form or another (Stawski et al., 2014). Torpor use in bats is complex, it may occur throughout the year, and is related to a suite of energetic and environmental pressures. Depending on roosting strategy and climate, patterns of torpor expression vary among species.

The most studied bats are temperate zone insectivores, which hibernate over winter, generally inhabiting caves and buildings in large colonies and maintaining low T_b for extended bouts of torpor (Jonasson and Willis, 2012). More recently, hibernation with a sequence of multiday torpor bouts has been found even in bats inhabiting mild climates, such as tropical/subtropical forests and caves and even deserts (Stawski et al., 2009; Cory Toussaint et al., 2010; Liu and Karasov, 2011; Bondarenco et al., 2013), demonstrating that hibernation in bats is not restricted to cold climates. The majority of bats inhabit the mid to low latitudes and many of these species exhibit short bouts of torpor, with some exhibiting only daily heterothermy (Geiser and Stawski, 2011). Torpor use in the Pteropodidae is currently only known for species <50g, with the larger species likely to be strict homeotherms (Bartholomew et al., 1964; Bartholomew et al., 1970; Bartels et al., 1998). Torpor use in bats appears to be related to energy balance, water conservation (Bondarenco et al., 2013), reproductive fitness benefits (Klug and Barclay, 2013), predator avoidance (Stawski and Geiser, 2010) and may even be beneficial during development of young bats, but this has yet to be established.

As mentioned previously, when animals enter torpor the cardiovascular system exhibits profound changes. However, little is known regarding electrophysiological changes of the hearts of bats during torpor. In addition bats' susceptibility to, or protection from, temperature related cardiac arrhythmias during torpor is unknown. This makes investigation pertinent as bats may enter torpor on a daily basis and often more than once/day regardless of season (Hock, 1951), and therefore regularly expose themselves to intense fluctuations of cardiac function. Furthermore bats frequently express short bouts of hibernation in the wild, making them ideal for studies of comparative physiology and the distinction between torpor in daily heterotherms and hibernation.

STUDY SPECIES

Gould's long-eared bat (*Nyctophilus gouldi*)

Nyctophilus gouldi are insectivorous bats from the family Vespertilionidae (Figure 2A). Their distribution extends along the east coast of Australia stretching from north Queensland to south Victoria and possibly the south coast of Western Australia (Figure 2B). These bats are widespread within their range and are also commonly found in urban areas, where they are restricted to remnant bush-land (Threlfall et al., 2013b). The average body mass of *N. gouldi* is 12.3g with little sexual dimorphism (Churchill, 2008). Females give birth in austral spring from October to November and wean the young at around six weeks of age. A captive study showed a twinning rate of ~55% (Phillips and Inwards, 1985), but it is likely most free-ranging females give birth to a single young in the wild where an abundance of food is less freely available. Individuals begin to learn how to fly around five weeks of age, however juveniles are not captured in the wild population until mid-summer (January) when they are around eight weeks old (Phillips and Inwards, 1985).

Figure 2A) Picture of *Nyctophilus gouldi*. **B)** Distribution map of *N. gouldi* across Australia (from van Dyck and Strahan, 2008)

This species roosts primarily in trees, under exfoliating bark or in tree hollows (Churchill, 2008) and has been suggested to occasionally roost in caves at the north western aspect of its range (Kutt, 2003). *N. gouldi* primarily forage in the mid to understory, capturing prey on the wing or gleaning insects from the ground or surrounding foliage. They are specialized for slow, low level flight amongst vegetation and around tree trunks and prefer to roost in areas of dense canopy cover (Brigham et al., 1997; Threlfall et al., 2013a). Individuals shift roosts frequently (sometimes daily) but show strong fidelity to a collection of roosts (Lunney et al., 1988). This species has been suggested to be prone to urban extinction due to light pollution and restricted roosting and foraging habitat (Threlfall et al., 2013b).

Roost selection and colony size in this species, as with most small bats, is directly related to energetic benefits, not only from social thermoregulation (as in maternity colonies) but also with regards to torpor use and maximizing energy savings associated with torpor. *N. gouldi* hibernate between April and September and continue to use torpor when inactive during the summer months (Phillips and Inwards, 1985). However, the depth and frequency of torpor use is greatly influenced by T_a and hence roosting ecology (Turbill, 2006). A number of studies have been undertaken on this species with regard to torpor use in the wild, measured via skin temperature (T_{sk}) telemetry (Turbill, 2006; Turbill and Geiser, 2008), as well as measurements of metabolism and behaviour in captivity (Phillips and Inwards, 1985; Geiser and Brigham, 2000). To date, data for heart rate during normothermy and torpor are non-existent for this species.

Common blossom bat (*Syconycteris australis*)

Syconycteris australis are amongst the smallest bats from the family Pteropodidae (Figure 3A). Within Australia, this species is found from the northern most tip of Queensland south along the east coast to northern New South Wales (Figure 3B). Their range is restricted to tropical/sub-tropical regions with limited populations in mild temperate areas, and is directly influenced by food abundance (Law, 1994a; Law, 1994b). In New South Wales populations of *S. australis* feed exclusively on nectar and pollen, with a preference

for *Banksia* spp., however in tropical Queensland bats tend to be more opportunistic feeders and will regularly ingest fruit (Law, 1992). This species has been suggested to play an important role in pollination of the rainforest Myrtaceae, *Syzygium cormiflorum* in north Queensland, carrying a greater quantity of pollen that birds and shifting between trees more frequently (Law and Lean, 1999).

Figure 3A) Picture of *S. australis* **B)** Map of the Australian distribution of *S. australis* (from van Dyck and Strahan, 2008)

Individuals roost solitarily in exposed foliage of the rainforest subcanopy, generally amongst large (sometimes dead) leaves 2-5m above the ground (Law, 1993; Drury and Geiser, 2014). Bats have been shown to shift roost locations within rainforest patches between seasons, possibly related to relative changes in microclimate temperature, as the microclimate amongst roost leaves is generally cooler than surrounding T_a in summer and similar to surrounding T_a in winter (Law, 1993). Foraging and roosting habitat types are generally distinct in New South Wales with animals showing high fidelity to foraging sites within coastal heathland, adjacent to patches of rainforest for roosting (Drury and Geiser, 2014).

Little information exists regarding the reproductive biology of this species. A single young is thought to be born in austral spring between October and November, with a second maternity season suggested between February and April (austral summer/ early autumn) (van Dyck and Strahan, 2008). This is supported by evidence from Geiser et al. (2001) of pregnant bats captured in winter, and observational data from Drury and Geiser (2014) which suggest females may still be caring for dependent young in winter (June).

S. australis has been shown to use daily torpor to offset high energetic demands, at least in the southern extent of its range with many previous studies investigating thermal energetics of this species in captivity (Geiser et al., 1996; Coburn and Geiser, 1998; Geiser et al., 2001; Geiser et al., 2005a). One study has also managed to measure field metabolic rate in a wild population of these bats in New South Wales showing the highest mass-specific field metabolic rate of any species to date (Geiser and Coburn, 1999). However, data regarding cardiac physiology for this species, either during normothermia or torpor, are non-existent.

THESIS AIMS

Torpor use in the Chiroptera is either expressed by daily heterotherms that undergo short shallow, daily torpor or hibernators that enter longer multiday torpor bouts. Although thermal biology and energetics of bats have been studied extensively in the field and laboratory, knowledge about the cardiovascular system is scarce, particularly with regard to torpor. There is a clear distinction between the two patterns of torpor with regard to duration of torpor bouts as well as minimum MR and T_b, however investigation into the likely differences in cardiac function has received less attention. The few bats for which cardiac function has been studied are primarily from the northern hemisphere and often hibernate in caves or man-made structures over winter. In contrast, Australian bats inhabit a wide variety of climates from the tropics to temperate zones and torpor use has been shown to occur throughout the year. In addition many forest dwelling species enter torpor in thermally labile environments under tree bark or among foliage, thus exposing

themselves to fluctuating temperatures and enabling passive rewarming from torpor (which can be common).

The aims of my study were to quantify the cardiac function of two species of bats that exhibit either daily torpor or hibernation. I aimed to assess cardiac electrical conduction and heart rate throughout steady-state torpor simultaneous to measures of oxygen consumption, ventilation frequency and T_b at a range of T_as. Further, my aim was to understand how these different physiological variables are interrelated during torpor, how they change in relation to one another during entry and arousal from torpor and whether there were significant differences between the patterns exhibited by a daily heterotherm compared to a hibernator.

The thesis is divided into five results chapters (Chapter 2-6) and contains a final discussion (Chapter 7).

Chapter 2: Passive integrated transponders as a non-invasive tool for measuring body temperature in bats

Current commonly used methods for remote measurement of T_b involve implantation of internal transmitters which can be too heavy for use in small species <50g. Therefore recording of T_b in these animals generally requires handling for rectal thermometry. This process is invasive and may result in stress of the animal, disrupting physiological conditions of interest such as steady-state torpor. As the bats used in my study are <20g, and thermocouple thermometry would disturb animals in torpor, small passive integrated transponders (PIT) were used to measure subcutaneous temperature (T_{sub}). As PITs allow remote measurement this ensures accuracy of recordings during all phases of a torpor bout, including entry and spontaneous arousal from torpor. I conducted this study to determine the accuracy, reliability and precision of PIT tags as a measure of T_{sub} in resting and torpid bats.

Chapter 3: Heart rate as a predictor of metabolic rate in heterothermic bats

While current methods for measurement of energy expenditure in free-ranging animals provide an accurate integration of metabolic output over time, they are unable to tease out fine scale energetic changes in small species. This is of particular importance for understanding the way heterothermic species budget their energy in the wild as integrated measurements do not give details regarding the timing and/or frequency of torpor use. HR has been shown to be a viable method for measuring energy expenditure over a range of activities in large homeotherms; however no data exist for heterothermic animals either at rest or in torpor. Thus, I aimed to assess the relationship between HR and MR in heterothermic bats and validate the use of HR as a measure of MR during different physiological states.

Chapter 4: Passive rewarming from torpor in hibernating bats: minimizing metabolic costs and cardiac demands

Rewarming from torpor is extremely energetically expensive and the arousal process exposes individuals to highly toxic reactive oxygen species as well as increased pressures on the heart, particularly during arousal from low T_b. The costs of rewarming from torpor can be reduced by the use of passive rewarming. In the wild, bats have been shown to actively select roosts which enable them to rewarm passively from torpor, either by exploiting increasing T_a or via direct solar radiation. To date, there are no data for HR during passive rewarming from torpor. As such I provide the first evidence of the influence of increasing T_a on HR and aimed to investigate the relationship between HR, MR and T_{sub} throughout this phase.

Chapter 5: Heart rate and metabolism in heterothermic bats: Comparison of a daily heterotherm and a hibernator

Torpor is frequently used by many bat species throughout the year, however the depth and duration of torpor may differ within and between species. Hibernation or multiday torpor in the hibernators has been shown to be distinct from short, shallow daily torpor in

the daily heterotherms with regard to minimum T_b and MR. However, there has been little investigation into the differences between these two strategies in terms of cardiac function. The purpose of this chapter was therefore to quantify HR, MR and T_{sub} during steady-state torpor in hibernating *N. gouldi* in comparison to *S. australis* during daily torpor. Further, I aimed to investigate the effect of T_a on the relationship between these variables between the two species.

Chapter 6: The effects of temperature on cardiac electrophysiology and respiratory function of hibernating long-eared bats (*Nyctophilus gouldi*)

The cardiovascular and respiratory systems are both responsible for ensuring adequate supply of oxygen throughout the body during different physiological states. During torpor the hearts of hibernators continue to function well below the levels at which non-hibernators experience fatal arrhythmias. Previous investigations of electrophysiology in hibernating bats have been invasive due to technological limitations of sampling methods, however new technologies now enable less invasive methods and improved quality of recordings. My aim therefore was to provide data on cardiac conduction during steady-state torpor simultaneous with measurements of ventilation frequency at a range of T_a in order to assess the inter-relations between cardiac and respiratory function.

THESIS STRUCTURE

Most of the chapters of this thesis, from Chapter 2 through to Chapter 6, have been written in the format of journal articles. Two of these chapters (Chapters 3 and 4) have already been published in journals, confirmed by statements at the beginning of each of these chapters. The aim is to publish the remaining result chapters also as journal articles.

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Chapter 2

Passive integrated transponders as a non-invasive tool for measuring body temperature in bats

ABSTRACT

Measurements of body temperature (T_b) in laboratory settings are often undertaken via rectal thermometry, which requires handling and restraint of animals and causes stress. More recently, remote recording has been developed using implantable temperature-sensitive devices. In small mammals however, implantation of devices can be difficult due to the large relative size of available equipment. Therefore, I investigated whether small (0.13g) temperature sensitive passive integrated transponders can be used as a viable tool for measuring T_b in small bats (<20g). The precision of transponders was investigated as a function of temperature and the accuracy of implanted transponders as a measure subcutaneous temperature (T_{sub}) was quantified against rectal T_b (T_{rec}) in two species of bats (*Nyctophilus gouldi, Ng* and *Syconycteris australis, Sa*). Transponders functioned well outside the manufacturer's recommended range, down to ~5°C. At rest T_{sub} and T_{rec} were strongly correlated for both bat species (*Ng* r^2=0.88; *Sa* r^2=0.95) and this was also true for *N. gouldi* in torpor (r^2=0.93). During induced rewarming from torpor transponders showed that T_{sub} warms faster than T_{rec} in both species of bat. My results demonstrate that transponders can be used provide accurate measurement of T_b in two species of bats during different physiological states both during steady-state conditions and throughout dynamic phases such as rewarming from torpor.

INTRODUCTION

Animals used in laboratory settings are subject to an array of stressors that alter their behaviour and physiology and possibly impede the quality of the work. This is even more likely to be the case for wild-caught animals kept in captivity for short periods. Therefore it

is important, particularly for studies of animal physiology, to minimize the exposure of captive animals to unnecessary stressors.

Previous work on laboratory rats showed that handling and cage movements resulted in circulatory changes and elevated blood serum levels linked with shock and stress, which were not evident when animals were exposed to familiar non-tactile stimuli of regular animal house workers (Gartner et al., 1980). In particular the handling and discomfort associated with rectal thermometry has been shown to be a relevant stressor resulting in a rise in T_b of laboratory animals (van der Heyden et al., 1997; Dallmann et al., 2006; Bae et al., 2007). The elevation of T_b associated with stress (stress induced hyperthermia) is a repeatable and sometimes chronic response, lasting 30-60 minutes (van der Heyden et al., 1997) to which animals do not habituate following repeated day to day testing (Bae et al., 2007).

Historically, rectal thermometry has been the most reliable method for measurement of core T_b of small animals. Although it has been shown that rectal temperatures are less accurate than those derived from intraperitoneally implanted recording devices (Dallmann et al., 2006), the large size of many devices and limits to battery life makes implantation in animals <50g difficult. Miniaturization of external temperature telemeters has provided great insight into temporal changes in T_b but does not allow for more precise reliable T_b measurement (Willis and Brigham, 2003). More recently, small temperature-sensitive transponders have been shown to be a reliable tool to measure subcutaneous temperature (T_{sub}) in small marsupials ~25g (Wacker et al., 2012).

Therefore, I aimed to quantify the accuracy of temperature-sensitive passive integrated transponders (PIT) across a range of temperatures and to assess the validity of this method as a measure of T_b in small bats (<20g) capable of daily torpor (*Syconycteris australis*) or hibernation (*Nyctophilus gouldi*). *N. gouldi* are insectivorous bats that weigh between 5.2 and 16.5g, with an average head and body length of 47.4mm (Churchill, 2008). Minimum rectal T_b during torpor is approximately 2°C. *S. australis* are nectar feeding bats which weigh between 13.7 and 23.0g and have a mean head and body

length of 62.5mm. Minimum rectal T_b in this species is around 17°C. Both species inhabit the east coast of Australia and are capable of entering torpor throughout the year.

METHODS

PIT calibrations

Temperature-sensitive PITs (IPTT-300, Bio Medic Data Systems, Delaware, USA) are small (14mm × 2mm) and lightweight (0.13g). All transponders continued to function below the manufacturer's recommended range of use (32-43°C) down to approximately 10.0°C, and around 16% continued to function at 5.0°C. Forty transponders were calibrated to the nearest 0.1°C with a precision reference thermometer traceable to a national standard in a water bath at temperatures between 5.0°C and 40.0°C. Calibrations were taken at approximately 5.0°C increments and at each temperature 3 readings were taken, each at 5 minute intervals to assess precision and thermal inertia of transponders. Drift over time has been shown to be minimal in these devices, with <0.5°C change over several days (Wacker et al., 2012). Transponder signals were read with a DAS-7009S Handheld Reader (Bio Medic Data Systems, Delaware, USA) modified for connection to a PC trigger system to schedule PIT readings at 1 minute intervals associated with respirometry. Transponders were selected for implantation into bats based on the functional temperature range, correlation coefficient, and intercept of the calibration equation.

Thermocouple calibration

To measure rectal T_b of bats a fine gauge (42 S.W.G) copper constantan thermocouple was used. The thermocouple and digital thermometer were calibrated in a water bath against a precision thermometer traceable to a national standard, following similar methods as for PIT calibrations above, and over the same temperature range. Thermocouple temperature was read using a handheld digital thermometer (HH81A, OMEGA Engineering, Connecticut, USA).

Study animals and PIT implantation

Eighteen *Nyctophilus gouldi* (*Ng*; 10.5 ± 1.4g) individuals were captured in mist nets at local bushland surrounding the University of New England (UNE) or at Imbota Nature Reserve and Newholme Stations near Armidale, NSW (30°35'S, 151°44'E). On the night of capture bats were transferred to UNE and were fed mealworms and provided water before being housed in large outdoor aviaries (3m×1.5m×2m). The cage was fitted with hessian cloth for bats to roost and animals were provided mealworms *ad libitum.* Four male *Syconycteris australis* (*Sa*; 18.7 ± 1.0g) were trapped in mist nets at Iluka Nature Reserve on the north coast of NSW, Australia (29°24'S, 153°22'E). Bats were initially kept indoors in a large tent for four nights before being transferred to UNE. Bats were hand-fed to ensure they maintained body weight, but were also given a fruit and protein mixture (for more detail regarding recipe see Chapter 5) *ad libitum* while in the tent. At UNE bats were housed in a large indoor flight cage (2m×2m×2m) equipped with branches and large stands of foliage for bats to roost in. The room was kept at T_a around 20°C with relative humidity greater than 55%. Individuals were given a minimum of three days (up to 14 days) to ensure animals were feeding on their own and a stable weight was maintained before implantation of transponders.

For implantation bats were anaesthetized with general isoflurane/oxygen anesthesia (0.5-4%). A small (~3mm) incision was made in the skin of the upper back for transponder insertion between the shoulder blades. The skin and transponder were sterilized with 70% ethanol prior to insertion. One or two sutures (4/0 chromic gut, Ethicon, Somerville USA) were used to close the incision site. The entire process took <15 minutes. Following the minor surgery bats were placed in individual cages in a warm room (~24°C) and given 48h to recover before being returned to their respective holding cages.

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

Nyctophilus gouldi

PIT tag readings were compared with rectal temperatures (T_{rec}) to assess accuracy of T_{sub} measurements and how this correlated to core T_b. Core T_b were taken using a calibrated thermocouple inserted rectally to a depth of at least 2cm. For comparisons of resting T_{rec} to T_{sub} animals were placed in individual calico bags within a temperature-controlled cabinet maintained overnight at T_a between 5.0 and 20.0°C. Bats were transferred from their holding cages to the cabinet during their active phase following lights off in the evening and given access to a few mealworms. Individuals were not disturbed for at least 45 minutes prior to initial measurement to ensure they were calm and had adjusted to the T_a. Following exposure to each T_a for at least 1h, T_{rec} and T_{sub} were recorded within 30 seconds of one another, always in the same sequence. T_a was increased in 5°C increments and animals were exposed to maximum of 3 temperatures overnight, and were returned to outdoor cages before midnight each night of measurement.

To assess the relationship between T_{rec} and T_{sub} during torpor animals were kept in individual calico bags in a temperature-controlled cabinet overnight without access to food at T_a of 5.0-20.0°C to induce torpor. *N. gouldi* individuals have been shown to enter torpor overnight or in the early morning in laboratory settings (Geiser and Brigham, 2000) and therefore measurements of T_{sub} and T_b were taken the following morning after lights on to ensure animals were torpid. Again, recordings of T_{rec} and T_{sub} were taken within 30 seconds of each other.

Syconycteris australis

The relationship between resting T_{rec} and T_{sub} was investigated in normothermic *S. australis* placed in a temperature-controlled cabinet in individual calico bags in the evening at T_a between 12.0 and 30.0°C. To assess the relationship between T_{rec} and T_{sub} during torpor, bats were exposed to T_a of 12.0°C overnight and T_{sub} and T_{rec} temperatures were measured mid-morning. In previous studies *S. australis* entered into torpor following

lights on in the morning in respirometry chambers and therefore bats were left to become torpid and reach steady-state minimum T_b before T_{rec} measurements.

Arousal from torpor

Rewarming rates and the relationship between T_{sub} and T_{rec} during arousal from torpor were quantified in five bats, four *N. gouldi* and a single *S. australis*. Rewarming of *N. gouldi* individuals either took place following respirometry measurements (details in Chapters 3 and 5) when animals were kept in a temperature-controlled cabinet or in the flight cages in the early morning. Rewarming was induced by opening the respirometry chamber or removing bats from their hessian roosts in the aviary. In either case, a thermocouple was inserted at least 2cm rectally and as the thermocouple did not move following insertion, animals were not handled for the remainder of the rewarming process. Measurement of T_{rec} during rewarming in *S. australis* required more handling and restraint, so in order to minimize the influence of heat transference to the PIT tag the bat was loosely held in a gloved hand. The first measurements of T_{rec} in all bats were taken within 1 minute of disturbance regardless of location. T_{sub} and T_{rec} were recorded once every minute until animals became too active for accurate measurement. In all cases T_a did not vary over the rewarming period and was approximately 10.0°C for *N. gouldi* and 18.0°C for *S. australis*.

Statistical analyses

Statistical analyses were performed using R v3.1.0. Linear mixed effects models were used to calculate regressions of T_{sub} against T_{rec} at rest for both species and during torpor for *N. gouldi* using the *nlme* package with animal as a random factor (Pinheiro et al., 2014). Ordinary least squares regressions were used to calculate calibration equations of each PIT tag against water bath temperature. Rates of rewarming were calculated from the initial T_{rec} reading until animals were too active or T_{rec}/T_{sub} stabilized. For average rewarming rates the first and last T_{rec}/T_{sub} were subtracted from one another and divided by time. Maximum rewarming rates were either calculated as the maximum value

between two consecutive minutes (one minute maximum) or the maximum value over a 10 minute period. Paired t-tests were used to compare the maximum rate of rewarming over one minute, 10 minute maximum and average rewarming rates between T_{sub} and T_{rec} for *N. gouldi*. Paired t-tests were also used to compare maximum rewarming rate over one minute to the maximum calculated over 10 minutes.

RESULTS

PIT Calibrations

Twenty-two PITs were selected for implantation into 18 *N. gouldi* individuals and 4 *S. australis* individuals. Transponders continued to function below the minimum temperature of factory calibration (32°C, Bio Medic Data Systems) down to a water bath temperature of ~5°C (minimum water temperature=4.3°C, minimum PIT temperature=0°C) (Table 1). Accuracy of the transponders decreased at the lower temperatures, however, the coefficients of determination in all calibration equations were still >0.995 (Table 1) and there was minimal change (<0.2°C) over 10 minutes. Transponders had low thermal inertia and equilibrated to water bath temperature within a few seconds.

Table 1. Slope, intercept and r^2 of calibration equations and PIT temperature range in transponders calibrated in water baths prior to implantation in *N. gouldi* or *S. australis*.

Bat ID	PIT Temperature Range (°C)	Slope	Intercept	r^2
NG01	2.4 - 38.4	0.8801	3.421	0.9983
NG02	1.7 - 41.1	0.8939	3.4117	0.998
NG03	0.5 - 41.2	0.8735	4.2861	0.9983
NG04	0.8 - 40.4	0.9055	3.343	0.9949
NG05	0.1 - 39.4	0.9028	3.4416	0.9972
NG06	1.5 - 39.8	0.9213	2.4528	0.9973
NG07	0.9 - 40.9	0.8981	3.3993	0.9966
NG08	0.0 - 40.3	0.8845	3.7317	0.997
NG09	1.3 - 40.4	0.9256	2.369	0.9984
NG10	2.0 - 40.3	0.9335	1.9409	0.9983
NG11	2.4 - 40.3	0.9684	0.9027	0.9982
NG12	2.3 - 40.5	0.9559	1.236	0.9983
NG13	2.5 - 40.4	0.9465	1.6982	0.9988
NG14	2.2 - 40.0	0.9348	1.8843	0.998
NG15	1.6 - 40.7	0.9246	2.3452	0.9983
NG16	2.5 - 39.9	0.9203	2.5447	0.9983
NG17	1.3 - 40.5	0.9197	2.5288	0.9987
NG18	1.5 - 40.1	0.9148	2.6737	0.9983
SA01	4.9 - 40.5	0.8858	3.2609	0.9963
SA02	5.9 - 40.2	0.9385	1.8229	0.9966
SA03	5.8 - 40.1	0.9071	3.0883	0.9981
SA04	6.8 - 40.4	0.9037	3.5961	0.9972

Over all T_a measured, the T_{sub} of both normothermic and torpid animals was within 3°C of T_{rec}. There was a strong correlation (*Ng* r^2=0.88, *Sa* r^2=0.95, $p<0.001$) between T_{sub} and T_{rec} for both species when normothermic at rest. This was also the case for *N. gouldi* during torpor (r^2=0.93, $p<0.001$) (Figure 1). Average resting T_{sub} was 36.6 ± 1.9°C for *N. gouldi* and T_{rec} was 37.4 ± 1.3°C. For *S. australis* the average resting T_{sub} was 36.0 ± 1.8°C with corresponding T_{rec} of 36.5 ± 1.7°C. There was no significant effect of T_a on either T_{rec} or T_{sub} in normothermic bats of either species (Figure 2). However, measurements of T_{sub} in torpid *N. gouldi* were closely related to T_a as was T_{rec} and, on

average, T_{sub} was within 2°C of T_a. On occasion T_{sub} of *N. gouldi* appeared to fall below T_a, whereas torpid *S. australis* always maintained a T_{sub} >2°C above T_a (Figure 2).

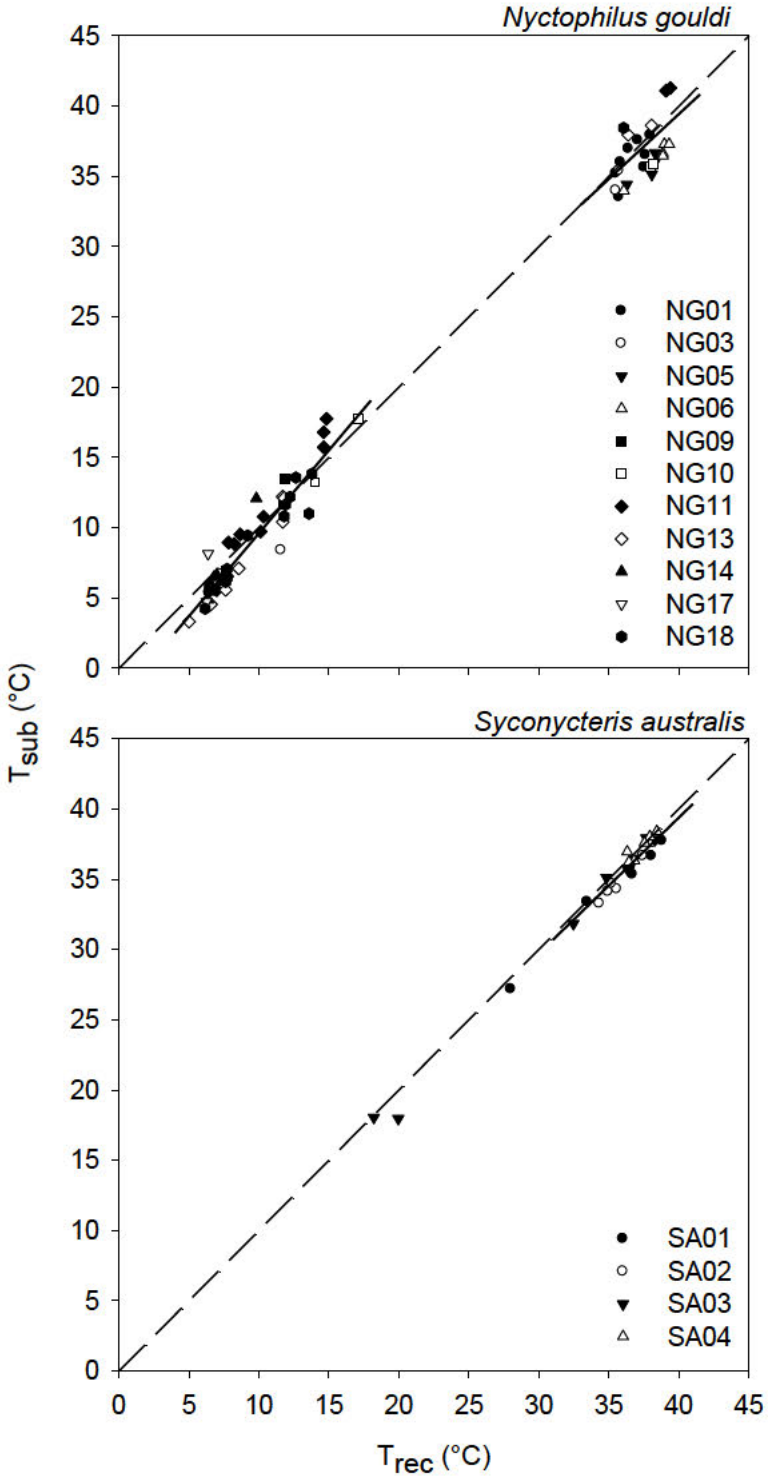

Figure 1. Subcutaneous PIT temperature (T_{sub}) as a function of rectal temperature (T_{rec}) for individual *N. gouldi* and *S. australis* during rest and torpor. Individuals with a T_{rec} <30°C were considered torpid. The dashed line represents the line of equality (T_{sub}=T_{rec}). In *N. gouldi* individuals T_{sub} was strongly correlated to T_{rec} at rest and during torpor (Rest: T_{sub}=0.92(T_{rec}) + 2.52, r^2=0.88, p<0.001; Torpor: T_{sub}=1.18(T_{rec}) + 2.22, r^2=0.93, p<0.001). The correlation between T_{sub} and T_{rec} for *S. australis* at rest was also significant T_{sub}=0.97(T_{rec}) + 0.67, r^2=0.95, p<0.001.

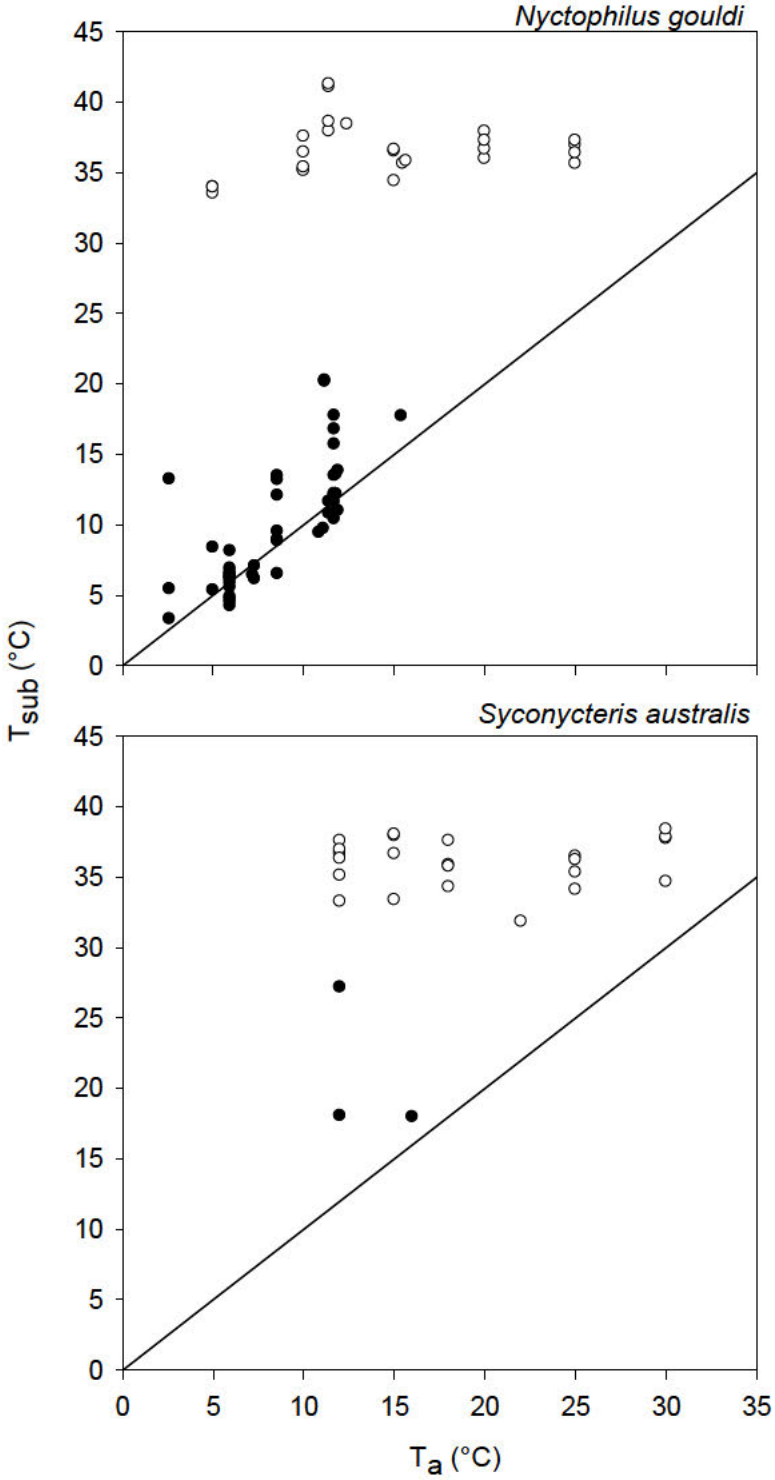

Figure 2. T_{sub} measurements as a function of T_a in resting (circles) and torpid (filled black circles) *N. gouldi* and *S. australis*. The threshold for torpor was T_{sub} <30°C. Solid black line indicates $T_{sub}=T_a$.

Rewarming

For all animals in torpor, the slightest touch or noise initiated the rewarming process. T_{rec} was taken within the first minute of disturbing bats and therefore was representative of the

T_b of animals in torpor. There was no difference in the rate of rewarming between T_{sub} and T_{rec} in *N. gouldi* calculated either as overall average rate (paired t-test, t=0.56, df=4, p=0.61) or as maximum rate over 10 minutes (paired t-test, t=-1.37, df=4, p=0.24). This was also similar for *S. australis* with only a 0.03°C min^{-1} difference in average rewarming rate (T_{rec}=0.89°C min^{-1} and T_{sub}=0.92°C min^{-1}) and 0.1°C min^{-1} difference in 10 minute maximum rate (T_{rec}=1.03°C min^{-1} and T_{sub}=0.93°C min^{-1}).

However in *N. gouldi*, maximum rewarming rate calculated over one minute was significantly higher than the rate over 10 minutes (paired t-test, t=5.09, df=4, $p<0.01$) and was also different between T_{sub} (1.86°C min^{-1}) and T_{rec} (2.45°C min^{-1}) (paired t-test, t=-5.36, df=4, $p<0.01$). During arousal from torpor T_{rec} lagged behind T_{sub} in both species with an average difference of 3.27°C and a range of 0.11-7.75°C. This difference was most pronounced during the middle of the arousal phase and towards the end of arousal the difference between T_{rec} and T_{sub} fell again to an average of 1.17°C.

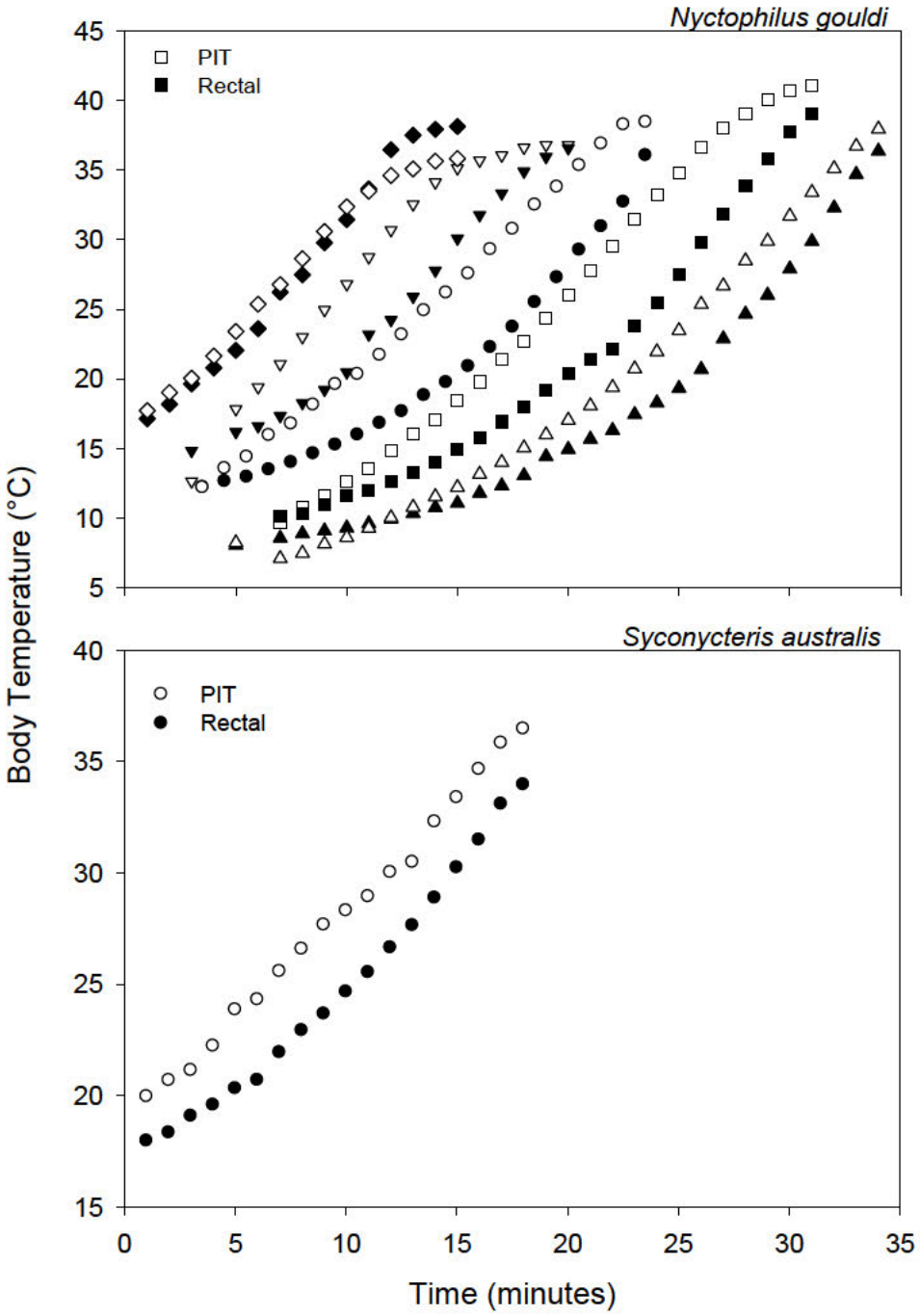

Figure 3. Simultaneous recordings of subcutaneous PIT temperature (white symbols) and rectal temperature (black symbols) during individual arousals from torpor in *N. gouldi* and *S. australis*. Note the different y-axis scaling.

DISCUSSION

My study shows that PIT tags provide reliable measurements of T_{sub} in two species of small heterothermic bats. Previously the measurement of T_b in small mammals required the invasive process of rectal thermometry, which requires handling and often restraining the animal being studied. Past investigations of T_b in undisturbed small animals <20g

have primarily been undertaken using external transmitters that measure skin temperature (T_{sk}). Although there is a correlation between T_{sk} and T_b, external transmitters are also affected by T_a and may not always provide precise measures of T_b especially when the relationship between T_b and T_a changes, as is the case for heterothermic species during torpor (Barclay et al., 1996; Willis and Brigham, 2003). In addition lightweight external transmitters have a limited battery life and are often shed by animals within a short period of time (from a few days to around one month) making long-term T_b measurements of small animals very difficult. The development of miniaturized, lightweight temperature sensitive transponders enables investigators to not only minimize the stress associated with animal handling but record T_b continuously in unrestrained animals over a range of physiological conditions. My results show that although the accuracy of IPTT-300 transponders is reduced at low temperatures, calibrations of individual transponders still enable reliable measures of T_{sub} during torpor in small bat species, which has not previously been possible. As the drift of transponders is minimal, with <0.5°C difference over four days (Wacker et al., 2012) and PITs require no battery, there is new scope for regular monitoring of individuals over long times scales, with retained function of PITs over several years (C.B. Wacker pers. comm.). Thus PITs provide an exciting opportunity to gain insight into the thermal biology of very small heterothermic species, not only during periods of torpor but during periods of activity which can be extremely energetically demanding, such as hovering flight in small hummingbirds that only weigh ~3g.

Although the current commercially available transponder system enables us to remotely measure T_b of undisturbed animals, which has previously been very difficult, the lack of automation of the scanner does not allow for regular or continuous recording. This is a particular issue when recording torpid animals within a respirometry chamber where even the smallest disturbances associated with use of the scanner may result in arousal. As investigation of steady-state torpor was an essential facet of my study, modification of the scanner to enable remotely triggered recordings was necessary. In addition, the small

range of even the scanner with the largest antenna (<5cm) restricted the scope of this method as a means of T_b measurement in active animals in larger enclosures and also meant that even within a small respirometry chamber (<300ml) animals could move out of range. For future development of transponder equipment, an extension of the recording range of scanners and automation of the system would greatly improve the scope of this tool for measurement of animals in different physiological states and possibly different environments.

During torpor animals are very sensitive to external disturbances (Thomas, 1995) and have even been shown to be able to undertake coordinated movement at T_b below normothermy (Rojas et al., 2012). Disturbances to torpid animals, whether directly through touch or even non-tactile means such as sound (Luo et al., 2014) or exposure to smoke, also increase energy expenditure in torpor (Speakman et al., 1991) or result in premature arousal (Stawski et al., 2014). As such the ability to remotely measure T_b in these animals is essential to ensure accurate and reliable representations of torpor use. Moreover, the premature induction of arousal from torpor has been shown to have significant effects on the rewarming process with increased rates of rewarming, increased variability of rate, and significant differences in the duration of arousal (Utz and van Breukelen, 2013). In golden mantled ground squirrels (*Spermophilus lateralis*) the effect of induced arousal (by mild shaking) was most pronounced at low T_a and had flow on effects to the amount of time spent normothermic during interbout arousals (Utz and van Breukelen, 2013). Both *N. gouldi* and *S. australis* were very sensitive to disturbance in this study as simply opening the temperature controlled cabinet often resulted in arousals. Although premature induction of arousal likely impacts some features of rewarming, the effect may not be as pronounced in small species, such as bats, that naturally rewarm very quickly. However, regardless of the initial disturbance handling and discomfort associated with rectal measurements of T_b would be most likely to confound results and as such should be minimized.

PITs showed low thermal inertia, unlike larger implantable and external transmitters, which enables more precise measurement of animals during dynamic phases such as entry into torpor and rewarming. Regardless of the difference in anteroposterior rewarming temperature, the overall rate of induced rewarming and maximum rate of rewarming over 10 minutes were not found to be different between measurements of T_{sub} and T_{rec} for bats in my study. This suggests that PIT tags and measurements of T_{sub} are an accurate method for quantifying integrated rates of rewarming. During rewarming from torpor the anterior portion of the body and hence T_{sub} rewarmed faster than the posterior body/T_{rec}. These results support previous findings in heterothermic placental mammals that show that the anterior portion of the body rewarms first (Chatfield and Lyman, 1950; Rauch and Hayward, 1970). This was also reflected in the significant difference between maximum rewarming rates of T_{sub} and T_{rec} calculated over one minute. The temporal precision of PIT tags enables fine time scale measurements which demonstrate a differential restriction of blood flow during arousal. Brown adipose tissue is an essential thermogenic organ in small placental mammals and, as animals rewarm from torpor, there is a dramatic increase in blood flow to this region (Hayward and Ball, 1966; Mejsnar and Janský, 1970). Restriction of blood flow to the peripheries enables animals to effectively rewarm the most critical organs and slowly return perfusion to the rest of the body. This generally tends to occur towards the end of the rewarming process. My results support these findings and show that towards the end of arousal the difference between T_{rec} and T_{sub} declined in both bat species. This difference was minimal in normothermic bats at an average of 1.1 ± 0.8°C. Consequently small PIT tags enable quantification of alternate changes in different body regions associated with dynamic physiological transitions such as arousal from torpor.

My study is the first to assess the accuracy of small temperature sensitive transponders as a measure of T_{sub} in bats during different physiological states. I show that T_{sub} and T_{rec} were very strongly correlated in bats during both steady-state conditions of rest and torpor. T_{sub} measurements of normothermic bats in my study were not a function of T_a and

as such provide a more reflective measure of T_b than the commonly used measure of T_{sk}. This suggests that PITs are a viable method for regular recording of T_b across an array of physiological states including the dynamic phase of rewarming, and may enable investigation of thermal physiology previously undocumented for very small species less than 5g.

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STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

	Author's Name (please print clearly)	% of contribution
Candidate	Shannon E Currie	80%
Other Authors	Gerhard Körtner	10%
	Fritz Geiser	10%

Name of Candidate: Shannon E Currie

Name/title of Principal Supervisor: Professor Fritz Geiser

[redacted]

Candidate

1/3/15

Date

[redacted]

Principal Supervisor

1/3/15

Date

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STATEMENT OF ORIGINALITY

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We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

Type of work	Page number/s
Text	41-57
Tables	48
Figures	49,50,52

Name of Candidate: Shannon E Currie

Name/title of Principal Supervisor: Professor Fritz Geiser

[redacted] 1/3/15

Candidate Date

[redacted] 1/3/15

Principal Supervisor Date

Chapter 3

Heart rate as a predictor of metabolic rate in heterothermic bats

This chapter has been published with slight modifications:

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Gerhard Körtner and Fritz Geiser provided assistance with equipment and project supervision. Drafts of the manuscript were commented on by both of the co-authors.

ABSTRACT

While heart rate (HR) 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 HR also predicts oxygen consumption ($\dot{V}O_2$) during torpor $\dot{V}O_2$, HR and subcutaneous body temperature (T_{sub}) of a hibernator, Gould's long-eared bats (*Nyctophilus gouldi*, 9 g, n=18), were measured simultaneously at ambient temperatures (T_a) between 0 and 25°C. At rest, HR of normothermic resting bats was negatively correlated with T_a, with maximum HR of 803 bpm (T_a=5°C). During torpor the relationship between HR and T_a was curvilinear, and at low T_{sub} (~6°C) HR fell to a minimum average of 8 bpm. The minimum average values for both $\dot{V}O_2$ and HR in torpor reported here were among the lowest recorded for bats. The relationship between HR 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 HR did not differ significantly from measured values during the different physiological states.

My study is the first to investigate the accuracy of HR 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, HR should only be used to predict $\dot{V}O_2$ within the range of activities for which robust correlations have been established.

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 is of particular interest to ecological and evolutionary physiologists as they provide an understanding of how animals budget their energy. In captivity energy use of endothermic animals is often quantified as basal metabolic rate (BMR) in resting animals under thermoneutral conditions, and it is often assumed to be proportional to field metabolic rates (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 adjustments 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 that is much higher than predicted (Geiser and Coburn, 1999).

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 (HR) methods (Nagy, 1987; Speakman, 2000; McCarron et al., 2001). The DLW method quantifies carbon dioxide 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 to up to 1-10% of BMR (Geiser, 2004). Bouts of torpor can last for short periods of time <24h 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 HR method, in contrast, relies on the intrinsic relationship between oxygen consumption ($\dot{V}O_2$) and HR, and can provide instantaneous and continuous measurements of metabolism over extended periods (~1yr; McPhee et al., 2003). This permits comparisons 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 HR method to standard respirometry show that HR 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 HR 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 HR 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 HR 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 HR telemetry, making measurements of HR 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 heterothermic species (Geiser, 2013), knowledge about whether the same relationship between $\dot{V}O_2$ and HR applies is highly desirable. It is known HR decreases with metabolic rate during torpor, but whether it can be used to estimate energy expenditure has not been established. I therefore aimed to determine the accuracy of HR 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, the precision of the HR method during entire torpor bouts was investigated, incorporating the transitional periods of torpor entry and arousal. Detailed knowledge of the relationship between $\dot{V}O_2$ and HR 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.

METHODS

Open-flow respirometry, ECGs and temperature-sensitive passive integrated transponders were used to measure the relationship between metabolic rate and HR of long-eared bats (*Nyctophilus gouldi*) during torpor at a range of T_a (0-25°C). Measurements were conducted on *n*=9 female and *n*=9 male *N. gouldi* (mass at capture: 10.5±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 approximately 1000 m elevation. Captured animals were transferred to the University of New England and kept in captivity for a maximum period of seven 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 1g of their body mass at the time of capture while in captivity.

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

Transponder Implantation

Subcutaneous body temperature (T_{sub}) was measured using temperature-sensitive transponders (IPTT-300 Bio Medic Data Systems Implantable Programmable Temperature Transponder, Delware, USA; 0.13 g, 14 mm x 2 mm) implanted interscapularly. For small mammals T_{sub} is closely related to T_b, particularly during torpor when T_b-T_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 sterilized 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 fifteen minutes. Bats were given 24h 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 ($\dot{V}O_2$), was monitored overnight and throughout the

following day(s) to allow animals to undergo their usual daily thermal cycle. Bats were weighed (±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 $\dot{V}O_2$ 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 randomized between measurements and the values obtained were not affected by chamber volume (ANOVA; $p>0.05$). The T_a (±0.1°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^{-1}) was adjusted based on chamber size to ensure that 99% equilibrium was reached within <11 minutes, controlled with rotameters and measured with mass flowmeters (Omega FMA-5606; Stamford, CT, USA).

Oxygen concentration was measured in a constant temperature room to minimize 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 minutes 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 $\dot{V}O_2$ was calculated using standardised gas volumes and Eq. 3a of Withers (1977). A respiratory quotient of 0.85 was assumed throughout.

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

temporarily stopped working when the T_{sub} of animals in torpor fell below 7°C. In these cases T_{sub} was estimated to be 0.5°C above T_a, as this was the average differential for animals with similar $\dot{V}O_2$ whose transponders continued to work at low T_a.

ECGs

Measurements of HR were recorded using the established methods of Zosky (2001). Individuals were placed in respirometry chambers in the evening and left until the following morning; when animals were torpid and $\dot{V}O_2$ 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, $\dot{V}O_2$ soon returned to similar or lower values than prior to the disturbance and did not differ significantly (paired t-test; $t=2.05$, $df=9$, $p>0.05$). The data were therefore considered representative of steady-state torpor.

Electrodes were fashioned from Kendall Care Resting ECG Electrodes (Tyco Healthcare Group, Mansfield, USA) 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, Mansfield, USA) fitted with customised clips at one end. ECGs were measured using either a FE132 BioAmp or ML135 Dual BioAmp (ADInstruments, Bella Vista, Australia) connected to a Powerlab 4/35 Data Acquisition System (ADInstruments, Bella Vista, Australia) and recorded with LabChart Pro v7.3 software (ADInstruments, Bella Vista, Australia). ECGs were analysed to calculate instantaneous HR, which was averaged per second using LabChart Pro v7.3 and exported to Microsoft Excel (Microsoft Corporation) for further analysis.

Statistical Analyses

For the purpose of my study, only data for normothermic resting after arousal from torpor and thermoconforming animals in steady-state torpor were used for regression analyses (bats that maintained a high T_{sub}-T_a differential when in torpor were considered to be thermoregulating, and were excluded from analyses). Mean minimum values of $\dot{V}O_2$, HR,

and T_{sub} during torpor were taken from times when all variables were lowest for at least 30 min. At T_as below 10°C periods of apnoea were generally longer than 30 min (S.E. Currie, 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 indirectly. Mean $\dot{V}O_2$ and HR during torpor were calculated for thermoconforming animals that entered torpor for <24h exposed to constant T_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; Figure 1- time between the arrows). On occasion, torpor lasted for >24h or bats were exposed to more than one T_a during a torpor bout. Animals were exposed to a maximum of three different T_a values for ≥1.5hrs 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 $\dot{V}O_2$ and HR of thermoconforming torpid bats was calculated using the following equation: $Q_{10}=(value_1/value_2)^{10/(Tb1 - Tb2)}$. Values for resting $\dot{V}O_2$, HR, and T_{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 ~ 5cm, and therefore T_{sub} was occasionally unavailable. This resulted in more HR values of resting normothermic bats against T_a than T_{sub}.

Statistical analyses were performed using R v2.15.2. Standardized major axis regressions were performed using the smatr package (Warton et al., 2012) and pseudo-replication was accounted for 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 $\dot{V}O_2$ before and after disturbance associated with ECG lead attachment, and predicted $\dot{V}O_2$ values with measured values. Analysis of covariance (ANCOVA) was used to compare slopes of regression equations. Means are reported ± SD for the number of measurements (*N*) and individuals (*n*).

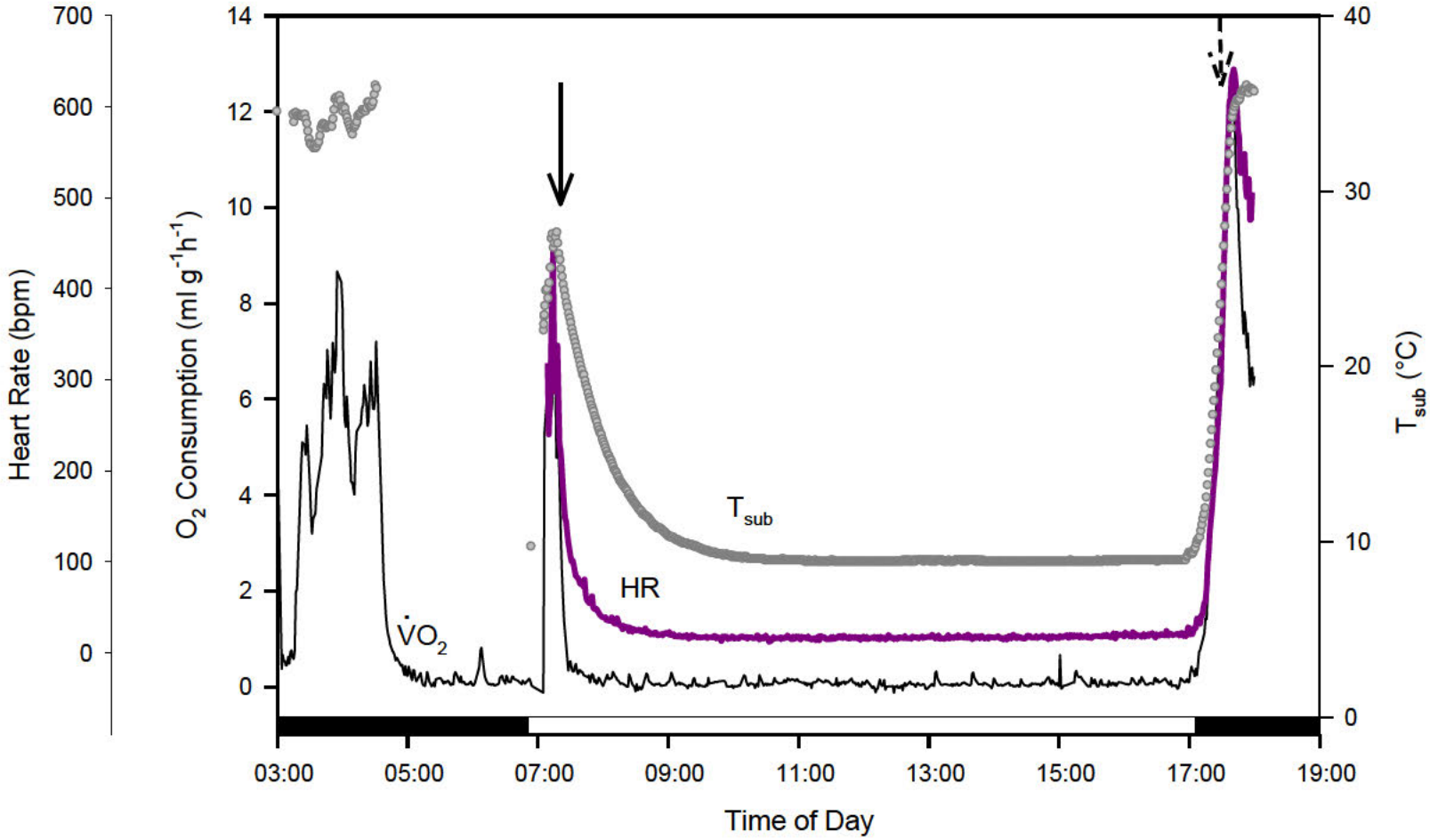

Figure 1. Representative HR (purple line), $\dot{V}O_2$ (solid line), and T_{sub} (grey circles- missing data are where the bat moved out of scanner range) of *N. gouldi* at T_a of 10°C; the dark bar on the horizontal axis represents scotophase. The animal entered torpor in the early morning prior to lights on, exhibited a partial arousal associated with 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).

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 HR fell concurrently and reached steady-state minima when subcutaneous temperature (T_{sub}) had declined to within ~2°C of minimum T_{sub} (Figure 1). Disturbance did not affect minimum $\dot{V}O_2$ values (paired t-test; t=2.05, df=9, p>0.05). At T_a below 20°C, bats remained torpid until shortly after lights off when $\dot{V}O_2$, HR and T_{sub} increased beginning with an increase in $\dot{V}O_2$ and HR associated with evening arousal.

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

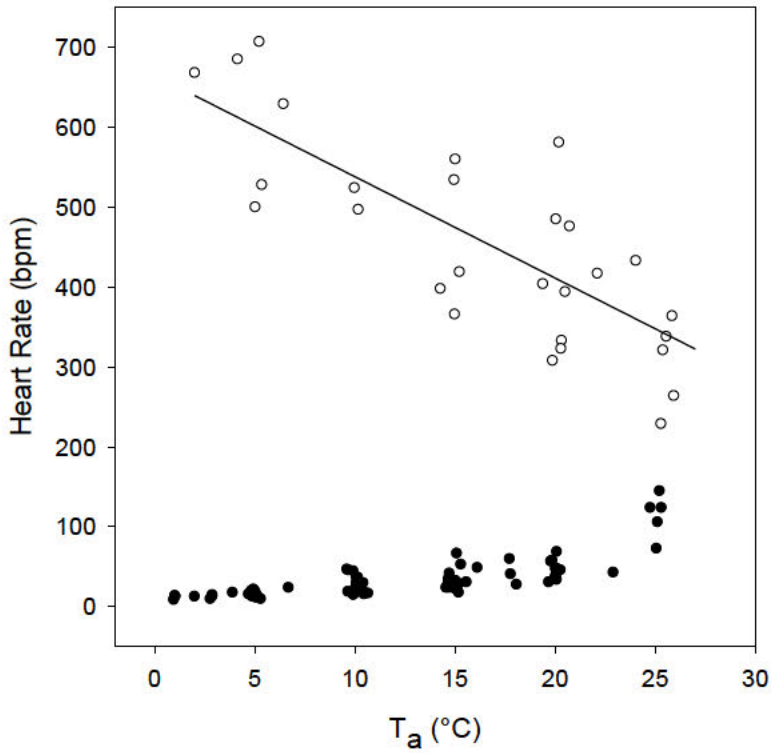

Figure 2. HR of normothermic resting and torpid *N. gouldi* as a function of ambient temperature. Each point represents an individual measurement taken from 18 individuals in total. HR in resting normothermic bats increased linearly with decreasing T_a: HR(bpm)=664.8 – 12.68T_a(°C), r^2=0.82, p<0.01.

At T_a between 1 and 25°C, the mean HR of torpid bats over 30 mins ranged from 8 to 144bpm with corresponding $\dot{V}O_2$ from 0.02 to 0.46 ml g^{-1} h^{-1}. Even at T_a of 25°C, mean HR during torpor was only 35% that of normothermic bats. HR during torpor was a curvilinear function of T_{sub} when plotted on a linear scale, with a Q_{10} of 2.0 (Figure 3). Mean HR of normothermic bats ranged from 1.3-fold to 4-fold the values predicted by the extrapolated curve of torpid bats against T_{sub} (Figure 3). The minimum HR recorded in a torpid bat over 1 min was 5 bpm at T_a=0°C.

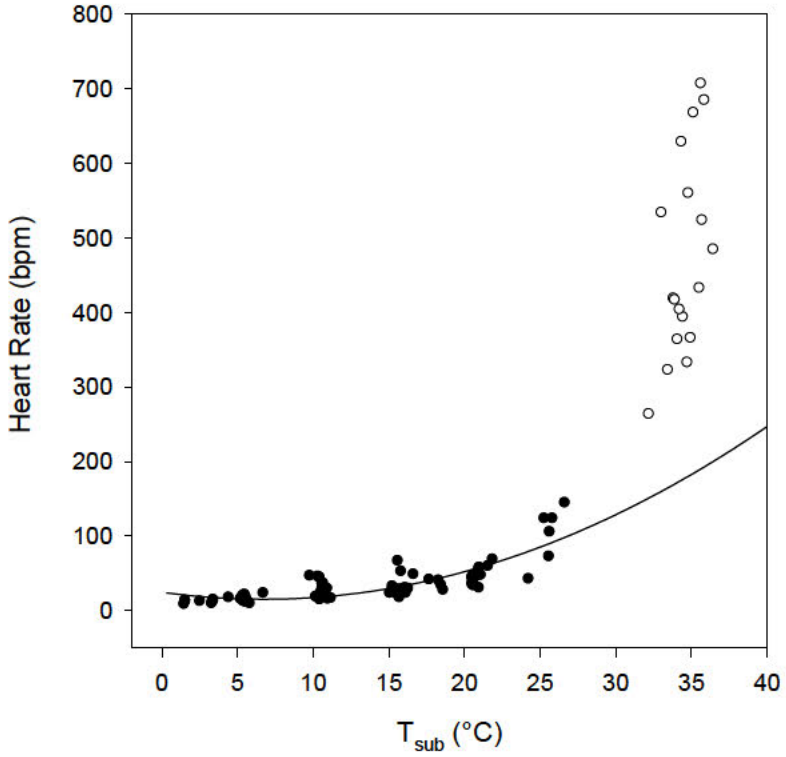

Figure 3. HR of normothermic resting and torpid *N. gouldi* as a function of T_{sub}. HR increased in a curvilinear pattern with T_{sub}; however the extrapolated curve for HR data in torpid bats fell below values obtained for normothermic resting individuals.

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

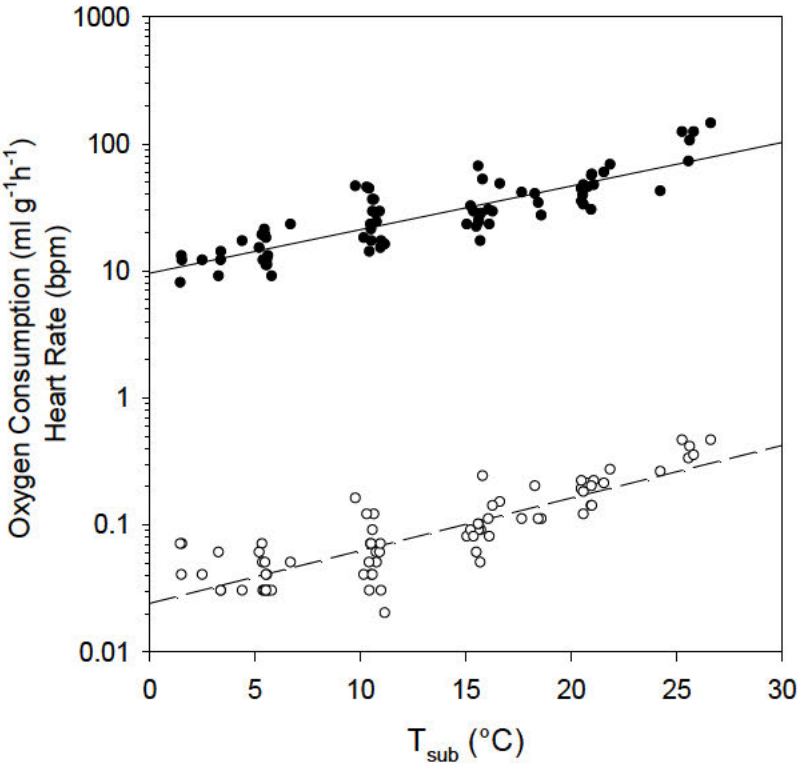

Figure 4. Mean HR and $\dot{V}O_2$ of torpid bats plotted against T_{sub}. Note the logarithmic scale. Linear regressions for $\log_{10}HR=0.03(x) + 0.98$, $r^2=0.81$ (solid line); and $\log_{10} \dot{V}O_2=0.04(x) - 1.62$, $r^2=0.72$ (dashed line).

$\dot{V}O_2$ and HR were strongly correlated at rest and during torpor (Figure 5). However, extrapolation of the line derived from bats during torpor fell below values for normothermic bats and the slopes differed enormously (ANCOVA; $p<0.01$) (Figure 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 HR in resting normothermic bats is described by the equation:

$$\dot{V}O_2 = 0.02(HR) - 3.458 \qquad (1)$$

($r^2=0.64$, $p<0.001$) where HR is in bpm and $\dot{V}O_2$ is measured in ml $g^{-1}h^{-1}$. For each individual, estimates of resting $\dot{V}O_2$ did not differ significantly from direct measurements at the same HR (paired t-test; $t=0.33$, $df=9$, $p=0.749$). This was calculated by sequentially removing data from one individual from equation 1 and recalculating the regression using

data from the remaining bats. During torpor the relationship between $\dot{V}O_2$ and HR was described by the equation:

$$\dot{V}O_2 = 0.004(HR) - 0.013, \qquad (2)$$

(r^2=0.84, p<0.001). There was also no significant difference between $\dot{V}O_2$ values estimated from modified versions of equation 2 and those measured directly at the same HR (paired t-test; t=-1.62, df=17, p=0.124).

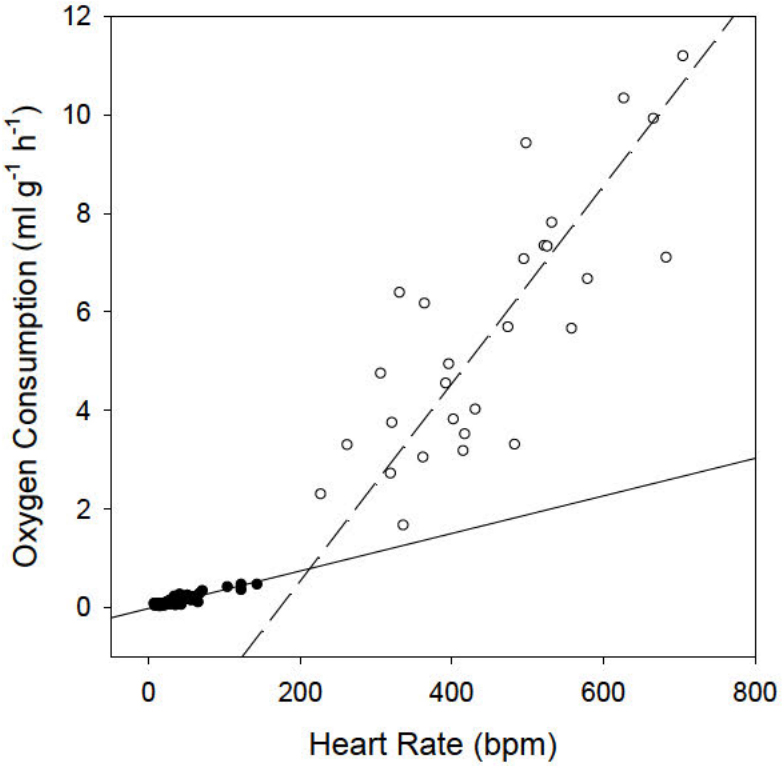

Figure 5. $\dot{V}O_2$ as a function of HR in normothermic resting and torpid *N. gouldi* at T_a between 1 and 25°C. Dashed line represents the regression equation for resting individuals ($\dot{V}O_2$ = 0.02(HR) - 3.458, r^2=0.64, p<0.001, *N*=34) and the solid line represent the regression equation for torpid individuals ($\dot{V}O_2$ = 0.004(HR) - 0.013, r^2=0.84, p<0.001, *N*=74).

DISCUSSION

My study is the first to provide continuous quantitative data on HR, MR and T_{sub} simultaneously and as a function of T_a for a microbat. My results demonstrate a strong positive correlation between metabolism and cardiac function at rest and during torpor.

The data suggest that HR 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 HR measurements, exhibited similar temporal patterns of torpor use to those described for the same species in previous studies (Geiser and Brigham, 2000). In addition, my results showed that the relationship between $\dot{V}O_2$, HR 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 HR 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-sample t-test, $t=0.78$, $df=21$, $p>0.05$). Minimum mean HR (8 bpm) in particular was well below values reported for unrestrained northern hemisphere bats of a similar mass (40 bpm) (Kulzer, 1967). Moreover, the absolute minimum of 5 bpm was similar to that measured in much larger hibernators such as woodchucks (*Marmota monax*, 3-5kg) Lyman, 1958) and dormice (*Glis glis*, ~150g) (Elvert and Heldmaier, 2005).

Interestingly, reported HR values of ~40bpm in other studies were similar to those of thermoregulating individuals (bats that maintained a T_{sub}-T_a differential >2°C when in torpor) at the same T_a in my study (not shown). This suggests that bats in previous investigations were not thermoconforming 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 HR data in torpor.

The maintenance of higher T_b-T_a differentials in thermoregulating torpid bats will reduce the energy savings associated with torpor when compared to animals that are thermoconforming 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 thermoregulating 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 HR for bats in different physiological states are required if the HR 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 thermoregulatory heat production is used in free-living animals.

I show that during steady-state torpor and at rest the relationships between HR 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, HR 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 HR 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 HR and $\dot{V}O_2$. I therefore investigated whether a strong linear correlation remains between $\dot{V}O_2$ and HR when averaged across a complete torpor bout (i.e. from peak values after partial arousal before torpor, to peak values following final arousal; refer to arrows in Figure 1). My results show that the relationship between $\dot{V}O_2$ and HR 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 (Figure 6). This signifies the precision of HR as a predictor of $\dot{V}O_2$, not only during steady-state conditions but also throughout the transition between physiological states.

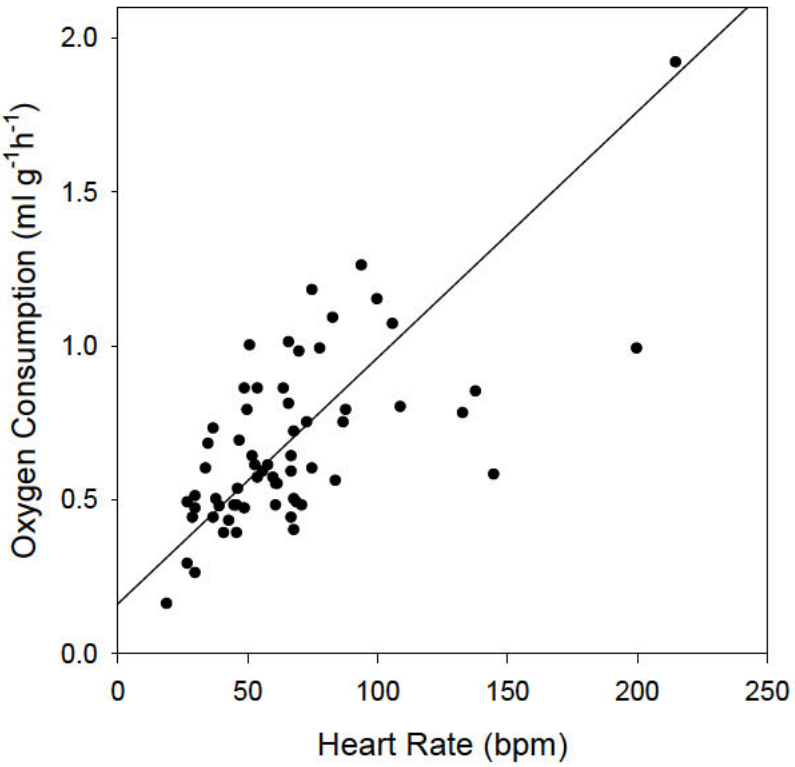

Figure 6. $\dot{V}O_2$ as a function of HR averaged for an entire torpor bout for *N. gouldi* at T_a between 1 and 25°C. The torpor bout includes entry after partial arousal and final arousal; each point represents one bout. The regression equation was; $\dot{V}O_2 = 0.008(HR) + 0.163$, $r^2=0.44$, $p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

Nevertheless, HR 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 heterothermic animals which as I have demonstrated, display a distinct difference between resting and torpor regressions, with no overlap between the two states. At rest, both HR 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 HR against $\dot{V}O_2$ in normothermic resting bats. My 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 HR 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%, emphasizing 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 (5g) flying bats has been shown to be >16 times higher than that at rest (Voigt and Lewanzik, 2012). Strong correlations between HR 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, HR doubled at the onset of flight, while oxygen consumption increased 4-fold and both HR and $\dot{V}O_2$ returned to resting levels within 30 seconds 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 HR 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 I show that regardless of torpor bout length there is a strong correlation between HR and $\dot{V}O_2$ across a complete torpor bout and at rest, consistent with the potential for the HR method to reliably measure field energy expenditure. Although it has been suggested in the past that the HR method becomes prohibitively expensive when applied to animals smaller than 1kg (Butler et al., 2004), miniaturized heart rate transmitters are becoming more readily available and can be used on animals as small as 10g (Dechmann et al., 2011). My 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 HR similar to those used for T_b or metabolic rate in most studies of heterothermy in free-living mammals and birds.

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(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

	Author's Name (please print clearly)	% of contribution
Candidate	Shannon E Currie	80%
Other Authors	Gerhard Körtner	10%
	Fritz Geiser	10%

Name of Candidate: Shannon E Currie

Name/title of Principal Supervisor: Professor Fritz Geiser

[redacted]

Candidate

1/3/15

Date

[redacted]

Principal Supervisor

1/3/15

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Name of Candidate: Shannon E Currie

Name/title of Principal Supervisor: Professor Fritz Geiser

[redacted] 1/3/15

Candidate Date

[redacted] 1/3/15

Principal Supervisor Date

Chapter 4

Passive rewarming from torpor in hibernating bats: minimizing metabolic costs and cardiac demands

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Kodie Noy assisted with data collection and Fritz Geiser provided assistance with equipment and project supervision. Drafts of the manuscript were commented on by both of the co-authors.

ABSTRACT

Endothermic arousal from torpor is an energetically costly process and imposes enormous demands on the cardiovascular system, particularly during early stage arousal from low body temperature (T_b). To minimize these costs many bats and other heterothermic endotherms rewarm passively from torpor using solar radiation or fluctuating ambient temperature (T_a). Because the heart plays a critical role in the arousal process in terms of blood distribution and as a source of heat production it is desirable to understand how the function of this organ responds to passive rewarming and how this relates to changes in metabolism and T_b. I investigated heart rate (HR) in hibernating long-eared bats (*Nyctophilus gouldi*) and its relationship to oxygen consumption ($\dot{V}O_2$) and subcutaneous temperature (T_{sub}) during exposure to increasing T_a in comparison to endogenous arousals at constant low T_a. During passive rewarming, HR and $\dot{V}O_2$ remained low over a large T_{sub} range and increased concurrently with increasing T_a (Q_{10}

2.4 and 2.5, respectively). Absolute values were higher than during steady-state torpor, but below those measured during torpor entry. During active arousals, mean HR and $\dot{V}O_2$ were substantially higher than during passive rewarming at corresponding T_{sub}. In addition, partial passive rewarming reduced the cost of arousal from torpor by 53% compared to entirely active arousal. My data show that passive rewarming considerably reduces arousal costs and arousal time; and this may also contribute to minimizing exposure to oxidative stress as well as demands on the cardiovascular system.

INTRODUCTION

Torpor is central to the biology of many small mammals and birds worldwide and involves the coordination of a complex array of costs and benefits that change seasonally and with climate, body condition and age (Ruf and Geiser, 2014; Stawski et al., 2014). Torpor is characterised by the controlled and reversible reduction of metabolic processes, body temperature (T_b), ventilation, and cardiac function. While energy requirements of torpid animals are substantially reduced, adequate perfusion of essential organs is still required and it has long been established that animals capable of entering torpor possess a suite of physiological adaptations to preserve coordinated functioning of the cardiovascular system at low T_b (Swoap and Gutilla, 2009). Increased peripheral resistance and reduced venous return, related to increased viscosity of cold blood, and low heart rates (HR) are offset by an increase in stroke volume and alterations of contractile proteins in the myocardium (Nelson and Rourke, 2013). Anti-arrhythmic protection ensures that coordinated conduction across all chambers of the heart is maintained throughout torpor (van Veen et al., 2008). These adaptations are also likely to enable a safe return to normothermia during arousal from torpor which is extremely demanding and can occur frequently.

Endothermic rewarming from torpor is extremely energetically expensive, and can deplete most of the energy needed during the hibernation season (Thomas et al., 1990). During arousal the massive increase in metabolic rate (MR) along with reperfusion of dormant tissues exposes animals to oxidative stress, as increased production of reactive oxygen

species (ROS) may out-weigh the animal's antioxidant defenses (Carey et al., 2003). In addition, the cardiovascular system must work to support the increasing oxygen demands of thermogenic organs and surrounding tissues. Rewarming is controlled by the sympatheticoadrenal system which drives HR and metabolism at maximum rates corresponding to rising T_b (Lyman et al., 1982). In addition rapid beating of the heart provides a vital source of heat for rewarming (Milsom et al., 1999). Intricate coordination of the circulatory system during this phase results in different rates of oxygen consumption in the body, with significantly greater perfusion of heart, brain, liver and brown adipose tissue in comparison to the posterior body, peripheral tissues and digestive tract (Rauch and Hayward, 1970). The restriction of blood to the anterior portion of the body increases systemic vascular resistance and results in a rapid increase in blood pressure, which reaches maximum levels early in arousal (Lyman et al., 1982). The heart however, is rate limited by low T_b and forced to work against high blood pressures, reducing its efficiency as a pump and likely introducing mechanical stress. Therefore, it can be argued that early stage arousal from low T_b imposes the greatest demands on the cardiorespiratory system. As such it is important for arousing animals to maintain a balance, reducing stresses on the heart and exposure to reactive oxygen species while still producing enough energy to rewarm.

The energy demands of rewarming can be off-set to some extent by the use of passive rewarming which often reduces arousal costs by >50%, and can result from heat transfer between nest/roost mates, increases in ambient temperature (T_a), or direct exposure to solar radiation (basking) (Geiser et al., 2004). The benefits of passive rewarming are obvious for daily heterotherms that must rewarm from torpor to forage on a daily basis. In contrast, accounts of passive rewarming in hibernating species are rare, which is largely due to the fact that many hibernators roost or nest in thermally stable microclimates. Bats however, hibernate in an array of microhabitats from caves to exposed foliage and can spend more than half of their lives in torpor (Kunz and Lumsden, 2003). In particular, tree-dwelling bat species generally roost under exfoliating bark and in hollows or rock crevices

and may even overwinter in these areas, exposing themselves to large fluctuations in T_a (Hamilton and Barclay, 1994; Turbill and Geiser, 2008).

The cost of endothermic arousals in bats may, in some cases, be proportionately greater than for other species, considering the higher relative surface area for heat loss attributed to wing membranes and the solitary nature of many tree-dwelling species. During torpor at low T_b, bats maintain average HRs below 40bpm and are capable of returning to normothermic T_b (~35°C) and HRs upwards of 600bpm in less than 1h (Kulzer, 1967; Rauch and Beatty, 1975; Currie et al., 2014). As many of these hibernating bats use torpor year round, often regardless of food availability or weather conditions (Stawski et al., 2014), energy savings can be maximised by the regular use of passive rewarming, particularly in summer when arousals are frequent, daily fluctuations in T_a are large and radiant heat is generally available (Bondarenco et al., 2014).

Many studies of free-ranging bats have shown that body temperature (T_b) during torpor fluctuates widely with T_a, from ~7°C in northern hemisphere bats (Halsall et al., 2012) up to 20°C in Australian vespertilionids (Turbill, 2006). Although for most bats passive rewarming is partial and complete arousal includes an active component, recent studies provide evidence that some desert dwelling bats rewarm entirely passively; using passive rewarming to increase T_b by more than 20°C (Bondarenco et al., 2013). Some bats select thermally labile roosts often choosing areas with afternoon sun exposure enabling them to maximize energy savings associated with fluctuating T_a (Hamilton and Barclay, 1994; Turbill, 2006). Thermal lability of roost microclimates allow bats to exploit cool morning T_a to minimize T_b and maximize energy savings, and warming temperatures/radiant heat later in the day to passively rewarm and reduce costs of normothermic thermoregulation (Willis and Brigham, 2005; Turbill et al., 2008). Although passive rewarming has been documented in a number of species, there are currently no published data detailing cardiovascular changes during passive rewarming from torpor or the relationship between HR, MR and T_b during this phase of arousal.

As passive rewarming may be important to minimize the energetic costs of endogenous arousal as well as oxidative and mechanical stress on the heart, I aimed to provide the first data on HR and MR during passive arousal from torpor in captive insectivorous long-eared bats (*Nyctophilus gouldi* ~10g). Bats exposed to fluctuating T_a profiles were compared with individuals maintained at a constant low T_a and required to rewarm actively. *N. gouldi* is a hibernating bat that uses torpor throughout the year in thermally labile roosts and often but not always passively rewarms from torpor in the wild (Turbill, 2006). As these hibernators maintain low MR during torpor through metabolic inhibition I was also interested in the interrelations between HR, MR and T_b during i) entry into torpor and ii) passive rewarming in comparison to iii) steady-state minimum values during torpor. During passive rewarming I predict a synchronized increase in HR and MR with increasing temperature qualitatively similar to that shown during active arousal; however, rates should reflect minimal values at corresponding T_bs in torpor well below rates during active arousal, or follow an intermediate trajectory between steady-state torpor values and those during active rewarming.

METHODS

Open-flow respirometry, ECGs, and temperature sensitive passive integrated transponders were used to measure the relationship between HR, MR (measured as rate of oxygen consumption- $\dot{V}O_2$) and subcutaneous temperature (T_{sub}) during passive rewarming from torpor in *Nyctophilus gouldi*. A total of 15 individuals were used for measurements (mass at capture 10.1±1.0g). Bats were captured with mist nests at Imbota Nature Reserve and Newholme Field Station near Armidale, New South Wales (30°35'S, 151°44'E) and kept in outdoor aviaries at the University of New England. Bats were provided with meal worms and water *ad libitum* and this was supplemented by moths and other flying insects that were attracted into cages by a UV light. Twice weekly mealworms were dusted with a supplement of Wombaroo™ Insectivore Rearing Mix. Bats remained within 1g of their body mass at the time of capture while in captivity.

This study was conducted under a scientific license provided by the NSW Parks and Wildlife Authority (SL100084) and with Animal Ethics approval from the University of New England (AEC12-043).

Transponder Implantation

Subcutaneous body temperature (T_{sub}) was measured using temperature-sensitive transponders (IPTT-300 Bio Medic Data Systems, Delaware, USA, 0.13g, 14mm x 2mm) implanted interscapularly. 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 (Wacker et al., 2012).

Prior to implantation bats were given a minimum of 3 days to acclimate to captivity and ensure they maintained a stable body mass. Bats were anaesthetized using general isoflurane/oxygen anesthesia and the skin sterilized with 70% alcohol before a small (~3mm) incision was made just below the shoulder blades for transponder insertion. The insertion site was closed with a single suture (chromic gut, Ethicon, Somerville USA) and the entire process was complete within fifteen minutes. Bats were given 24h to recover in a warm room before being returned to outdoor flight cages.

Respirometry and T_a Profile

Bats were placed into respirometry chambers in the early evening and were fasted to ensure they were post absorptive. $\dot{V}O_2$ was monitored overnight and throughout the following day(s) to allow animals to undergo their usual daily thermal cycle. Information regarding open-flow respirometry equipment and techniques are detailed in Currie *et al*. (2014). Respirometry chambers (0.40 or 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. Flow rate (170-200ml min^{-1}) was adjusted based on chamber size to ensure that 99% equilibrium was reached within 11 minutes. $\dot{V}O_2$ measurements were time adjusted to correspond with measurements of HR, accounting for lag of the system.

Chambers were placed inside a temperature-controlled cabinet with an incandescent light source set to the natural photoperiod at the time of year. Bats were either measured at a single T_a (between 5 and 25°C, *n*=12) which remained constant throughout the torpor bout or individuals were exposed to a diurnal T_a increase (from 5 to 23°C, *n*=5) similar to that experienced in their roosts in the warm season (adapted from Turbill et al., 2008). Bats were either exposed to the T_a profile following entry into torpor on the first day of measurement or following up to four days of hibernation. One bat remained in torpor when the T_a had reached 23°C and in this case T_a was gradually increased to 30°C before active arousal was induced. Basal metabolic rate (BMR) and basal heart rate (BHR) were measured following arousal from torpor while animals were resting and within the thermoneutral zone previously determined for this species (29-34°C; Geiser and Brigham, 2000).

ECGs and Ventilation

Heart rate was recorded using ECG following the methods of Currie *et al.* (2014). Individuals were placed in respirometry chambers in the evening and ECG wires (Lead I arrangement) were attached to adhesive electrodes on the bat's forearm just after lights on the following morning. Ventilatory movements were measured using a pulse transducer (MLT1010, ADInstruments, Bella Vista, Australia) that lay flush with the bat's chest and was sensitive enough to also detect cardiac contractions during apnoeic periods.

Statistical Analyses

Average minimum values of $\dot{V}O_2$, HR, and T_{sub} during torpor were taken from times when all variables were lowest for at least 30 min. During torpor all bats exhibited an episodic breathing pattern, which ceased when individuals were actively rewarming; thus active arousal was determined from the start of continuous ventilation. Arousal was assumed to end following a peak (overshoot) in $\dot{V}O_2$, and was measured until $\dot{V}O_2$ fell to ≤ 75% of that maximum. Energy expenditure of arousal (kJ) was calculated from mean $\dot{V}O_2$ (l/h)

multiplied by the time taken to arouse (h) and a conversion factor of 20.083 (Schmidt-Nielsen, 1997). Total torpor bout energy expenditure was calculated from the peak $\dot{V}O_2$ following the induced partial arousal prior to entry in the early morning until the peak following active arousal (indicated by arrows in Figure 1). Total torpor energy expenditure had to be time adjusted for one animal that rewarmed following four days of hibernation. This was done by integrating energy expenditure over torpor entry and part of steady-state torpor on the first day added to passive rewarming and arousal on the final day, for an overall torpor bout $\dot{V}O_2$ value. Fat requirements were calculated from energy expenditure assuming that metabolism of 1 mg of fat releases 39.3 J (Thomas et al., 1990).

Average $\dot{V}O_2$ and HR during passive rewarming, active arousal and entry (following partial arousal T_{sub}>17°C) were averaged over the same T_{sub} intervals to enable comparison. Values for resting $\dot{V}O_2$, HR, and T_{sub} were taken from the period following arousal. Due to 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 ~ 5cm, and therefore T_{sub} was occasionally unavailable. The Q_{10} for rates of $\dot{V}O_2$ or HR (R) of thermoconforming torpid bats and passively rewarming bats was calculated using the following equation: $Q_{10}=(R1/R2)^{10/(Tsub1 - Tsub2)}$.

Statistical analyses were performed using R v3.1.0 (R Core Team, 2014). Two sample t-tests were used to compare individual's mean $\dot{V}O_2$ and HR following arousal as well as the time taken to reach maximum $\dot{V}O_2$ at different T_a. Repeated measures analysis of variance (ANOVA) was used to compare mean $\dot{V}O_2$ and HR between passive rewarming and corresponding values during active arousal and torpor entry. Analysis of covariance (ANCOVA) was used to compare slopes and intercepts of the curves fitted to passive rewarming and torpor values. Standardized major axis regression was performed to assess the relationships between HR and $\dot{V}O_2$ using the smatr package, I accounted for pseudo-replication by adjusting the degrees of freedom as for mixed effect linear

modelling that are adjusted for repeated measures (Warton et al., 2012) . Means are reported ± SD for the number of individuals (*n*).

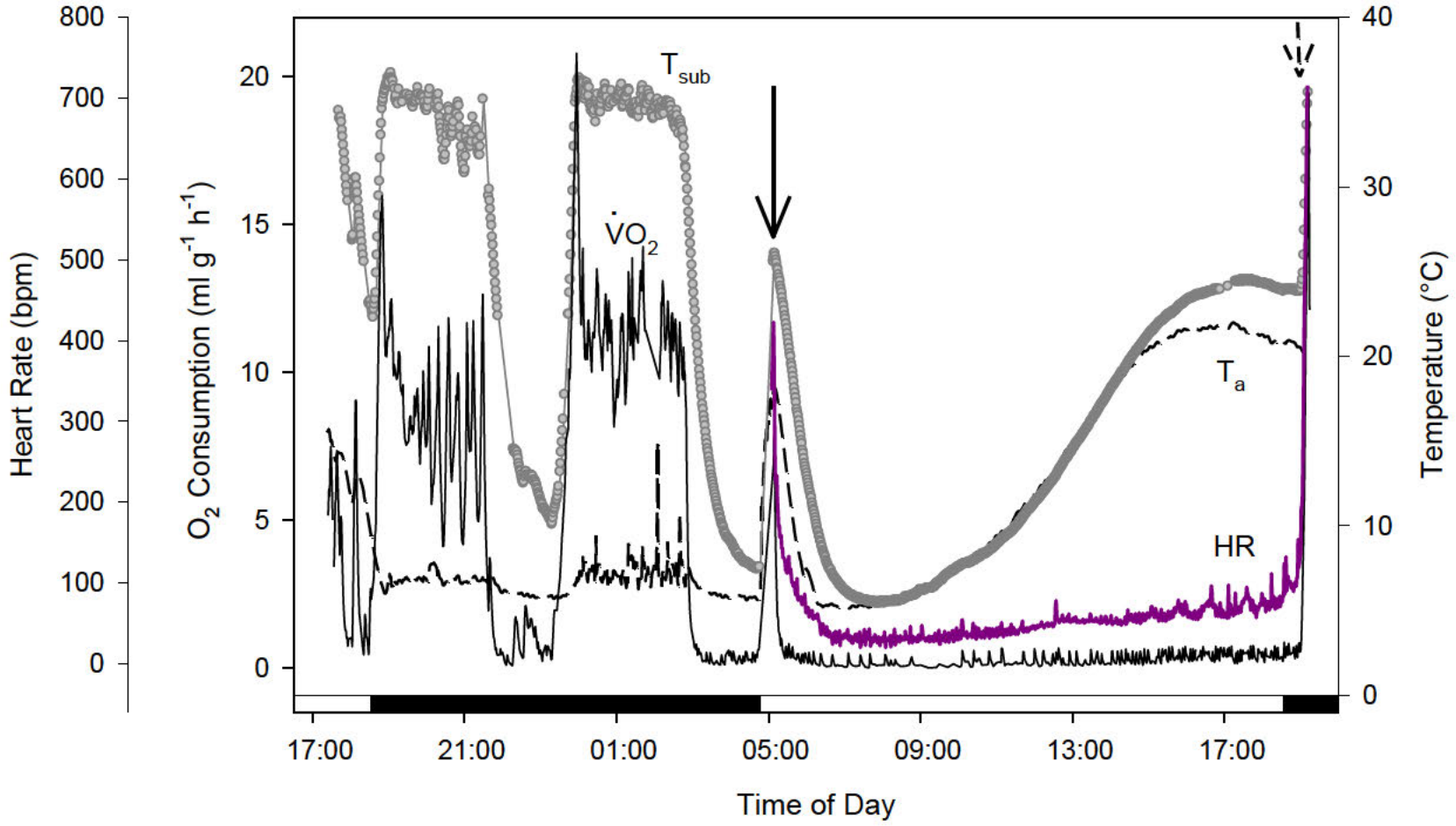

Figure 1. Representative example HR (purple line), $\dot{V}O_2$ (solid line), and T_{sub} (grey filled circles and line) of *Nyctophilus gouldi* exposed to an increasing T_a; the dark horizontal bar represents scotophase. The animal entered torpor in the early morning prior to lights on, exhibited a partial arousal associated with ECG lead attachment (indicated by solid arrow) and then proceeded to re-enter and remain in torpor until spontaneously arousing at lights off in the evening (indicated by dashed arrow).

RESULTS

Effects of T_a on resting HR and $\dot{V}O_2$

During periods of normothermy, metabolism and HR of bats declined linearly in a qualitatively similar pattern with exposure to increasing T_a (Figure 2). Average resting $\dot{V}O_2$ decreased from 10.37 ± 2.75 ml g^{-1} h^{-1} at a minimum average T_a of 5.9°C (*n*=7, T_{sub}=34.8 ± 1.1°C) to 4.86 ± 1.26 ml g^{-1} h^{-1} at an average T_a of 20.2°C (*n*= 8, T_{sub}=34.7 ± 1.1°C) (Figure 2). Correspondingly, resting HR fell inversely with T_a from an average 629 ± 84 bpm (*n*=7, T_{sub}=34.8°C) to 412 ± 95 bpm (*n*= 8, T_{sub}=34.7°C). When T_a increased to 30.5 ± 0.5°C HR fell to basal levels at 227 ± 34 bpm with corresponding BMR of 1.34 ± 0.16 ml O_2 g^{-1} h^{-1} (*n*=4, mass=9.9 ± 1.0g, T_{sub}=34.3 ± 0.6°C).

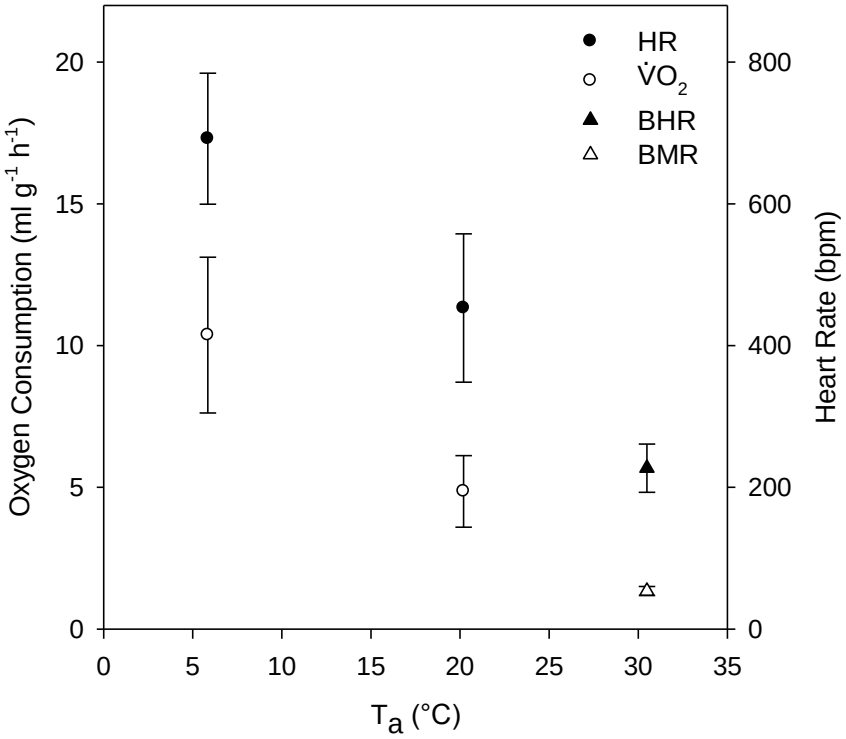

Figure 2. The relationship for HR (filled circles) and $\dot{V}O_2$ (open circles) against T_a in normothermic *N. gouldi* at rest and within the thermoneutral zone (filled and open triangles). Both HR and $\dot{V}O_2$ increased linearly as temperatures decreased outside of the thermoneutral zone.

HR and MR during entry into torpor

All bats, at all T_a tested, entered torpor either overnight or in the early morning following lights on (Figure 1). Following a partial arousal associated with attachment of ECG leads

in the morning all bats re-entered torpor. As animals re-entered torpor, HR and $\dot{V}O_2$ fell exponentially with decreasing T_{sub} (HR Q_{10}=2.2, $\dot{V}O_2$ Q_{10}=3.1) (Figure 3A & B) and steady-state minimum values of HR, $\dot{V}O_2$, and T_{sub} during torpor were reached within 2-3hrs of partial arousal. Figure 4 shows a representative ECG of one animal at T_a of 15°C at rest (Figure 4A) and during steady-state torpor (Figure 4B). When animals were in torpor at T_{sub} 16.5 ± 1.2°C HR fell to 31 ± 11bpm with average PR interval 0.11s, QRS width 0.03s and QT time 0.09s; it was not possible to derive these values from ECGs of normothermic bats. As expected, HR and $\dot{V}O_2$ were significantly lower during cooling than when animals actively rewarmed (ANOVA, $p < 0.05$).

Interestingly however, at any given T_{sub} during entry into torpor (T_a=8.1 ± 2.7°C) both HR and $\dot{V}O_2$ were significantly higher when compared to corresponding T_{sub} of animals in steady-state torpor (ANOVA, $p<0.001$). The slope of the relationship between $\log_{10}$HR and T_{sub} differed significantly for animals that were entering torpor compared to animals that passively rewarmed (ANCOVA, $p<0.01$). In contrast, there was no significant difference between torpor entry and passive rewarming with regards to the slope of $\log_{10}\dot{V}O_2$ against T_{sub} (ANCOVA, $p=0.08$), however the intercept for passive rewarming was significantly higher (ANCOVA, $p<0.05$).

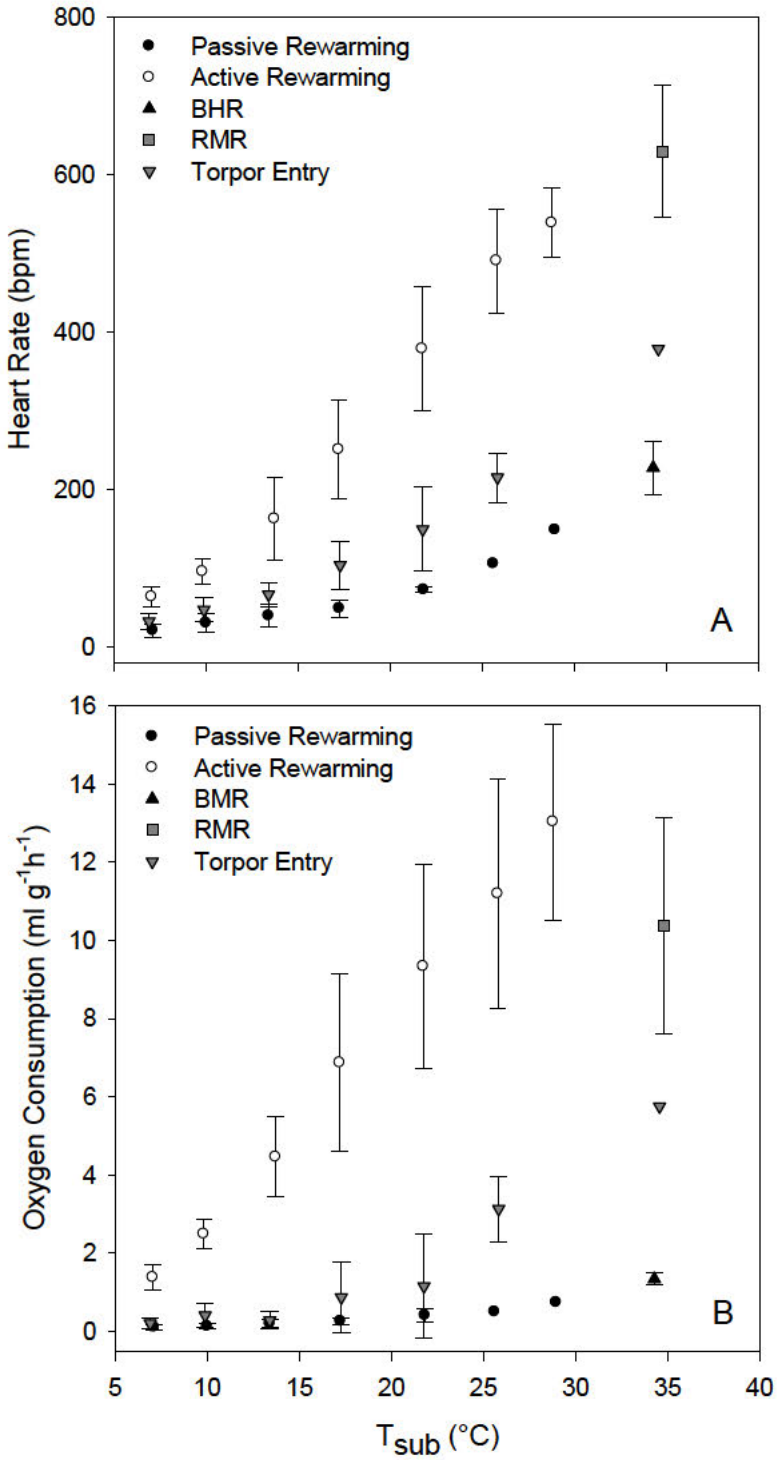

Figure 3A) The relationship between average HR and T_{sub} of *N. gouldi* during entry into torpor (grey inverted triangles), passive rewarming from torpor (filled circles), active arousal from torpor at average T_a=5.5°C (circles), RHR at T_a=5.9°C (grey square) and BHR (black triangle). Torpor entry and passive rewarming showed a curvilinear response to changing T_{sub} and were well below HR at the corresponding T_{sub} during active arousal. **B)** Corresponding $\dot{V}O_2$ averages as plotted against T_{sub} (symbols are as for A). $\dot{V}O_2$ showed a similar temperature dependent response as HR. Data points for passive rewarming beyond 20°C and entry beyond 25°C are averages taken from a single animal.

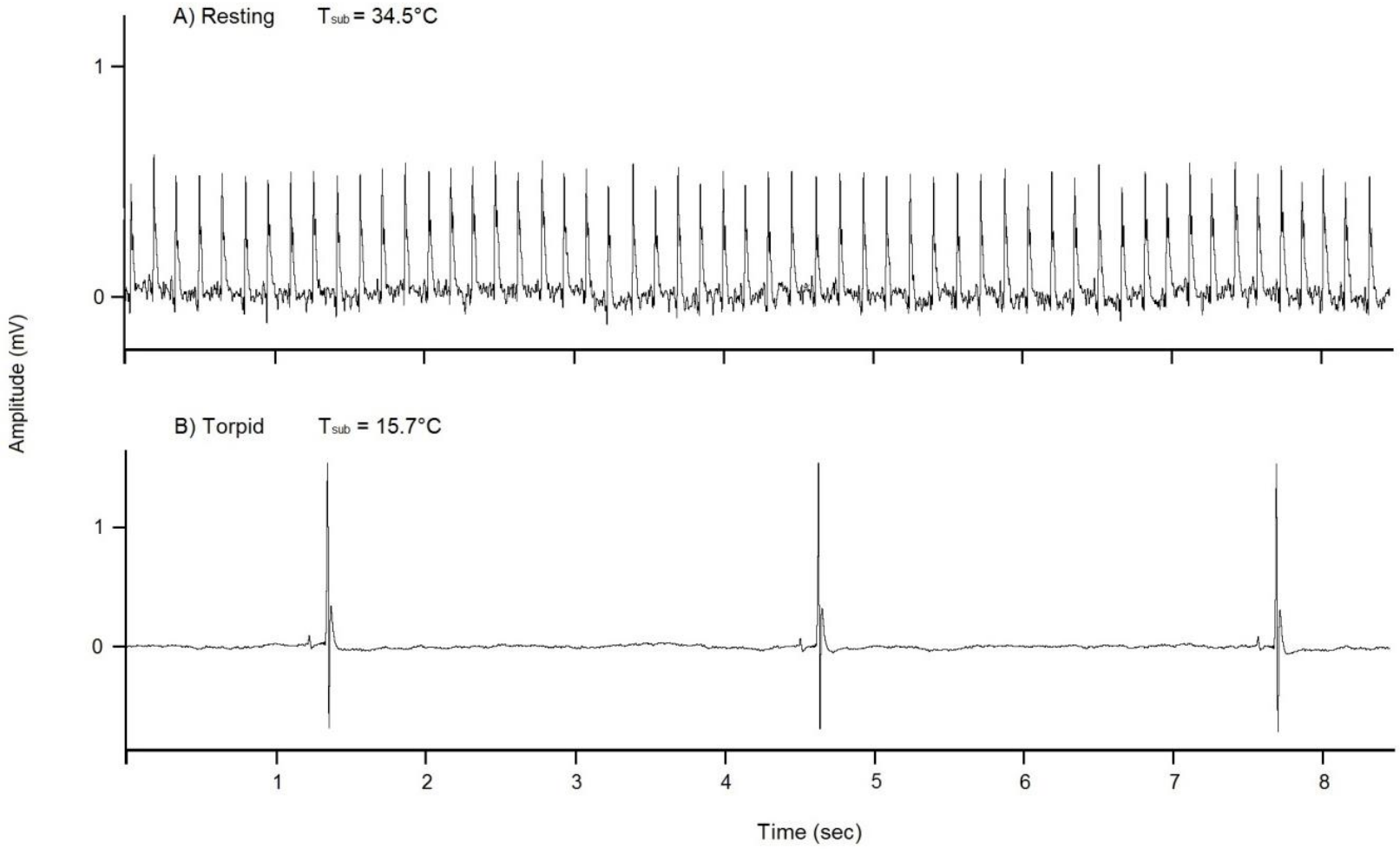

Figure 4. Representative trace of an ECG for one individual *N. gouldi* at T_a=15°C at rest (A; HR ~400bpm, T_{sub}=34.5°C) and during torpor (B; HR ~18bpm, T_{sub}=15.7°C). Note the y axis scaling.

Effect of increasing T_a on HR, $\dot{V}O_2$ and initiation of active arousal

When bats were exposed to an increasing T_a, 3 of 4 individuals aroused from torpor before the T_a profile reached a 23°C plateau; active arousal began at an average T_{sub} of 19.5 ± 1.2°C following passive rewarming. One individual rewarmed passively with the T_a profile but did not arouse actively until the stimulus of lights off when T_{sub} was 23.9°C and HR had reached 137bpm (Figure 1). Both $\dot{V}O_2$ and HR increased exponentially with increasing T_{sub} during passive rewarming with an average $\dot{V}O_2$ Q_{10} of 2.5 and HR Q_{10} 2.4 (Figure 5A & B). $\dot{V}O_2$ increased ~6-fold from a minimum of 0.06 ± 0.04 ml g^{-1} h^{-1} (*n*=5, T_a=7.0 ± 0.1°C, T_{sub}=6.2± 1.0°C) at the beginning of T_a increase to 0.35 ± 0.06 ml g^{-1} h^{-1} (*n*=3, T_a=18.8 ± 1.2°C, T_{sub}=19.5°C) just prior to endogenous arousal. Across the same temperature range HR only increased ~3-fold from 17 ± 5 bpm (*n*=5, T_{sub}=6.2°C) to 48 ± 18 bpm (*n*=3, T_{sub}=19.5°C). The Q_{10} of highest average $\dot{V}O_2$ during passive rewarming compared to BMR was 3.2 and for highest average passive rewarming HR to BHR it was 2.3 (Figure 5A & B).

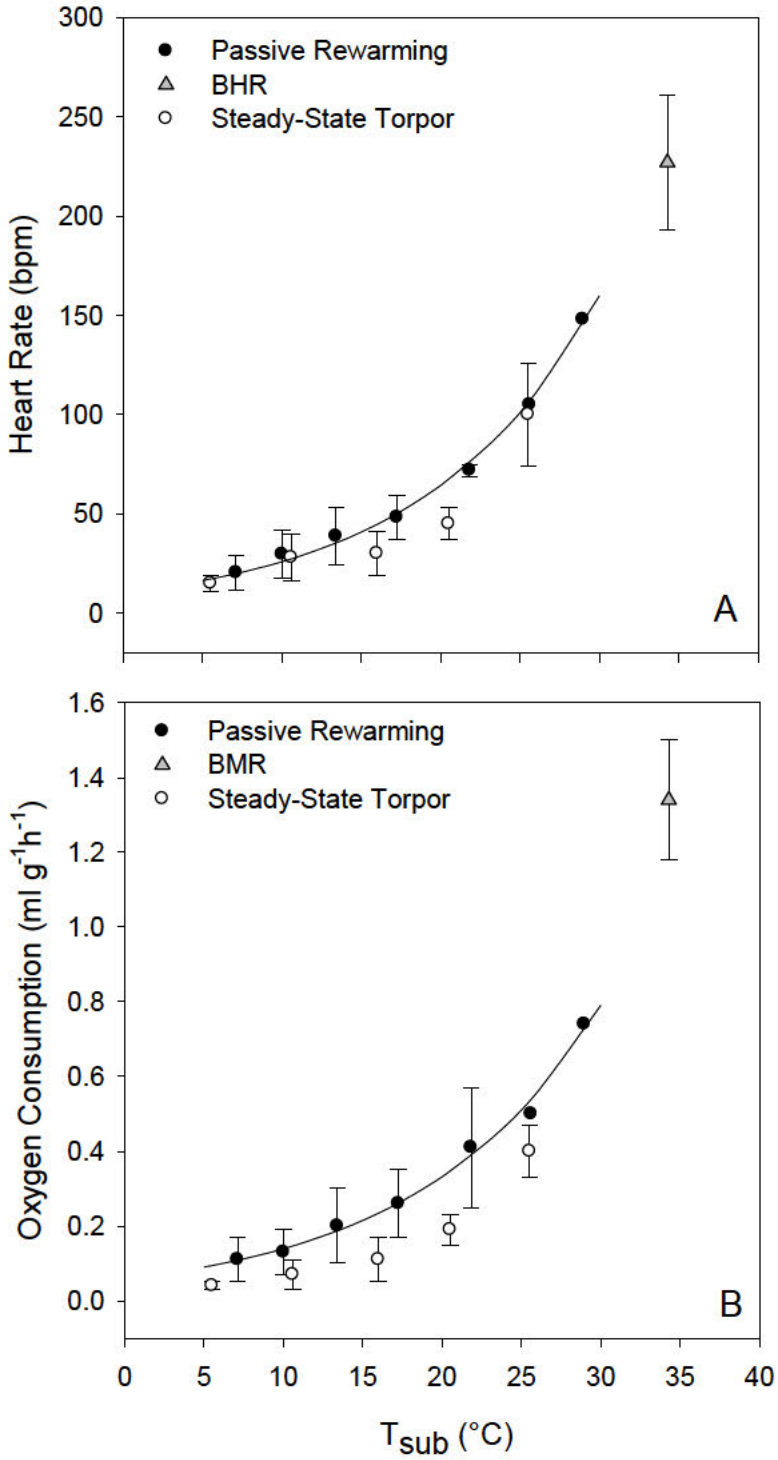

Figure 5A) Average HR against T_{sub} for steady-state thermoconforming *N. gouldi* (open circles), during passive rewarming (filled circles), and BHR (grey triangle): HR = 10.413*1.096^T_{sub}. **B)** Average $\dot{V}O_2$ against corresponding T_{sub} and BMR (symbols are as for A): $\dot{V}O_2$ = 0.058*1.091^T_{sub}. Both HR and $\dot{V}O_2$ showed a curvilinear response to increasing T_{sub} during passive rewarming and steady-state torpor. Data points for passive rewarming beyond 20°C are averages taken from a single animal.

The curve fitted to average $\dot{V}O_2$ during passive rewarming fell above values for animals that were thermoconforming in steady-state torpor at a similar T_{sub} (Figure 5B). There was no significant difference between slopes (ANCOVA, p=0.26), but the intercept for passive rewarming $\dot{V}O_2$ was significantly higher than for steady-state torpor (ANCOVA, p<0.001). In contrast both the slope and intercepts of the curve fitted to HR against T_{sub} for bats in steady-state torpor were not significantly different from passive rewarming (ANCOVA, p=0.64 and p=0.27 respectively) (Figure 5A).

Comparison of active and passive arousal and effect of T_a

On average, $\dot{V}O_2$ was 20.4-fold higher during active arousal compared to passive rewarming, with a maximum 26-fold difference in $\dot{V}O_2$ at T_{sub} 17.2 ± 0.4°C (Figure 3B). Although still significant, mean HR was only 4.2-fold higher on average in bats that actively aroused compared to passive rewarming and the maximum difference of 5.3-fold occurred at T_{sub} 21.8 ± 0.8°C (Figure 3A). During active arousal HR and $\dot{V}O_2$ differed in their relationship to increasing T_{sub} with HR increasing in a sigmoidal pattern while $\dot{V}O_2$ increased almost linearly (Figure 3A & B). Interestingly however, both HR and $\dot{V}O_2$ exhibited an overall 9-fold increase between T_{sub} 7.1 ± 0.5°C and 28.8 ± 0.3°C.

Following passive rewarming at the beginning of active arousal, clearly delineated by restoration of continuous breathing, HR increased substantially (>2 fold) over a one minute period (2 consecutive readings) from 70 ± 47 bpm to 150 ± 45 bpm (*n*=4). In contrast, when bats actively rewarmed from a low T_a (average 5.7 ± 1.2°C) the initial change in HR was negligible, increasing from 40 ± 9 bpm to only 54 ± 23 bpm (*n*=6) over one minute. Unlike HR, the initial minute increase in $\dot{V}O_2$ at active arousal was similar for both thermal conditions; increasing from 0.42 ± 0.13 to 0.56 ± 0.32 ml g^{-1} h^{-1} (*n*=4) after passive rewarming and from 0.35 ± 0.28 to 0.54 ± 0.33 ml g^{-1} h^{-1} (*n*=6) at constant T_a 5.7°C.

The peak arousal $\dot{V}O_2$ following passive rewarming was 10.96 ± 1.41 ml g^{-1} h^{-1} (*n*=3) and did not differ significantly from peak $\dot{V}O_2$ following entirely active arousal at T_a 20.6°C ±

1.1 (9.6 ± 0.99 ml g^{-1} h^{-1}, *n*=9; two-sample t-test, t=-1.37, df=7, p=0.21). There was also no significant difference between maximum arousal HR after passive rewarming (543 ± 45 bpm *n*=3) and the peak HR at constant T_a 20.6°C ± 1.1 (468 ± 95 bpm, *n*=9; two-sample t-test, t=-1.26, df=7, p=0.25). Not surprisingly, at low T_a (5.7°C) both maximum $\dot{V}O_2$ (14.32 ± 1.96 ml g^{-1} h^{-1}, *n*=6) and maximum HR (679 ± 65 bpm, *n*=6) were significantly higher following active arousal (two sample t-test, $\dot{V}O_2$: t=4.83, df=13, p<0.01, HR: t=4.19, df=13, p<0.01) than at T_a 20.6°C or following passive rewarming.

The time taken to reach maximum HR during active arousal did not differ significantly between animals that rewarmed passively and those kept at an average constant T_a of 20.6 ± 1.1°C (average 15 minutes, two sample t-test, t=-0.75, df=3.34, p=0.50). However, it took animals significantly longer (average 51 minutes) to reach maximum HR at T_a=5.7°C (two sample t-test, t=2.31, df=8, p<0.05). The time taken to reach maximum $\dot{V}O_2$ did not differ significantly from the time taken to reach maximum HR during all active arousals (paired t-test, t=0.19, df=15, p=0.85).

Energetic costs of rewarming

The cost of rewarming from torpor by *N. gouldi* kept at constant T_a of 5.7°C was 1.33 ± 0.39 kJ (*n*=5) which made up an average 62% (range 46%-75%) of total energy expenditure over the torpor bout (measured from the peak before entry through to arousal- indicated by arrows in Figure 1). When exposed to increasing T_a the total energy expenditure in torpor was reduced by 53% from 2.12 ± 0.31 kJ (T_a=5.7°C, *n*=5) to 1.0 ± 0.27 kJ (*n*=4). In addition, exposure to increasing T_a also reduced the cost of arousal by 68% to 0.42 ± 0.08 kJ (*n*=4). When only passive rewarming was considered, energy expenditure was 0.23 ± 0.21 kJ (*n*=4) and contributed only 34% to the total cost of arousal (0.70 ± 0.22 kJ, *n*=4), which included both passive and active portions.

DISCUSSION

Torpor imposes enormous demands on the cardiovascular system, especially during rewarming from low T_b when HR must increase dramatically from less than 10bpm to over

700bpm in a short time frame. My study is the first to quantify HR during passive rewarming from torpor and demonstrate the relationship between HR, $\dot{V}O_2$ and T_{sub} during entry into torpor and throughout passive and active arousal. As animals entered into torpor following an induced partial arousal, HR and $\dot{V}O_2$ decreased simultaneously followed by a drop in T_{sub} and this progressed in a qualitatively similar pattern to other hibernating species (Milsom et al., 1999). Torpor re-entry was characterized by the onset of regular apnoeas and a return to the episodic breathing pattern typical of hibernating bats. As anticipated, there was a similar exponential pattern of increase in both HR and $\dot{V}O_2$ during passive rewarming that corresponded to rising T_{sub}. However, these values represented an intermediate between active arousal and steady-state minimum values during torpor. When animals actively aroused, HR and $\dot{V}O_2$ increased at a substantially greater rate than at the same T_{sub} during both entry into torpor and passive rewarming.

This is also the first study to present BHR for an Australian insectivorous bat. Previous investigations report minimum HR of bats ranging from 128 to 235bpm in species weighing between 56 and 825g (T_a=19-35°C) (Bartholomew et al., 1964; Leitner, 1966; Leitner and Nelson, 1967). BHR recorded for *Nyctophilus gouldi* fell within this range at 227bpm, even though animals were much smaller (9.9 g), and was 50% of the BHR predicted from the allometric equation $HR= 816 \times BM^{-0.25}$ (Wang and Hudson, 1971). BMR reported in my study (1.34 ± 0.16 ml O_2 g^{-1} h^{-1}) was averaged over the same period as BHR and was indistinguishable from previously reported values for *N. gouldi* of 1.22 ml O_2 g^{-1} h^{-1} (Geiser and Brigham, 2000).

Heart rate showed a similar qualitative pattern to $\dot{V}O_2$ during both passive rewarming and entry into torpor, changing exponentially with T_{sub}. However, at each T_{sub} interval during passive rewarming HR and $\dot{V}O_2$ fell slightly above corresponding values when animals where in steady-state torpor, and this difference was even greater for torpor entry. It has been well documented that during steady-state torpor in hibernators metabolic inhibition maintains HR and metabolism at low levels (Storey and Storey, 2004). Although there is still an effect of temperature on cardiac and metabolic function, steady-state torpor values

fall below what would be expected if temperature was the primary driving force (Ruf and Geiser, 2014). It is not known however, how important metabolic inhibition is during entry into torpor or at which point inhibition is initiated. In ground-squirrels (*Spermophilus lateralis*) depression of mitochondrial respiration did not occur until animals were in deep torpor and this suggests that active mitochondrial inhibition may not be critical during entrance into hibernation (Martin et al., 1999). My results support this interpretation and indicate that the effects of metabolic inhibition may be relatively small early in torpor entry and increase as torpor entry proceeds and animals become deeply torpid. Furthermore, my data suggest that as animals begin to passively rewarm, metabolic inhibition is withdrawn resulting in higher $\dot{V}O_2$ and HR values that increase in a temperature-dependent fashion (Q_{10} ~2.5). As rewarming is a transitional period, the removal of metabolic inhibition would facilitate the onset of active arousal once the animal is warmed to a critical T_b. I would expect however, that if temperature did not continue to rise to a critical value for arousal, as was the case with the imposed T_a profile, animals would have remained torpid and metabolic inhibition would have been restored, reducing $\dot{V}O_2$ and HR to steady-state values.

When bats were rewarmed from 7°C after torpor bouts <24hrs, the critical T_{sub} inducing arousal was 19.5°C which was comparable to that found for *Nyctophilus geoffroyi* exposed to a similar T_a profile (T_{sk}=21.4°C) (Turbill et al., 2008) and free ranging *Eptesicus fuscus* passively rewarmed during the hibernation season (T_{sk}=17.9°C) (Halsall et al., 2012). At the onset of active arousal there was a doubling of $\dot{V}O_2$ when animals rewarmed from both the constant T_a and following passive rewarming. However, the immediate increase in HR at active arousal was substantially higher in bats that were passively rewarmed when compared to those at low T_a. This clear difference in cardiac capacity is likely directly related to temperature of the heart at the corresponding T_{sub}s. Although hibernators' hearts are well adapted to maintain coordinated activity at low T_b, low temperatures still impede many aspects of cardiac function. For example, isolated hearts of *Myotis lucifugus* showed a limited scope for HR increase at low temperatures

but clear improvement in scope above 20°C (Michael and Menaker, 1963). In addition, contractility of isolated myocardium of a number of hibernators declined rapidly below 15°C, with maximum contractility observed between 15.5 and 24°C (Smith and Katzung, 1966; South and Jacobs, 1973; Caprette and Senturia, 1984). As the critical T_{sub}/T_{sk} for active arousal in a number of temperate bat species is around this 20°C threshold it suggests that rewarming the heart prior to active arousal may be beneficial to reduce physiological stress of arousal. The importance of the heart as a thermogenic organ during arousal has been demonstrated in a number of hibernating species (Lyman et al., 1982), and therefore maximizing cardiac performance at the start of active arousal would clearly be advantageous. Also, considering that early stage arousal from low T_b likely imposes the greatest demands on the cardiovascular system, warming the heart prior to active arousal may reduce mechanical stresses as well. As such, I suggest that optimal T_b for peak cardiac capacity may also influence the critical threshold temperatures for active arousal following passive rewarming.

Because the cost of arousal increases with the time taken to arouse (McKechnie and Wolf, 2004) it would be worthwhile for bats to increase HR to maximal levels as quickly as possible during active arousal in order to shorten arousal time. During arousal the risk of oxidative stress is at its highest as metabolism increases dramatically and peak concentrations of ROS have been shown to correspond to periods of maximum oxygen consumption during rewarming (Carey et al., 2003). Although hibernators possess mechanisms to combat oxidative stress, such as up-regulation of antioxidants (Morin and Storey, 2007), it is possible that during arousal these defences may not be entirely sufficient. As such, reducing arousal times through passive rewarming may also help to minimize exposure to ROS and/or increase efficiency of antioxidant defences.

Passive rewarming resulted in substantial energy savings when compared to the cost of active arousal at a low T_a. When this is extrapolated to fat usage, approximately 16.2 mg less fat is required on average when bats are exposed to increasing T_a than when forced to actively arouse. Free-ranging male *N. gouldi* were shown to employ torpor twice per

day on 80% of tracking days during late spring and regularly rewarmed passively from torpor (Turbill, 2006). Here, I exposed bats to similar thermal conditions and demonstrate that a single arousal (including passive rewarming) requires the metabolism of ~18 mg of fat in this species. Therefore, the overall extrapolated cost of rewarming in these animals would equate to ~36 mg of fat per day for the two arousals. This amounts to a savings of >225 mg of fat per week compared to the cost of wholly active arousal. In addition, the savings attributed to passive rewarming in this study mirror those shown for other bat species in captivity (*Nyctophilus geoffroyi*, Turbill et al., 2008) and in the wild (*Eptesicus fuscus*, Halsall et al., 2012).

Measurements of the energy savings attributed to torpor use and costs of arousal are crucial to the study of bat biology as torpor is so widely used by these animals. Heart rate has been suggested as a viable method for predicting oxygen consumption in the field, and this has been supported by a study on torpid and resting bats (Currie et al., 2014). As heart rate can provide information regarding energy expenditure over small time scales, such as the rewarming phase of torpor, and this is the most energetically expensive phase of torpor, one aim of my study was to examine whether or not a strong correlation exists between HR and $\dot{V}O_2$ for both passive and actively rewarmed bats. My data show a strong positive correlation for both active ($p<0.01$, $r^2=0.94$) and passive arousal ($p<0.05$, $r^2=0.74$) with no overlap between the two states (Figure 6). This suggests that HR may be a good predictor of $\dot{V}O_2$ during these phases, but that they must be distinguished from one another, indicating the importance of simultaneous measurement of T_{sk} and T_a.

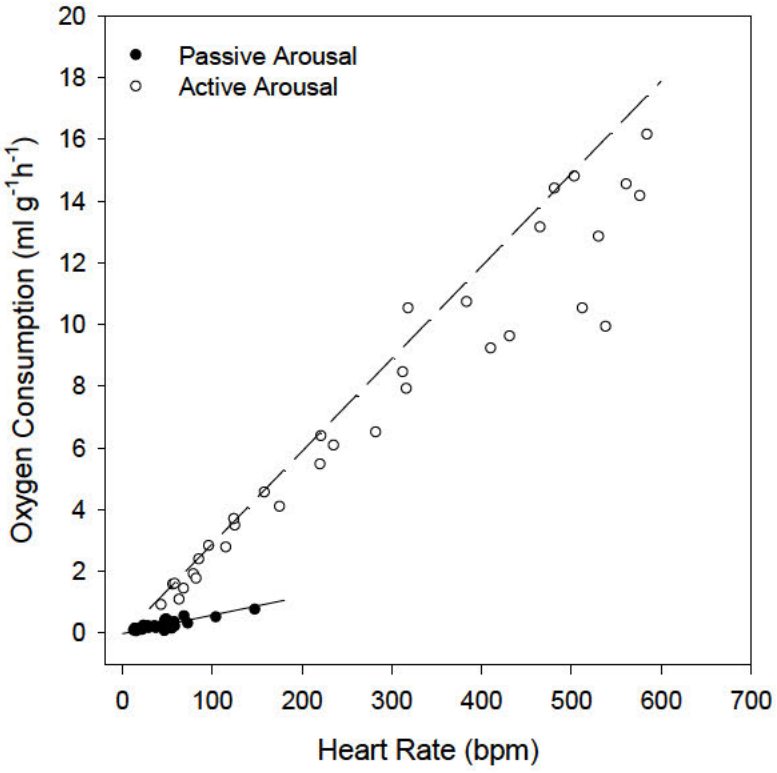

Figure 6. $\dot{V}O_2$ as a function of HR during active arousal (open circles) and passive arousal (filled circles) for *N. gouldi*. Dashed line represents the regression equation for active arousal ($\dot{V}O_2$ = 0.03(HR) – 0.11, r^2=0.94, p<0.01) and the solid line represents the regression equation for passive arousal ($\dot{V}O_2$= 0.005(HR) - 0.016, r^2=0.74, p<0.05).

Passive rewarming is used by many bat species to reduce energetic costs of arousal from torpor (Willis et al., 2006; Rambaldini and Brigham, 2008; Stawski et al., 2009; Bondarenco et al., 2013). My study demonstrates the importance of temperature on cardiac capacity during this phase and the possible role this plays in signalling arousal from torpor and reducing stress on the cardiovascular system. I suggest that the ability to rewarm passively may enable individuals to raise T_b to optimal levels for maximal cardiac performance and swift arousal without the need for excessive energy expenditure initially. My results also show that metabolic inhibition may not be fully applied until late stage entry into torpor and that it is at least partially withdrawn during passive rewarming as a response to temperature increases and as a precursor to active arousal. Unfortunately, little information exists regarding changes in metabolism associated with torpor use under natural conditions, including fluctuating T_a. My results show a strong correlation between HR and MR throughout different phases of arousal. Therefore I suggest further study to

examine the possibility of HR telemetry as a means of predicting MR in free-ranging heterothermic animals. The extensive and flexible use of torpor in bats has been suggested to play a vital role in long-term survival of these animals (Stawski et al., 2014), and as such the understanding of energy use in the wild has strong implications for their future management and survival.

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STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

	Author's Name (please print clearly)	% of contribution
Candidate	Shannon E Currie	80%
Other Authors	Kodie Noy	10%
	Fritz Geiser	10%

Name of Candidate: Shannon E Currie

Name/title of Principal Supervisor: Professor Fritz Geiser

[redacted]

Candidate

1/3/15

Date

[redacted]

Principal Supervisor

1/3/15

Date

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Name of Candidate: Shannon E Currie

Name/title of Principal Supervisor: Professor Fritz Geiser

[redacted] 1/3/15

Candidate Date

[redacted] 1/3/15

Principal Supervisor Date

Chapter 5

Heart rate and metabolism in heterothermic bats: Comparison of a daily heterotherm and a hibernator

ABSTRACT

Torpor is essential to the survival of many bat species. However the patterns of torpor among bats vary between strict daily heterothermy, seasonal hibernation and short-term hibernation. Although previous studies have demonstrated significant differences between daily heterotherms and hibernators with regard to the duration and depth of torpor, little information is known about the differences in cardiac function between these two groups. I therefore aimed to quantify the cardiac physiology of two small heterothermic bat species that express either strict daily heterothermy (18g *Syconycteris australis; Sa*) or hibernation (10g *Nyctophilus gouldi; Ng*) and show habitat overlap along the east coast of Australia. Comparisons were made between HR, $\dot{V}O_2$, and T_{sub} measured simultaneously at rest and during torpor for each species at a range of T_a. At T_a around 15.0°C HR of normothermic resting bats was similar for both species at 482 ± 42 bpm (*Sa*; n=3, T_{sub}=33.8 ± 0.9°C) and 431 ± 88 bpm (*Ng*; n=7, T_{sub}=34.4 ± 1.5°C); whereas during torpor at the same T_a, HR only fell to a minimum of 74 bpm in *S. australis* (T_{sub}=16.3°C) while *N. gouldi* HR fell as low as 14 bpm (T_{sub}=15.6°C). Similarly, corresponding resting $\dot{V}O_2$ was comparable between the species at 4.31 ± 0.46 ml $g^{-1}h^{-1}$ (*Sa*; n=3, T_{sub}=33.8 ± 0.9°C) and 4.79 ± 1.91 ml $g^{-1}h^{-1}$ (*Ng*; n=7, T_{sub}=34.4 ± 1.5°C). However, minimum $\dot{V}O_2$ in torpor was again substantially higher for *S. australis* (0.26 ml $g^{-1}h^{-1}$, T_{sub}=16.4°C) than for *N. gouldi* (0.04 ml $g^{-1}h^{-1}$, T_{sub}=15.6°C). Although the thermal response of the heart of torpid *S. australis* was qualitatively similar to that of hibernating *N. gouldi*, the HR of the hibernator was only 35% of that in the daily heterotherm at the same T_{sub}. Moreover, the slope and

the intercept of the relationship between HR and $\dot{V}O_2$ differed significantly. My study provides the first quantitative data of HR as a function of temperature for a 'fruit bat' during torpor and demonstrates a clear difference between HR and $\dot{V}O_2$ and T_{sub} during daily heterothermy and hibernation, at least in bats.

INTRODUCTION

Bats are unique amongst mammals as the only order capable of powered flight. Coupled with this expensive form of locomotion, bats have a limited capacity for fat storage and the large surface area of their wings and bodies promotes heat loss making the cost of living extremely high, particularly for small species. Moreover these animals are active at night when, at higher latitudes, ambient temperatures (T_a) are low and in harsh conditions endothermy may prove prohibitively expensive. Consequently, many bats use torpor, primarily to minimize energy expenditure during rest, and it is highly likely that the majority of small bat species are capable of entering torpor in one form or another (Stawski et al., 2014).

Torpor is expressed in two main forms; daily, short-term torpor in daily heterotherms or multiday hibernation in hibernators. In general the delineation of pattern is most pronounced for torpor bout duration with daily heterotherms only entering torpor for <24hrs and hibernators capable of remaining torpid for up to many days. In bats this is confounded by the common use of short torpor bouts by hibernators that temporally resemble daily torpor, but are in fact metabolically indistinguishable from hibernation at the same ambient temperature (T_a) (Geiser and Brigham, 2000). During torpor, body temperature (T_b) generally falls to within 1-3°C of T_a depending on thermal conditions. Above a critical threshold, T_b will follow T_a and during this time animals are considered to be thermoconforming (Geiser, 2004), however below this critical threshold T_b animals begin to thermoregulate in torpor. Minimum T_b during torpor has therefore also been used to differentiate between torpor patterns, however, this has been suggested to be misleading as a sole predictor as it is highly influenced by body mass and T_a conditions (Geiser and Ruf, 1995). More recently metabolic rate (MR) in torpor and its relationship to

basal metabolic rate (BMR) have been incorporated into these comparisons using phylogenetic analyses and these show a clear bimodal distinction between the two types of torpor expression (Ruf and Geiser, 2014). Although metabolism has been shown to be crucial in understanding the ways in which animals express torpor, very little attention has been paid to the likely differences in cardiac parameters of daily heterotherms and hibernators. Moreover, information regarding the relationship between MR, heart rate (HR) and T_b are entirely lacking in the former.

Torpor use in bats has previously been assumed to be restricted to temperate zone species (Stones and Wiebers, 1965). However, more recently evidence has demonstrated extensive use of torpor in both tropical and subtropical bat species (Geiser and Stawski, 2011). Although these bats are exposed to mild temperatures year round, supposedly counterintuitive to torpor use, even multiday bouts of torpor have been observed in tree roosting (Stawski et al., 2009), house dwelling (Cory Toussaint et al., 2010) and cave dwelling species (Liu and Karasov, 2011), and daily torpor is commonly expressed even in some small frugivorous/nectarivorous pteropodids (Geiser et al., 1996; Bartels et al., 1998). Although the greatest energy savings attributed to torpor occur at low T_a and in hibernating species, torpor use even at mild temperatures can result in energy savings of >85% (Geiser and Stawski, 2011). Moreover, many of these tropical or subtropical bats will use torpor almost daily, even in conditions of high food abundance (Stawski and Geiser, 2010) suggesting that other factors may be influencing the patterns we see. It is possible then, that the driving pressures for torpor use in these species relate to evaporative water loss, predator avoidance, and seasonality of food sources (particularly in the specialist nectarivores), as well as the restriction of foraging times, rather than simply harsh temperature conditions. While the majority of research on torpor in bats has been on insectivorous species in the temperate zone, which hibernate and express multiday torpor, detailed knowledge about daily heterothermy in the Pteropodidae ('fruit bats') is scant.

My study compared HR, MR and subcutaneous temperature (T_{sub}) throughout torpor and at rest for two species of bat that express either daily torpor (the blossom bat, *Syconycteris australis*), or hibernation (the long-eared bat, *Nyctophilus gouldi*). I aimed to investigate whether cardiac function differs between daily heterotherms and hibernators of a similar size from the same mammalian order and how this relates to metabolism. *S. australis* are nectarivorous blossom bats from tropical and subtropical eastern Australia and have been shown to use daily torpor throughout the year. These bats use deeper and longer torpor bouts in summer related to reduced availability of nectar from flowering *Banksia* spp. (Law, 1993; Coburn and Geiser, 1998). Torpor bouts are generally restricted to <12h and minimum T_b during torpor is ~18°C (Geiser et al., 1996). The propensity of these bats to enter torpor has been suggested to have enabled *S. australis* to extend its range into higher latitudes where it overlaps with temperate species such as *N. gouldi* (Law, 1994; Bonaccorso and McNab, 1997). *N. gouldi* are insectivorous long-eared bats that hibernate in winter and often express short-term torpor bouts throughout the year (Turbill, 2006). These bats maintain a low T_b during both short and long torpor bouts with minimum T_b ~2°C (Geiser and Brigham, 2000).

METHODS

Adult male *S. australis* (*Sa*) were caught in mist nets at Iluka Nature Reserve on the north coast of NSW, Australia (29°24'S, 153°22'E) at sea level in June 2011. Bats were kept indoors in a large tent for four nights before being transferred to the University of New England (UNE), Armidale. Bats were initially hand-fed to ensure they maintained body weight, but were also given food *ad libitum* while in the tent. Their diet consisted of a blended mixture of fruit, juice and protein supplements (recipe and methods of dilution available in Geiser et al., 1996). Bats were housed in a large indoor flight cage (2×2×2m) at UNE, which was equipped with branches and large stands of foliage for bats to roost in. Temperature of the room was maintained at 20-22°C, with relative humidity >55% and animals were exposed to natural light. Food was available *ad libitum* in modified plastic syringes that acted as feeders and were placed among branches, often near to *Banksia*

sp flower heads that were replaced every few days. Feeders were refilled daily and washed/soaked overnight in Milton antibacterial solution to minimize microbial growth. Water was available in birdfeeders.

Adult *N. gouldi* (*Ng*) were netted at Imbota Nature Reserve and Newholme Field Station near Armidale, NSW (30°35'S, 151°44'E) at 1000m elevation between May 2011 and October 2013. Bats were transported to UNE on the night of capture, given water and hand fed. Individuals were kept in large outdoor flight cages (3×1.5×2m) with a maximum of eight animals per cage, and provided with mealworms and water *ad libitum*. Mealworms were dusted with a supplement of Wombaroo™ Insectivore Rearing Mix twice a week. Additional food was supplied in the form of moths and other flying insects, and these were attracted into cages by a UV light.

All bats were kept in captivity for a maximum period of seven months and each individual remained within 1g of their body mass at the time of capture while in captivity. This study was conducted under a scientific license provided by the NSW Parks and Wildlife Authority (SL100084) and with Animal Ethics approval from the University of New England (AEC11-016 and AEC12-043).

Transponder Implantation

Temperature-sensitive transponders (IPTT-300 Bio Medic Data Systems, Delaware, USA) were used to measure subcutaneous body temperature (T_{sub}). 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 interscapularly under general Isoflurane/oxygen anaesthesia. The skin was sterilized with 70% alcohol before a small (~3 mm) incision was made in the skin just below the shoulder blades for insertion. The insertion site was closed with a single suture (chromic gut, Ethicon, Somerville, MA, USA) and the entire process was complete within fifteen minutes. Bats were given 24h to recover in small

individual cages a warm room before being returned to either outdoor flight cages (*N. gouldi*) or the large indoor cage (*S. australis*).

Respirometry

Bats were placed in respirometry chambers in the late afternoon/early evening and $\dot{V}O_2$ was measured for the following day (at least ~24 h). Bats were weighed (to 0.1g) before the start of experimentation and immediately after being removed from respirometry chambers. A linear rate of mass loss was assumed to calculate mass-specific $\dot{V}O_2$ values. Bats were only exposed to T_as within the typical range of their natural habitat, as such the *N. gouldi* were measured from T_a 33°C down to 0°C and *S. australis* were only measured to minimum T_a 12°C. During this period bats were exposed to a natural photoperiod adjusted to suit their native habitat at that time of year.

Oxygen concentration of excurrent air was measured using either a FC-1B Oxygen Analyser or FOX Field Oxygen Analyser (Version 1.01, FXO301-01R, Sable Systems, Las Vegas, NV, USA). Measurements were taken from airflow through the chamber every minute for 15min and then switched to outside air for reference readings (3min) using solenoid valves. Chamber T_a was measured to the nearest 0.1°C using a calibrated thermocouple placed ~5mm within the chamber. Outputs of the digital thermocouple thermometer, flowmeter and oxygen analyser were recorded using custom-written data-acquisition software (Gerhard Körtner) onto a personal computer and $\dot{V}O_2$ calculated using equation 3A of Withers (1977). T_{sub} was read from each animal with a DAS-7006/7R/S Handheld Reader (Bio Medic Data Systems, Delaware, USA) which was connected to a personal computer and programmed to take readings every minute, concurrent with respirometry measurements.

As body mass and roosting posture differed between the two species, respirometry chambers were of different sizes and roosting material was also different. *N. gouldi* were placed in rectangular polycarbonate chambers (0.26, 0.40, or 0.53 L) where they roosted flush with the back wall of the chamber and clung to a small patch of hessian cloth. *S.*

australis preferred to hang in the centre of the chamber (glass jar 0.75 L) where they were free hanging and not touching the glass. Individuals roosted from plastic mesh supported horizontally near the roof of the chamber by a wire frame. Flow rate was adjusted (180-290 ml min^{-1}) to ensure that 99% equilibrium was reached in <15min (in most cases this was <12min). $\dot{V}O_2$ measurements were time adjusted for lag of the system, but not washout characteristics of the chambers, to correspond with measurements of HR and T_{sub}.

ECG measurements

For both species of bat ECGs could only be measured during the day as the animals did not tolerate electrode wires during their active phase (overnight). Following lights on in the morning, most bats were either already torpid or had begun to enter torpor (depending on T_a and species) and at that point ECG electrode wires were attached to the bats' forearms (lead I arrangement). This disturbance resulted in a partial arousal and bats either remained normothermic and resting for the remainder of recording or returned to torpor, either with or without ECG electrodes attached.

N. gouldi ECG

Electrodes were cut from adhesive Kendall Care Resting ECG Electrodes (Tyco Healthcare Group, Mansfield, USA) into strips of appropriate length and width to fit the forearm of the *N. gouldi*, and the animals showed no aversion to them during the rest phase. More detail regarding ECG wires etc. is available in Chapter 3 or Currie et al. (2014).

S. australis ECG

S. australis individuals have a much shorter forearm area which was not suitable for attachment of adhesive electrodes; as such a different method for obtaining ECGs was used. ECG electrodes for the *S. australis* were made from metal ear tags for mice that were modified to fit around the forearm of the bats in a similar fashion to bat ID bands

(Figure 1). Bands were kept on the animals throughout time spent in captivity and only one bat experienced irritation associated with the bands; the band was removed from this animal and it was not used in experiments until the skin was completely healed. Electrode leads were made from modified Kittycat™ Paediatric Monitoring Electrodes (Tyco Healthcare Group, Mansfield, USA) with stainless steel clips at one end. ECG electrode gel was applied to the metal clips to improve signal conduction only for *S. australis*. Animals were initially curious about the electrode gel and this resulted in some ingestion, but the product is non-toxic and the bats showed no ill side effects on consuming it.

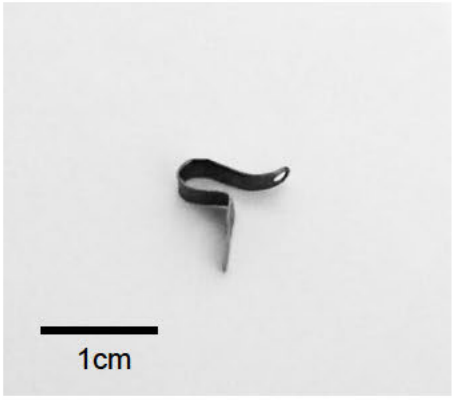

Figure 1. Image of metal ECG electrode band for *S. australis*

ECGs were measured using either a FE132 BioAmp or ML135 Dual BioAmp (ADInstruments, Bella Vista, Australia) connected to a Powerlab 4/35 Data Acquisition System and recorded with LabChart Pro v7.3 software. ECGs were analysed to calculate instantaneous HR, which was averaged per second using LabChart Pro v7.3 and exported to Microsoft Excel (Microsoft Corporation) for further analysis.

Statistical Analysis

In order to assess whether there is a difference between torpor in a daily heterotherm and that in a hibernator analyses were restricted to short torpor bouts of less than 24h. Minimum values of T_{sub}, HR, and $\dot{V}O_2$ were averaged over 30 minutes from animals in steady-state torpor. On occasion transponders temporarily stopped working when the T_{sub} of *N. gouldi* in torpor fell below 7°C, well below the specified temperature range of the

manufacturer (32-43°C). In these cases T_{sub} was estimated to be 0.5°C above T_a, as this was the average differential for animals with similar $\dot{V}O_2$ whose transponders continued to work at low T_a.

RMR values for *S. australis* were either taken from the period in the afternoon when animals were first placed in the respirometry chamber, from individuals that didn't re-enter torpor after disturbance on the following day or following arousal from torpor. Data of RHR for both species were either taken from animals that did not enter torpor during the day, or from the period following arousal. Basal metabolic rate (BMR) and basal heart rate (BHR) were measured following arousal from torpor while animals were resting and within the thermoneutral zone previously determined for each species (Geiser et al., 1996; Geiser and Brigham, 2000).

Entry into torpor was calculated from the point at which T_{sub} began to continuously decline, to the first point at which the average minimum T_{sub} during torpor was reached. During entry into torpor animals cool in a non-linear fashion down to an equilibrium temperature in steady-state torpor. This equilibrium temperature is a function of T_a, thermal conductance and $\dot{V}O_2$ in thermoconforming individuals, and it can take large animals hours to reach this level (Nicol and Andersen, 2007). The calculation of a cooling constant, derived from natural log transformation of cooling curve, requires animals to reach equilibrium T_b for accurate calculations (Nicol and Andersen, 2007). In daily heterotherms the period of time spent in torpor may be too short for equilibrium temperature to be reached, making calculation of cooling constants difficult. Therefore, in my study the initial phase of cooling during torpor entry was used to calculate cooling rate as it is most comparable between the two species. Cooling rates during the initiation of torpor were calculated from the start of the cooling curve for 10 minutes, following a drop in T_{sub} below 32°C (average max T_{sub} 32.2 ± 0.3°C), and standardised to °C per hour. This phase showed the steepest decline in temperature and was approximately linear. As the rate of cooling is likely to be effected by surface area to volume ratios, and this in turn relates to body mass (BM), cooling rates were corrected for mass and approximate

surface area by dividing values by $BM^{0.67}$ to ensure the validity of comparisons between bats.

Rewarming rate and duration of arousal were calculated from the point at which T_{sub} began a steady continuous increase until maximum T_{sub} was reached (average 34.9 ± 1.5°C, range 32.1-37.9°C) and averaged to °C per minute. Maximum rewarming rates were calculated as either the maximum rate per minute or maximum rate over 10 minutes, standardised to per minute. For *S. australis* rewarming data was difficult to obtain as animals often moved outside of the range of the PIT scanner. Repositioning of the scanner generally resulted in a disturbance of the animal and arousal from torpor. However, average rewarming rates from spontaneous and induced arousals were within the same range and as such data were pooled. As for cooling rates, the effects of body mass and surface area on rewarming were accounted for by dividing values by $BM^{0.67}$ (Calder, 1996).

The percentage contribution of RHR to increased oxygen transport needs associated with thermoregulation of normothermic individuals at decreasing T_a, was calculated using the equation of Bartholomew and Tucker (1963);

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

using HR and oxygen pulse (OP; $\dot{V}O_2$/HR) at T_a 1 and T_a 2. HR contribution was calculated between the TNZ (average T_a=30.4 ± 0.8°C) and a low T_a measured for both species (12.0°C). Values for the TNZ were taken from average BMR and BHR and these were compared with values derived from the regression equations for RHR and RMR at T_a 12°C. Alternatively comparisons were made between measured BMR/BHR and resting data recorded at T_a ~12°C per individual (*Ng, n*=2; *Sa, n*=1).

Statistical analyses were performed using R v3.1.0. Standardized major axis regressions were performed to assess the relationship between HR and $\dot{V}O_2$ using the *smatr* package (Warton et al., 2012). Pseudo-replication was accounted for by using the degrees of

freedom as for mixed effect modelling. Linear mixed effects models were used to interpret and compare (ANCOVA) the relationship between physiological variables and T_a/T_{sub} using the *nlme* package (Pinheiro et al., 2014). Paired t-tests were used to compare different methods of rewarming rate measurements, as well as rewarming rate between species at the same T_a.

RESULTS

Effect of T_a on heart rate and metabolism in resting bats

The BMR measured for *S. australis* was 1.58 ± 0.26 ml O_2 $g^{-1}h^{-1}$ with a corresponding BHR of 337 ± 45 bpm (*n*=4 T_a=30.9 ± 1.3°C, T_{sub}=34.7 ± 1.2°C, mass=18.7 ± 0.6g) (Figure 2A & B). My results for BMR were 87% of the MR predicted from body mass following the equation BMR (ml O_2 g^{-1} h^{-1}) = 0.676×$BM^{-0.25}$ (Schmidt-Nielsen, 1997) while BHR was 85% of the HR predicted from body mass using the equation HR= 816×$BM^{-0.25}$ (Wang and Hudson, 1971). The BMR measured for *N. gouldi* (1.34 ± 0.16 ml O_2 g^{-1} h^{-1}) was 63% of the value predicted from body mass, while the BHR of 227 ± 34 bpm was only 50% of the predicted value from body mass (*n*=4, T_{sub}=34.3 ± 0.6°C, mass=9.9 ± 1.0g) (Figure 3A & B).

When bats were normothermic (*Sa, n*=4, T_{sub}=33.8 ± 0.9°C; *Ng*, *n*=13, T_{sub}=34.4 ± 1.2°C) MR increased linearly with decreasing T_a below of the thermoneutral zone (Figure 2A, 3A). Over a comparable temperature range of 15 to 25°C for both species, resting $\dot{V}O_2$ showed a similar level of decline; from mean maximum of 4.31 ± 0.46 to mean minimum of 2.72 ± 0.41 ml g^{-1} h^{-1} in *S. australis* (Figure 2A), and from 5.19 ± 1.75 ml g^{-1} h^{-1} to 2.83 ± 0.81 ml g^{-1} h^{-1} in *N. gouldi* (Figure 3A). However, the relationship between T_a and $\dot{V}O_2$ was significantly different between the two species (ANCOVA, $p<0.05$) with a significantly steeper slope in the *N. gouldi* compared with *S. australis* reflecting the difference in size and hence thermal conductance.

Heart rate also showed a linear increase in normothermic bats exposed to decreasing T_a (Figure 2B and 3B) and as for $\dot{V}O_2$, the relationship between RHR and T_a differed

significantly between the two species (ANCOVA $p<0.05$). For *N. gouldi,* RHR slowed by 331bpm between T_a of 15.1°C and 25.3°C (Figure 3B). In contrast, for *S. australis* there was little change in HR across a similar T_a gradient, with only a 155bpm difference between the maximum RHR measured at T_a=15.3°C and the minimum RHR measured at T_a=22.3°C (Figure 2B).

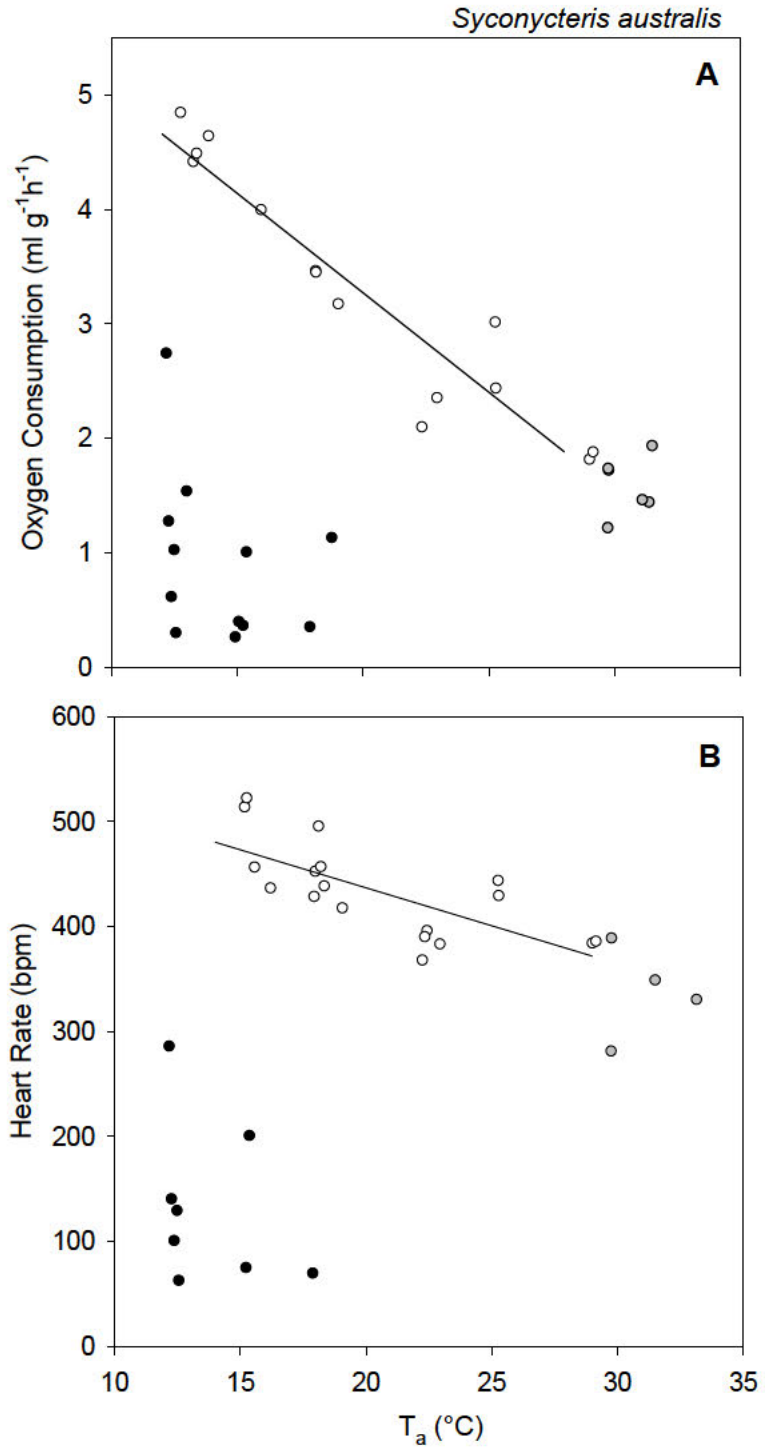

Figure 2. Heart rate and $\dot{V}O_2$ as a function of T_a for *S. australis*. **A)** BMR (filled grey circles) was recorded in the thermoneutral zone 29.5-34°C. Below this resting $\dot{V}O_2$ (circles) increased linearly; $\dot{V}O_2 = 6.74\text{-}0.17(T_a)$, $r^2=0.90$, $p<0.001$. Bats entered torpor (filled black circles) when exposed to T_a below 20°C. **B)** HR showed a qualitatively similar response as $\dot{V}O_2$ to T_a for both resting and torpid states (symbols as for A). HR (bpm) = $581.8\text{-}7.24(T_a)$, $r^2=0.50$, $p<0.01$.

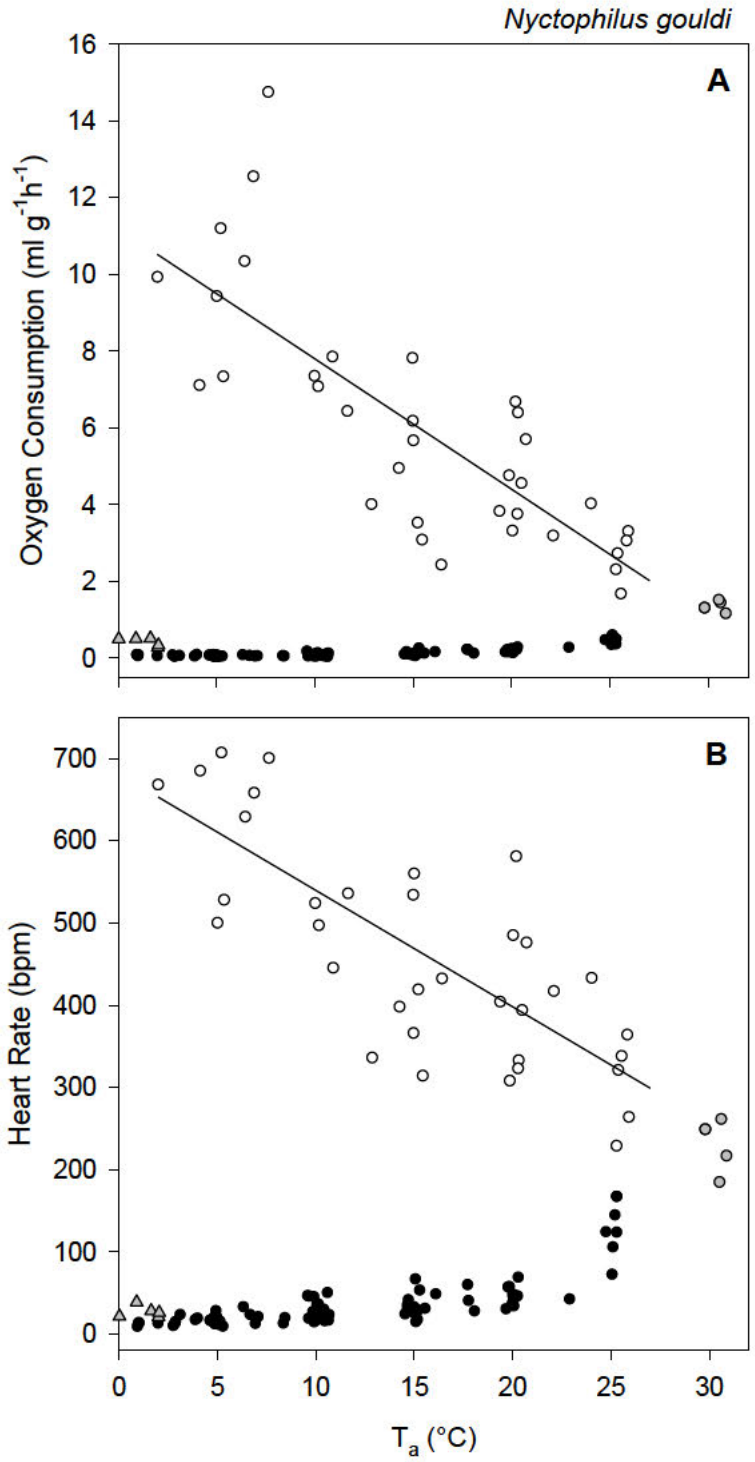

Figure 3. Heart rate and $\dot{V}O_2$ as a function of T_a for *N. gouldi*. **A)** Basal $\dot{V}O_2$ (filled grey circles) was recorded in the thermoneutral zone 29.5-33°C. Below this resting $\dot{V}O_2$ (circles) increased linearly; $\dot{V}O_2 = 11.2\text{-}0.34(T_a)$, r^2=0.64, p <0.001. Bats entered torpor (filled black circles) when exposed to T_a below 25°C and $\dot{V}O_2$ decreased in a curvilinear fashion with decreasing T_a. Below T_a 3°C *N. gouldi* individuals began thermoregulating when torpid (grey triangles). **B)** HR showed a qualitatively similar response as $\dot{V}O_2$ to T_a for both resting and torpid states (symbols as for A). HR (bpm) = $681.4\text{-}14.16(T_a)$, r^2=0.74, p <0.001.

When interrelations between resting $\dot{V}O_2$ and HR were assessed it was revealed for both species that resting $\dot{V}O_2$ showed a strong positive linear correlation with RHR (*Sa* r^2=0.56, p<0.05; *Ng* r^2=0.69, p<0.001) (Figure 4). However, both the slopes and intercepts differed significantly between the two species (ANCOVA, p<0.05).

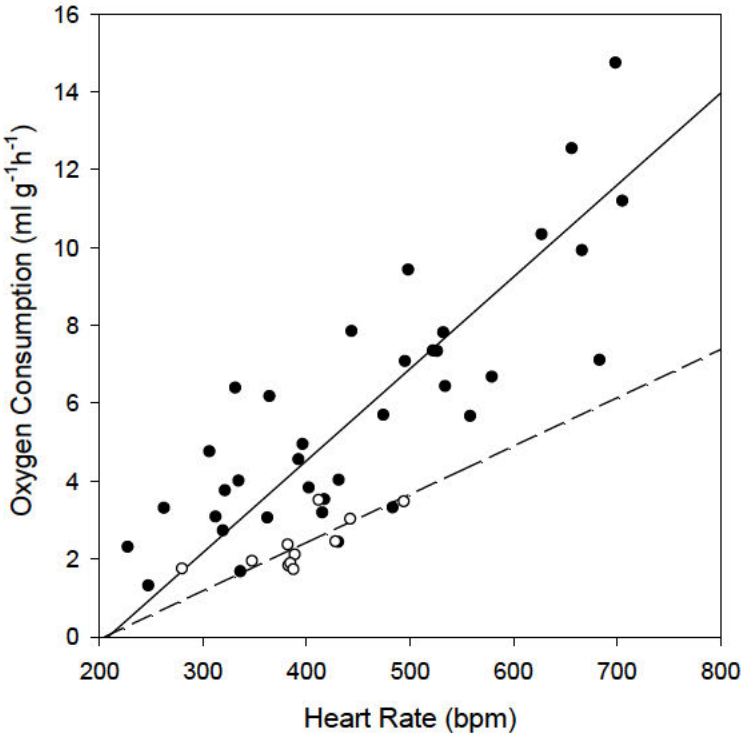

Figure 4. The relationship between RHR and $\dot{V}O_2$ for *N. gouldi* (filled circles- solid line) and *S. australis* (circles- dashed line). *N. gouldi*; $\dot{V}O_2$ = 0.024(HR) – 4.915, r^2=0.69, p<0.001. *S. australis*; $\dot{V}O_2$ = 0.012(HR) – 2. 539, r^2=0.56, p<0.05.

The different relationships of RHR and RMR for the two species were also reflected in calculations of oxygen pulse. Below the TNZ OP increased from 1.12×10^{-4} to 2.47×10^{-4} ml O_2 g^{-1} $beat^{-1}$ at T_a 11.3°C for two individual *N. gouldi* where data were available at both T_a, which equates to an average 54.4% increase in HR relative to increased $\dot{V}O_2$. For *S. australis* data were available for only one animal with OP both within the TNZ and at low T_a. For this individual, the measured OP only increased from 1.03×10^{-4} ml O_2 g^{-1} $beat^{-1}$ at T_a 29.8°C to 1.41×10^{-4} ml O_2 g^{-1} $beat^{-1}$ at T_a 12.3°C and increasing HR accounted for 55.8% of the increased oxygen transport required to meet the increased metabolic demands at low T_a. When OP was calculated using the alternative method, deriving values from the regression equations of RHR and RMR, in *N. gouldi* (*n*=11) values

increased from average 1.01×10^{-4} ml O_2 g^{-1} $beat^{-1}$ in the TNZ to estimated 2.32×10^{-4} ml O_2 g^{-1} $beat^{-1}$ at T_a 12°C. This represents a 49.1% increase of HR to account for increased $\dot{V}O2$, similar to the average calculated from measurements of individual bats. Interestingly however, when the average measured basal OP was compared with values calculated from regression equations from all *S. australis* (*n*=4) OP increased from 0.78×10^{-4} to 1.57×10^{-4} ml O_2 g^{-1} $beat^{-1}$, which equated to a contribution of HR of only 31.8% and is much lower than the value derived from the single bat.

Heart rate and metabolism in torpor

S. australis individuals were often found torpid in their holding cage in the presence of *ad libitum* food and entered into torpor frequently at T_a <20.0°C in respirometry chambers (Figure 5). Torpor generally commenced following lights on, however, very occasionally animals entered torpor during the dark phase in the early morning or even close to midnight. Torpor bouts near midnight were shorter than 3h and T_{sub} fell to between ~23.0 and 28.0°C. Unfortunately, *S. australis* individuals were very sensitive to disturbance and rarely returned to torpor once ECG leads were applied in the early morning. However some individuals did return to torpor following detachment of ECG leads. Consequently, it was very difficult to obtain HR data for *S. australis* in torpor, particularly for animals that were thermoconforming. *N. gouldi* on the other hand showed a strong proclivity to enter torpor in the respirometry chambers and were generally unaffected by the disturbance and/or partial arousals associated with attachment of ECG leads.

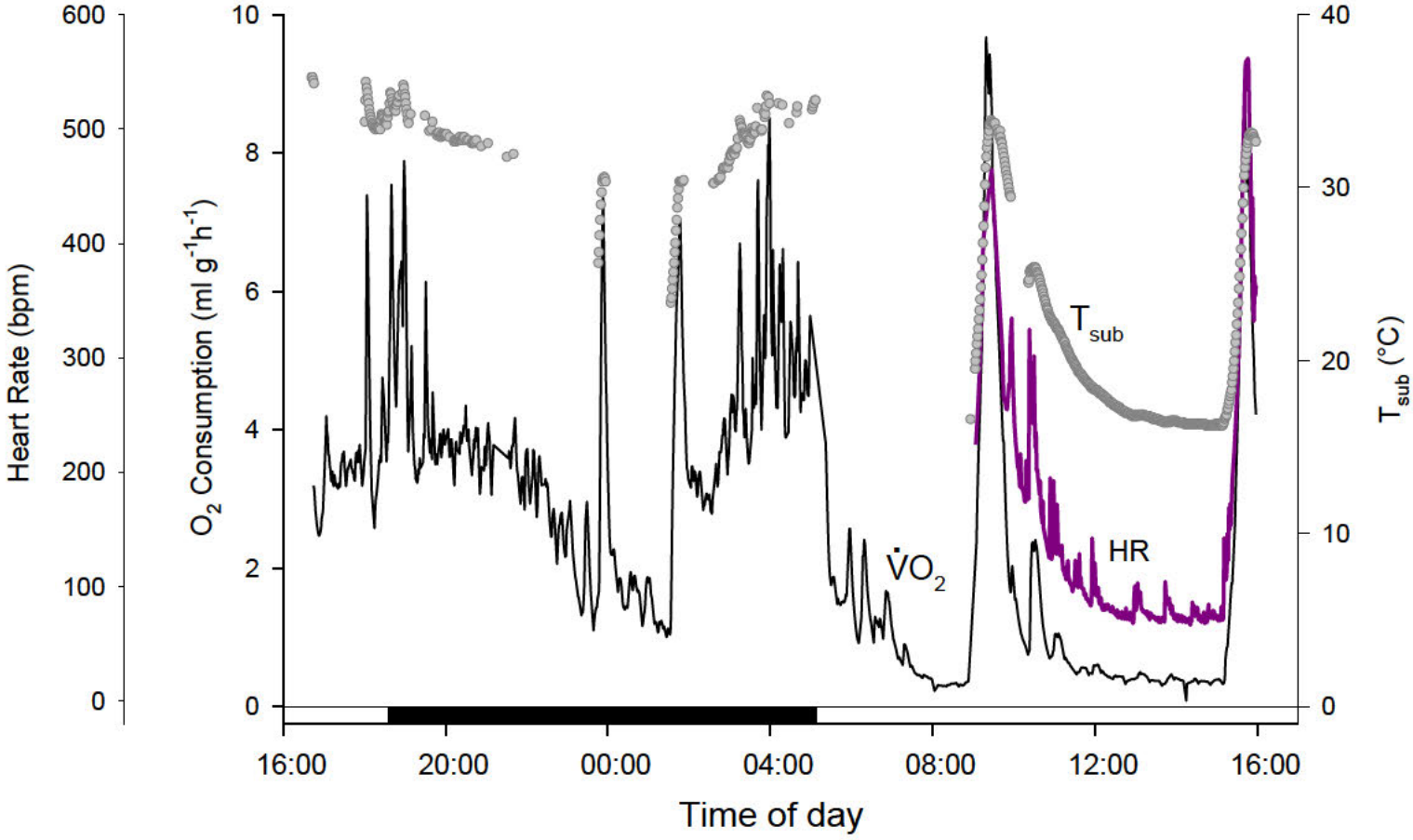

Figure 5. Representative trace of *S. australis* showing HR (purple line), $\dot{V}O_2$ (black line) and T_{sub} (grey circles- missing data are where bats moved out of range of the scanner) over a ~24 hr period (black bar represents scotophase). Animals remained fairly active overnight but occasionally entered torpor during the dark phase for short, shallow bouts. In general however, bats entered into torpor in the early morning following lights on. Attachment of ECG leads resulted in arousal and this bat subsequently returned to torpor before arousing again in the early evening prior to lights off.

In general, torpor bouts of *S. australis* were shorter than *N. gouldi* at any given T_a and *S. australis* individuals tended to arouse prior to lights off in the evenings (Figure 5). However, this was affected by the time of disturbance in the morning associated with ECG lead attachment, and whether or not bats re-entered torpor straight away.

At T_a around 15°C RHR was similar for both species at 482 ± 42 bpm (*Sa*; n=3, T_{sub}=33.8 ± 0.9°C) and 431 ± 88 bpm (*Ng*; n=7, T_{sub}=34.4 ± 1.5°C) (Figure 2B & 3B). Similarly, when bats were resting at 15°C, $\dot{V}O_2$ was comparable between the species at 4.31 ± 0.46 ml $g^{-1}h^{-1}$ (*Sa*; n=3, T_{sub}=33.8 ± 0.9°C) and 4.79 ± 1.91 ml $g^{-1}h^{-1}$ (*Ng*; n=7, T_{sub}=34.4 ± 1.5°C) (Figure 2A & 3A). However, when animals entered torpor at the same T_a (15°C) HR only fell to a minimum of 74 bpm in *S. australis* (T_{sub}=16.3°C) while minimum HR in *N. gouldi* fell as low as 14 bpm (T_{sub}=15.6°C). In addition, the absolute minimum $\dot{V}O_2$ in torpor was again substantially higher for the *S. australis* (0.26 ml $g^{-1}h^{-1}$, T_{sub}=16.4°C) than for *N. gouldi* (0.04 ml $g^{-1}h^{-1}$, T_{sub}=15.6°C).

Both bat species maintained a mean T_{sub} around 34°C (*Sa* 33.8±0.9°C; *Ng* 34.4±1.3°C) when normothermic (Figure 6A & B). As expected, when animals entered torpor the relationship between T_{sub} and T_a differed between the two species. *N. gouldi* entered torpor at T_a ≤25°C and T_{sub} remained within 2°C of T_a in most thermoconforming torpid individuals. The critical T_b set-point temperature (T_{set}) for thermoregulation in torpor in *N. gouldi* was around 5°C however, below T_a 3°C most animals no longer thermoconformed (Figure 6B) and this corresponded to increases in $\dot{V}O_2$ and HR (Figure 3A & B). In contrast, *S. australis* maintained a greater differential between T_{sub} and T_a when in torpor below 20°C (Figure 6A) and average T_{sub} during torpor was highly variable at any given T_a; for example T_{sub} ranged from 16.3 to 24.1°C at T_a 14.9 ± 0.9°C. The minimum T_{sub} was 15°C, recorded at T_a=12.6°C, and animals often maintained a T_{sub} >3°C above T_a regardless of T_a.

When *N. gouldi* were thermoconforming in torpor $\dot{V}O_2$ was reduced to as low as 2% of BMR, but when T_a was decreased below 5°C, $\dot{V}O_2$ increased >10-fold to a minimum

20.9% of BMR. In contrast *S. australis* only reduced $\dot{V}O_2$ to 16.9% of BMR when thermoconforming in steady-state torpor, and when animals maintained a T_{sub}-T_a differential >3°C, the minimum $\dot{V}O_2$ was double that of the *N. gouldi* and was 40% of BMR. Interestingly, the corresponding HR during thermoconforming torpor in *N. gouldi* was 4% of BHR but only increased to 9% of BHR when T_a was reduced below 5°C. Similarly, the increase in HR from thermoconforming *S. australis* to thermoregulation during torpor was also smaller than for $\dot{V}O_2$ increasing from 18.4% to 29.6% of BHR. When examined at a similar T_a (15.1 ± 0.9°C) the Q_{10} between the average $\dot{V}O_2$ of thermoconforming torpid bats to BMR was much lower for *S. australis* (2.4) than for *N. gouldi* (4.1). Conversely, the Q_{10} between BHR and mean HR in torpor was similar for both species; *S. australis* 2.5 and *N. gouldi* 2.9.

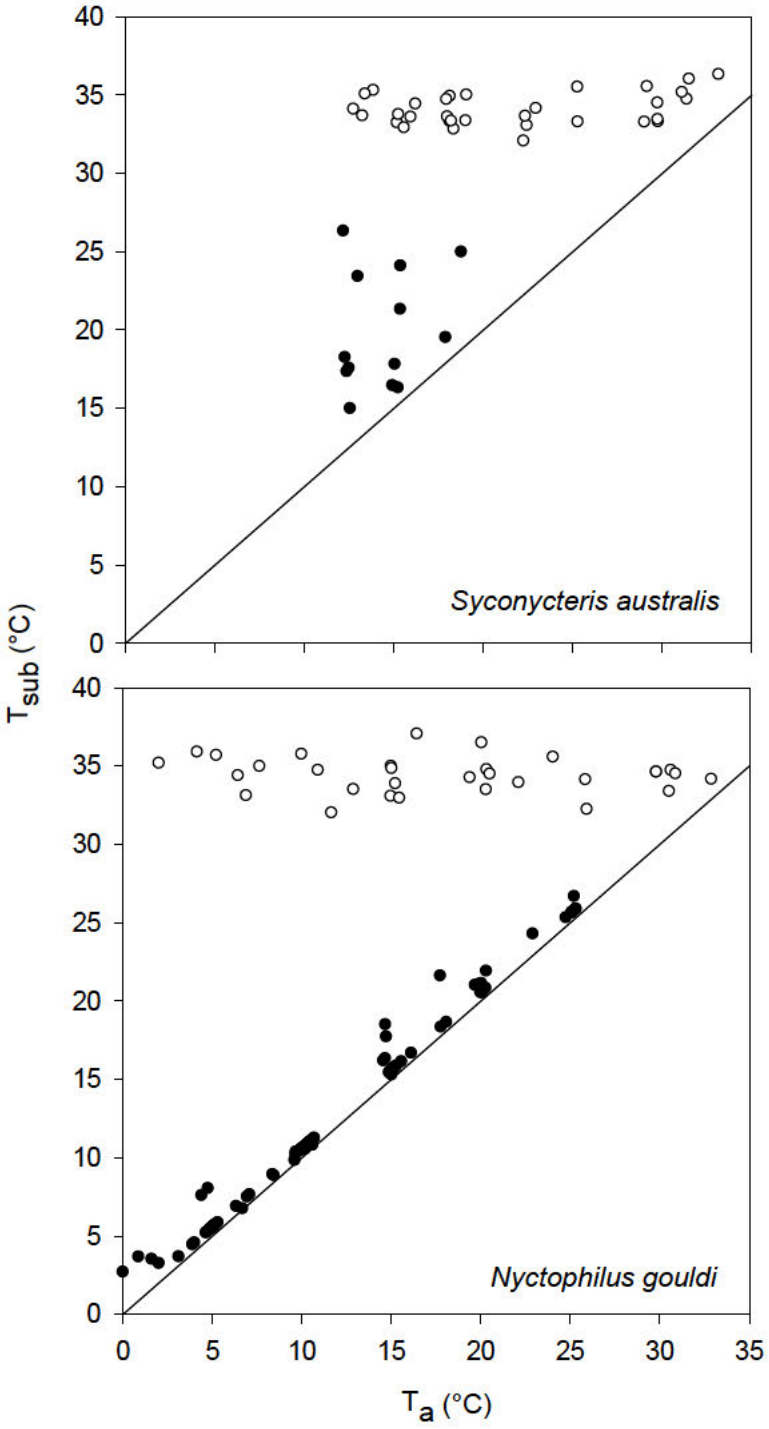

Figure 6. Subcutaneous temperature (T_{sub}) as a function of ambient temperature (T_a) during torpor (filled circles) and at rest (circles). Solid line represents the line of equality (T_{sub}=T_a) A) *S. australis* individuals entered torpor below T_a of 20°C maintained a T_{sub} in torpor >15°C B) *N. gouldi* individuals entered torpor at T_a ≤ 25°C and thermoconformed in torpor down to ~3-5°C (depending on the individual).

During steady-state torpor bats of both species showed strong linear correlations between HR and $\dot{V}O_2$ (*Sa* r^2=0.99, *Ng* r^2=0.84, p<0.001) (Figure 7). However, there was a

significant difference between the relationships for each species (ANCOVA, $p<0.001$) as $\dot{V}O_2$ inclined much steeper with HR in the torpid *S. australis* than for *N. gouldi*. Importantly, for all HRs, measured $\dot{V}O_2$ in *S. australis* was above that of *N. gouldi*.

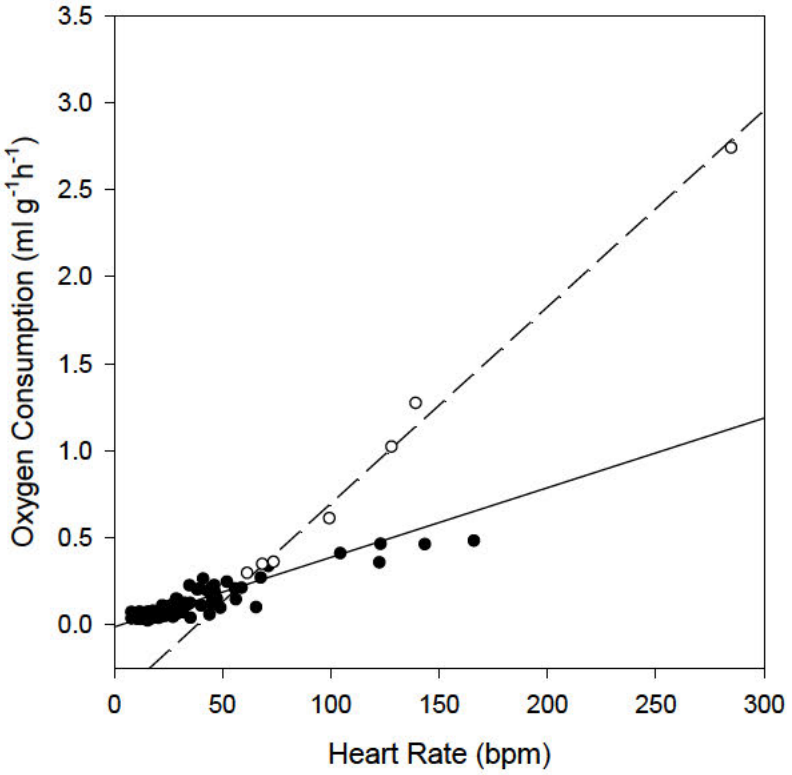

Figure 7. $\dot{V}O_2$ as a function of HR in torpid *N. gouldi* (filled circles) and *S. australis* (circles) as T_a between 1 and 25°C. Dashed line represents the regression equation for *S. australis* individuals ($\dot{V}O_2 = 0.011(HR) – 0.433$, $r^2=0.99$, $p<0.001$) and the solid line represent the regression equation for *N. gouldi* individuals ($\dot{V}O_2 = 0.004(f_H) - 0.012$, $r^2=0.85$, $p<0.001$).

HR showed a pronounced hysteresis with T_{sub} during entry and arousal from torpor in both species, although absolute values of HR differed substantially between the two species (Figure 8A & B). During entry into torpor HR was slower at each T_{sub} than at the corresponding T_{sub} during rewarming. Figure 8 also illustrates the pronounced drop in HR during torpor entry with little change in T_{sub} in *N. gouldi* as well as the rapid increase in HR during rewarming over a small T_{sub} change with an over shoot of HR at the end of arousal from torpor in both species. For example, at the initiation of arousal in *N. gouldi* HR increased ~8 fold over a rise in T_{sub} of only 1.6°C, from 24 to 200 bpm. Similarly, HR of *S.*

australis more than doubled when rewarming over a T_{sub} range of only 1.4°C from 70 to 173bpm.

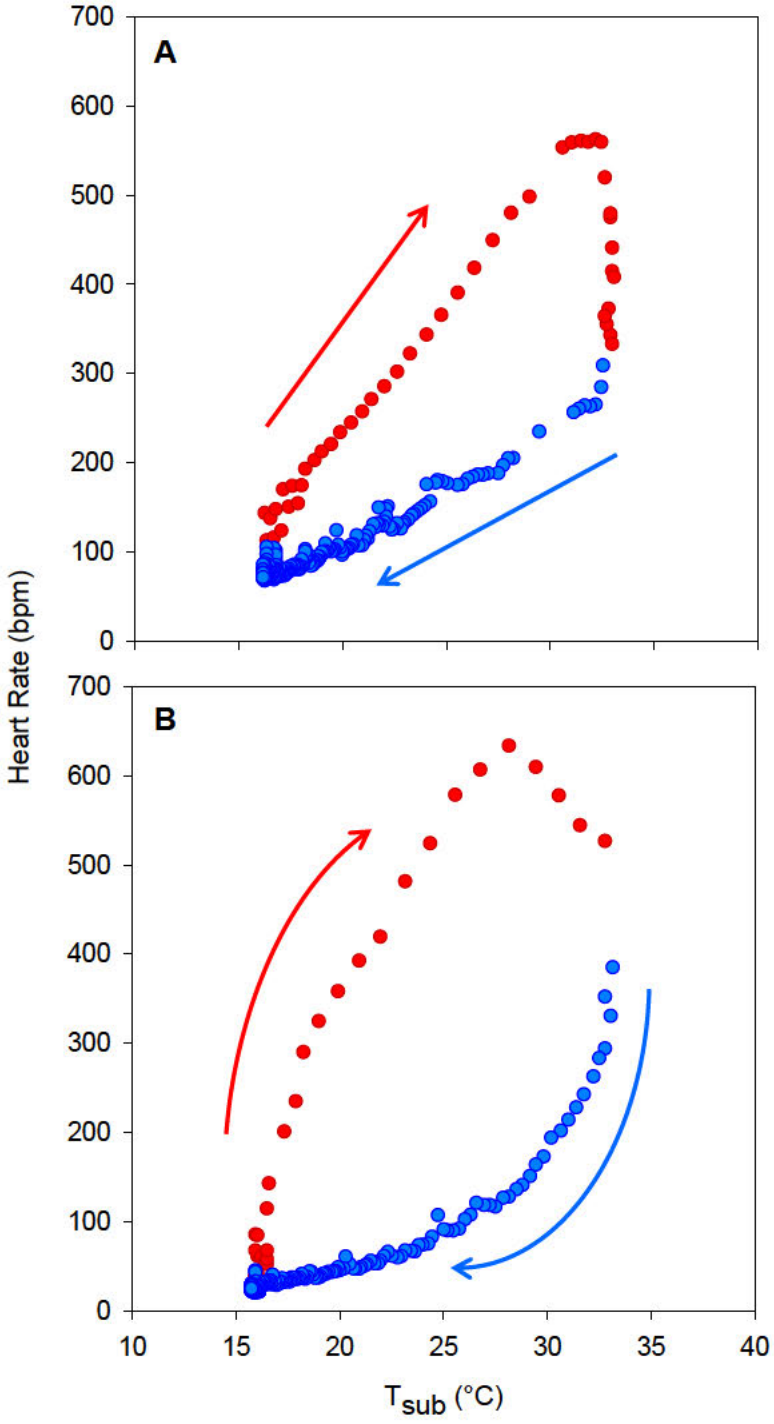

Figure 8. Heart rate as a function of subcutaneous temperature during entry (blue circles and arrow) and arousal (red circles and arrow) from a single torpor bout for *S. australis* (A) and *N. gouldi* (B) at an ambient temperature of 15°C. Arrows indicated the progression of the torpor bout in time.

The process of cooling during the initial stage of torpor entry proceeded in a different manner for the two species. The rate of cooling during entry into torpor was negatively

correlated with T_a ($p<0.0001$) in *N. gouldi* (Figure 9). In contrast, *S. australis* did not show a significant response of cooling rate to changing T_a ($p=0.205$). Moreover, the overall cooling rates for *S. australis* were significantly lower than for *N. gouldi* ($p<0.05$) (Figure 9). This was confirmed by a t-test to compare animals at a common T_a (15.0 ± 1.9°C), *S. australis* individuals showed a significantly lower mass-specific rate of cooling (1.77 ± 0.44°C $g^{-1}h^{-1}$) than *N. gouldi* (4.53 ± 0.94°C $g^{-1}h^{-1}$) (t-test, $t=8.59$, $df=15.35$, $p<0.001$). The effect of body mass on cooling rate however could only be examined for the *N. gouldi* as there was not enough data available for the *S. australis* of differing masses. At an average T_a of 15.03 ± 0.24°C and across a 5g mass range (9-13.9g) average cooling rate was not related to body mass ($p=0.891$). Regardless, it was ensured that cooling rate was comparable between the bat species by dividing values by $BM^{0.67}$ (i.e. mass-specific values).

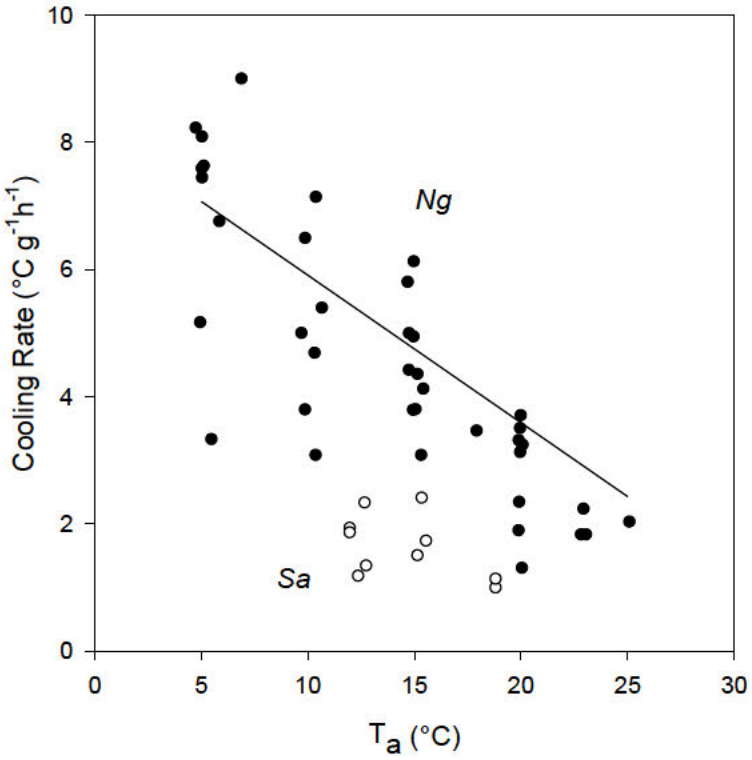

Figure 9. Mass-specific cooling rate against T_a. *N. gouldi* (filled circles) showed a negative response of cooling rate to increasing T_a ($CR= 3.829-0.124(T_a)$, $r^2=0.87$, $p<0.001$). *S. australis* (circles) had significantly slower rate of cooling than the *N. gouldi*.

When bats aroused from torpor HR and $\dot{V}O_2$ increased dramatically associated with endogenous heat production to swiftly return animals to a normothermic T_{sub}. Rewarming followed a sigmoid curve (for example see Figure 10). Maximum rewarming rates measured over 10 minutes were significantly lower than the maximum rate over 1 minute for both species (ANCOVA, $p<0.05$). The maximum rate of rewarming over 1 minute increased significantly with increasing T_a in *N. gouldi* and data were pooled for both species for linear regression ($r^2=0.85$, $p<0.05$) as there was no significant difference between rewarming rates for the species across the T_a (ANCOVA, $p=0.737$) (Figure 11). This was also the case for mass-specific rates of rewarming over 1 minute (Figure 12). However, when mass-specific maximum rewarming rate over 1 minute was examined at a similar T_a (14.9 ± 1.2°C) there was a significant difference between species (two-sample t-test, $t=9.02$, $df=10$, $p<0.001$).

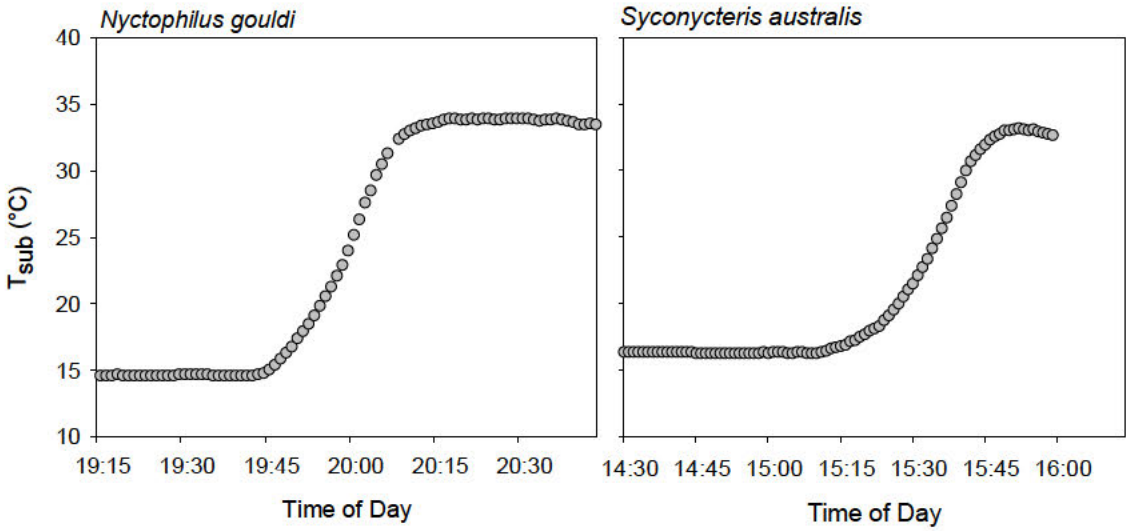

Figure 10. Representative example of rewarming curve for *N. gouldi* or *S. australis* individuals arousing from torpor at T_a 15°C.

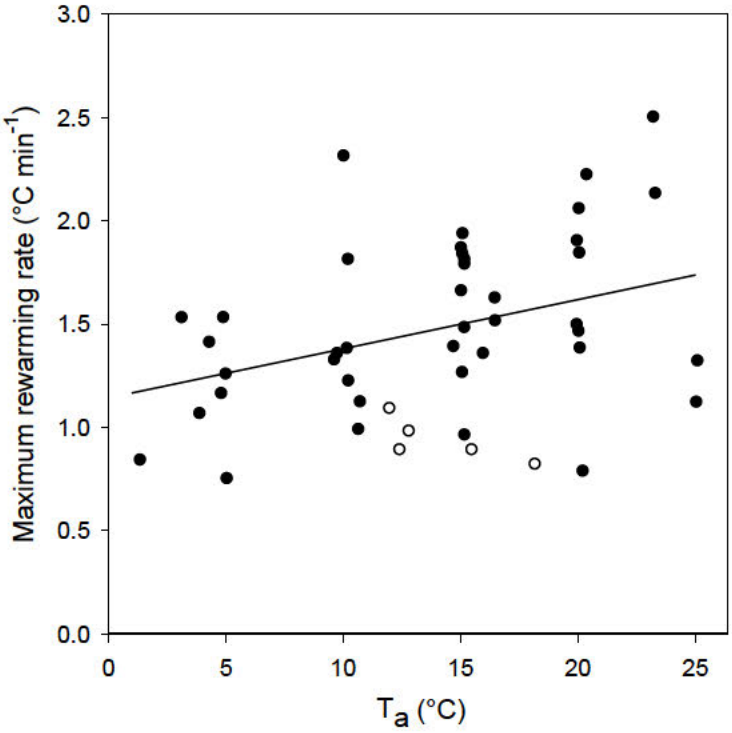

Figure 11. Maximum rewarming rate (MaxR) over 1 minute as a function of T_a (*N. gouldi*; filled circles, *S. australis*; circles). There was no significant difference in rate between species, therefore regression was calculated from pooled data. MaxR = 0.024(T_a) + 1.143, r^2=0.73, p<0.01.

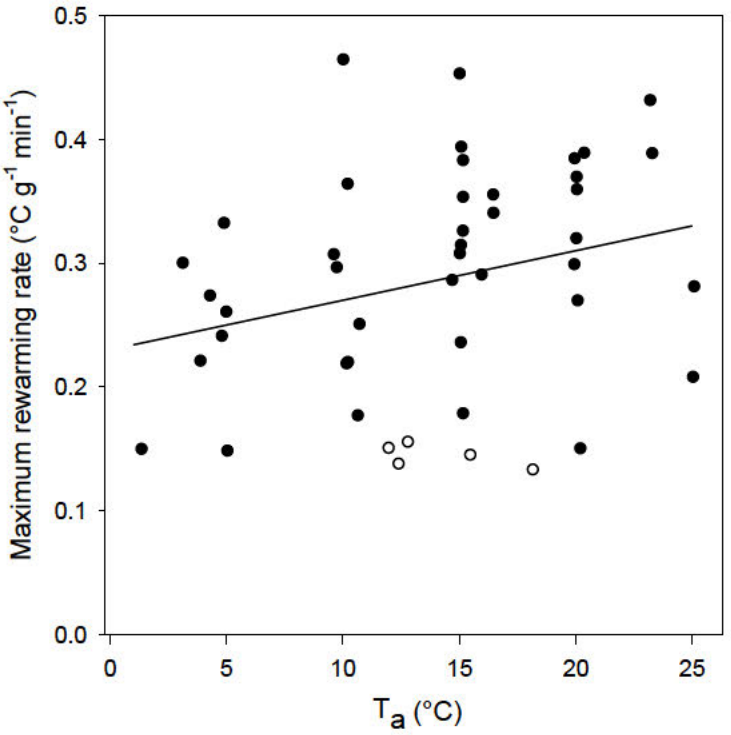

Figure 12. Mass-specific maximum rewarming rate (MaxR) over 1 minute as a function of ambient temperature (T_a) data were pooled for both species (*N. gouldi*; filled circles, *S. australis*; circles). MaxR = 0.002(T_a) + 0.103, r^2=0.85, p<0.01.

The average rate of rewarming integrated over the entire arousal showed a positive linear relationship when plotted against the minimum T_{sub} at arousal and this did not differ between the species (ANCOVA, p=0.549). Again, data were pooled for linear regression (r^2=0.64, p<0.001) (Figure 13). Time taken to rewarm showed a negative curvilinear response to increasing T_a. When data was log transformed a linear regression was significant (r^2= 0.83, p<0.001) and did not differ between the two species (ANCOVA, p=0.508) (Figure 14).

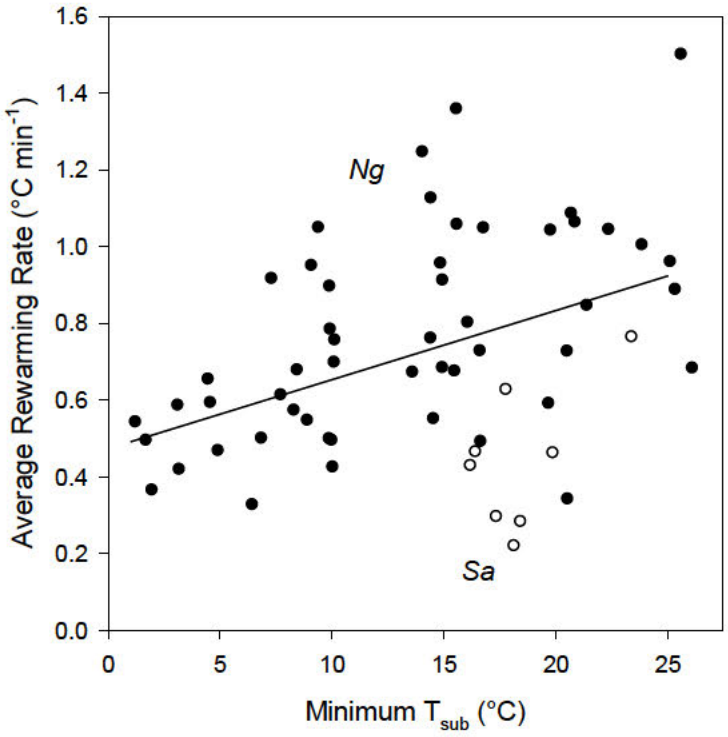

Figure 13. Average rewarming rate (AvgRW) as a function of minimum T_{sub} at the start of arousal. There was no significant effect of species and as such data were pooled. AvgRW =0.018(T_{sub}) + 0.474, r^2=0.64, p<0.001.

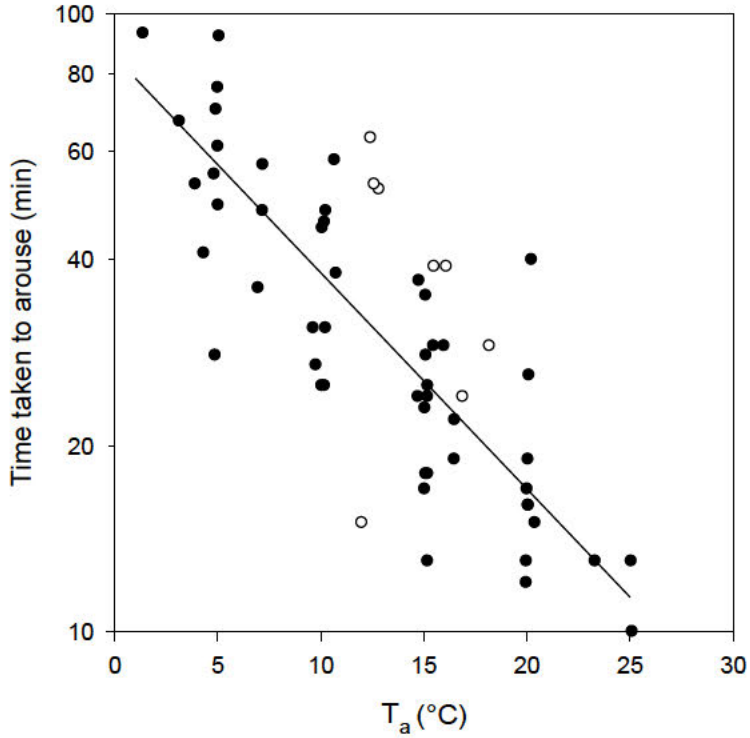

Figure 14. Time taken to arouse as a function of T_a for both species (*N. gouldi*; filled circles, *S. australis*; circles). There was no significant effect of species and as such data were pooled. $Log_{10}Time = 1.931 – 0.035(T_a)$, $r^2=0.83$, $p<0.001$.

DISCUSSION

Effect of T_a on heart rate and metabolism in resting bats

During periods of normothermy both bat species showed a qualitatively similar response of HR and $\dot{V}O_2$ to changes in T_a following the general endothermic response. Below the TNZ both HR and $\dot{V}O_2$ increased linearly however the slope of these relationships differed significantly between the two species. My results also demonstrate that the interrelations between HR and $\dot{V}O_2$ at rest vary between the two species reflected in both regression analyses and calculations of OP. Although the OP of both species increased with decreasing T_a, the percentage contribution of HR to oxygen transport differed between them. The proportional increase in HR for increased oxygen transport at low T_a was substantially lower in *S. australis* (31.8%) than *N. gouldi* (49.1%) across an equivalent T_a gradient. This suggests that differential alterations of other aspects of the cardiovascular system are contributing to changes in OP with T_a for the two species. This could be driven

by an increase in stroke volume (SV) in *S. australis* to maintain cardiac output at high MR, possibly related to a larger heart. It is possible that these differences relate to energy acquisition and partitioning associated with flight. Nectar feeding bats and birds are known to have the highest rates of oxygen consumption amongst animals, which is magnified by the use of hovering flight in some species (Suarez et al., 2011). Although *Nyctophilus* spp.are also known to hover when gleaning insects from vegetation, the energy reward from insects is greater than that received from nectar. As such nectarivorous bats have comparatively high field metabolic rates due to greater foraging activity necessary to meet energy demands (Geiser and Coburn, 1999; Voigt et al., 2006). Furthermore, adaptations of the cardiovascular system improve cardiac reserve and efficiency for flight, and foraging niche and flight dynamics have been shown to be highly correlated with heart mass (Bullen et al., 2009). Bullen et al. (2009) concluded that bats which forage in high density cluttered foliage and bats capable of hovering had significantly larger heart mass fractions than other bat species. This study also showed that the heart mass fraction of the northern blossom bat, *Macroglossus minimus*, was greater than that of *N. gouldi* (Bullen et al., 2009). *M. minimus* is a close relative of *S. australis* that shares most of its range, foraging habitat and is, for generalist purposes, effectively indistinguishable morphologically (Bonaccorso and McNab, 1997). Moreover, torpor depth and duration in *M. minimus* are similar to those described for *S. australis* (Bartels et al., 1998) and as such is it likely that their relative heart masses are proportionate to one another. Therefore, it is possible that the differences reported here for RHR and the relationship with $\dot{V}O_2$ in *N. gouldi* and *S. australis* relate to the size of the heart and a greater cardiac reserve necessary for longer foraging times in the nectar feeding bats.

Heart rate and metabolism in torpor

The only other pteropodid bat for which HR has been measured during torpor is *Nyctimene albiventer* a 28g nectarivorous/frugivorous bat. Only two HR measurements were presented and animals were unlikely to have been in steady-state torpor as they

were only measured for less than 4h. Regardless the values presented were low (88 and 96bpm at T_a 25°C) (Bartholomew et al., 1970). Minimum average HR recorded for *S. australis* in my study was only slightly lower, 62bpm at T_a 12.6°C which corresponded to a $\dot{V}O_2$ of 0.29 ml O_2 $g^{-1}h^{-1}$. The minimum $\dot{V}O_2$ measured in torpid *S. australis* without ECG electrodes attached in my study (0.26 ml O_2 $g^{-1}h^{-1}$) was not dissimilar to values simultaneous to ECG measurement. Although *S. australis* individuals were less tolerant of ECG electrodes than *N. gouldi*, these values are among the lowest $\dot{V}O_2$ recorded for a small pteropodid bat and this suggests that animals were not overly disturbed by the experimental protocol.

Data from previous captive studies have suggested that *S. australis* only entered torpor following lights on in the morning and never remained in torpor for more than 12hrs (Geiser et al., 1996; Coburn and Geiser, 1998). Interestingly it was found that on rare occasions torpor was induced near midnight or in the early morning during the dark phase in *S. australis*. It has been suggested that torpor use is often underestimated in laboratory settings (Geiser et al., 2000) and therefore torpor use overnight may occur more frequently than anticipated in this species. The lowest individual T_b previously measured in *S. australis* was 17.2°C (Coburn and Geiser, 1998) while the minimum T_{sub} recorded here was 15.0°C at T_a 12.6°C. T_{sub} was measured as <1°C of rectal T_b and as such the minimum T_b here is still less than the minimum previously recorded. This also suggests that the T_{set} for this species may likely be closer to 15°C than the previously reported 18°C. Moreover, at all T_a bats were capable of arousing without the need for external heat sources showing the minimum values reported here reflect actual torpor rather than simply hypothermia. Perhaps because these animals were in captivity for longer they may have been more willing to drop T_b. In contrast the *N. gouldi* regularly entered torpor overnight, occasionally within 1h of lights off in the evening. However, at or below T_a of 15°C some animals remained torpid for greater than 24h with the longest bout of four days only interrupted by an induced arousal.

Few studies have been undertaken directly comparing the physiological variables relevant to torpor use between a daily heterotherm and hibernator in a single study. While most comparisons have been made using data derived from the literature it may be more relevant to investigate torpor patterns within a single study using identical methods. Reviews have shown that there are substantial differences between the two groups in their ability to maintain low T_b and the mechanisms by which metabolism is lowered and maintained during steady-state torpor (Ruf and Geiser, 2014). However, to date these comparisons have not incorporated measurements of HR during torpor or how this is related to MR in heterothermic species. Unfortunately, most studies have compared data collected from seasonal hibernators during multiday torpor bouts at low T_a, with data from short term torpor bouts in daily heterotherms at mild T_a. Furthermore, the confusion surrounding the proposed delineations of torpor pattern arise from animals that appear to be capable of using both torpor strategies. However closer investigation indicates that in hibernators these bouts of 'daily torpor' are equivalent to short-term hibernation. In the few cases where differences have been observed (Wilz and Heldmaier, 2000) these differences are most likely related to the time spent in torpor and the inability of larger animals to reach steady-state low levels. My study is the first to directly compare cardiac physiology of an animal only capable of daily torpor to that of a hibernator during a temporally similar torpor bout of torpor less than 24h.

Both species entered torpor readily throughout the period of study. At all T_a measured, the hibernator *N. gouldi*, had lower $\dot{V}O_2$ and HR during torpor than corresponding measurements for the daily heterotherm, *S. australis*. The Q_{10} for average $\dot{V}O_2$ of thermoconforming torpid bats to BMR was 4.1 for *N. gouldi*, which is higher than expected from temperature effects alone and indicates possible active suppression of metabolism. When the Q_{10} of average $\dot{V}O_2$ for thermoconforming *S. australis* was examined at the same T_a (15°C) against BMR, it was only 2.4 which suggests that metabolism in torpor is an effect of low T_b in these bats. These results support the theory that metabolic inhibition is essential in maintaining low MR during torpor in some hibernators, while daily

heterotherms largely rely on temperature effects of low T_b to reduce MR below BMR in torpor.

During torpor *S. australis* also expressed shallower reductions of T_{sub} as the daily heterotherms rarely thermoconformed in torpor and maintained an average T_{sub}-T_a differential of 5.4°C (range 1.0-14.1°C). Maintenance of a greater T_{sub}-T_a differential in *S. australis* may be facilitated by their roosting posture. These bats wrap their wings around their bodies and tuck their heads in while they roost, trapping a pocket of warm air between the body and wing membranes. Whereas *Nyctophilus* spp. roost with their wings tucked up beside their bodies. The difference in temperature between the external air and the pocket between the body and wings was shown to be up to >10°C in other pteropodid bats (*Pteropus poliocephalus* and *P. scapulatus*) (Bartholomew et al., 1964). Unfortunately, although *S. australis* were investigated by Bartholomew et al. (1964), data were not presented for inside wing versus T_a but it is likely that some level of insulation is provided by the wing membranes in these small bats as well. My results show that *S. australis* have a lower thermal conductance at rest than *N. gouldi* and this appears to also be the case when animals are torpid. Active thermoregulation and the maintenance of a large T_b-T_a differential would likely impact on an array of cardiovascular features of torpid animals in contrast to individuals that thermoconform. It is possible that peripheral resistance and organ perfusion during this phase would change and it is unknown how this would affect conductance as well as cardiac output, which may or may not be reflected by HR.

In order to make comparisons of active thermoregulation during torpor between the two species, data for animals that had a T_{sub} ≥3°C (*Sa*) or ≥2°C (*Ng*) above T_a were selected and plotted against the T_{sub}-T_a differential (Figure 15). It is obvious that *S. australis* maintained a greater T_{sub}-T_a gradient and higher HR and $\dot{V}O_2$ than *N. gouldi* thermoregulating at a low T_a. For comparison, further data are shown for *N. gouldi* that maintained a large T_{sub}-T_a differential during torpor at mild T_a (~20°C) comparable to the proposed T_{set} for *S. australis*. Even at higher T_a there is a clear distinction of torpid $\dot{V}O_2$

and HR between the two species with very limited overlap. Interestingly, in *N. gouldi*, the effect of ΔT_{sub} on cardiac function is evident in thermoregulating torpid individuals at a mild T_a but this effect is not represented for $\dot{V}O_2$, with limited change as T_{sub}-T_a increases. Moreover, average $\dot{V}O_2$ in these bats was still only 33% of BMR. In contrast, *S. australis* individuals showed the same response of both HR and $\dot{V}O_2$ to increases in T_{sub}-T_a. There appeared to be no active suppression of $\dot{V}O_2$ below BMR in these animals as the average $\dot{V}O_2$ across thermoregulating animals was within the range of BMR. This further supports the theory that different mechanisms are working to maintain low MR in torpor in the two species.

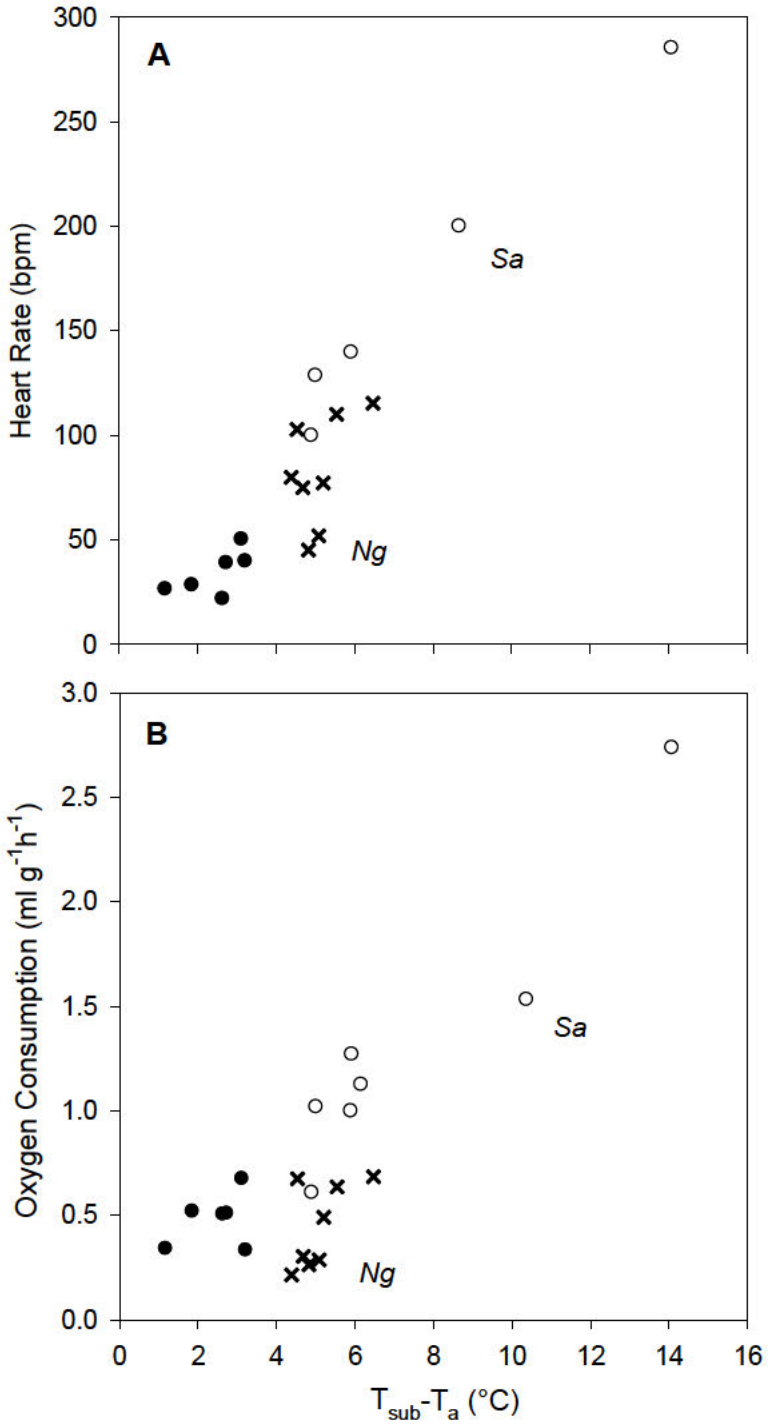

Figure 15A) Heart rate as a function of T_{sub}-T_a in actively thermoregulating *N. gouldi* during torpor at low T_a (filled circles), during torpor at mild T_a (crosses) and for *S. australis* during torpor at mild T_a (open circles). **B)** $\dot{V}O_2$ as a function of T_{sub}-T_a (symbols as for A).

Entry into torpor progressed more slowly in the larger daily heterotherms than the smaller hibernators and there was no effect of T_a on cooling rate during the initial phase of entry as these bats appeared to thermoregulate during cooling. During entry into torpor the

hypothalamic set point for thermoregulation gradually falls to the new low set point for steady-state torpor (Heller, 1979). My results suggest that the fall in T_{set} occurs more slowly in daily heterotherms as animals defend T_{sub} throughout or for part of cooling. This was also reflected by a slower decline in HR during cooling, as illustrated by a more gradual slope of hysteresis curve in *S. australis* compared to *N. gouldi* at the same T_a. The clear difference in cooling rates is also reflected by different torpor T_{set} for the two bat species. In hibernating *N. gouldi* active thermoregulation in torpor did not occur until a critical T_{sub} <3°C, while T_{set} is substantially higher during daily torpor in *S. australis* and is closer to T_{sub} ~15°C.

The ability to rewarm from torpor is a defining feature of heterothermic animals and the arousal process is extremely costly. Arousal costs are reduced when rewarming rate is maximised (McKechnie and Wolf, 2004) and the importance of this is evident in both species of bats investigated here as there was no significant difference in maximum rewarming rates for either bat. However, the period of time over which rewarming rates were calculated resulted in significantly different maximum values. As was the case for cooling, the rewarming process was not linear, and integration over longer time periods may be an oversimplification. Previous investigators have measured maximum rewarming at intervals of 10 minutes (Geiser and Baudinette, 1990), generally associated with equipment measurement interval and inertia of devices used to record T_b, but also for comparative review. In larger species of heterotherms that rewarm slowly (>1h) an interval of ~10 minutes may provide a reasonable measure of maximum rewarming rates, however small bats can rewarm very quickly (minimum 10min) and as such longer measurement intervals likely underestimate maximum rewarming capacity. When compared to the rewarming rates of other vespertilionid bats taken at T_a of 20°C (as reported in Willis, 2008) rewarming rates from T_{sub} for *N. gouldi* are generally high (1.64 ± 0.56°C min^{-1} compared to a range from 0.15-1.52°C min^{-1} of rectal T_b) and this is in part likely reflective of the time over which measurements were taken in the previous studies which is unfortunately not specified in many reviews. However it may also relate to the

positioning of subcutaneous transponders. PIT tags were implanted interscapularly which, in bats, is near to the largest deposit of brown adipose tissue, the thermogenic organ essential to the rewarming process (Hayward and Ball, 1966).

Both species of bat in this study roost in thermally labile environments exposing themselves to fluctuating T_a and passive rewarming. *N. gouldi* are known to select roosts to enable frequent passive rewarming in the wild (Turbill, 2006) and this is likely true for *S. australis* as well, as they have been shown to select roosts on the outer, more thermally labile edges of their habitat in winter (Drury and Geiser, 2014). As previously shown for *N. gouldi* (Currie et al., 2015) maximising rewarming rates and minimizing arousal times through passive rewarming saves substantial energy. Considering that the time taken to rewarm did not differ in *S. australis* from *N. gouldi* similar significant energy savings are likely to also occur if these daily heterotherms passively rewarmed. My results also support previous findings that there is no effect of body mass on rewarming rate in bats (Willis, 2008) and that rewarming is not effected by torpor pattern (Figure 16) (Geiser and Baudinette, 1990). However, this most likely related to the fact that heterothermy in bats only occurs in animals <50g and the range of body masses may be too small to show a significant relationship.

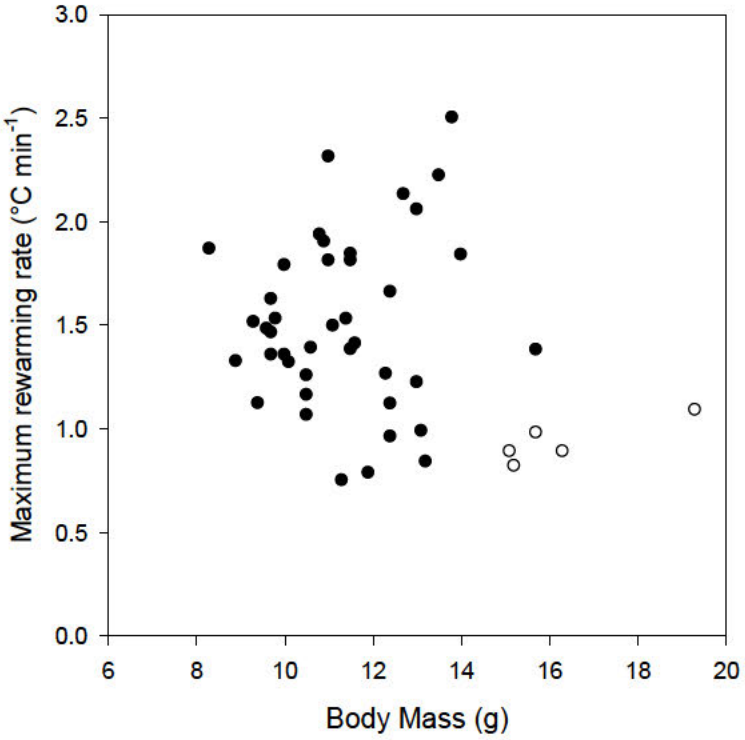

Figure 16. Maximum rewarming rate over 1 minute as a function of body mass for *N. gouldi* (filled circles) and *S. australis* (circles). There was no significant effect of body mass on rewarming.

My study is the first to present HR at rest and during torpor at a range of T_a for a chiropteran daily heterotherm in comparison to another small bat during hibernation. I show that torpor progressed in a similar manner for both bat species however the depth, duration and mechanisms reducing HR, MR and T_b in torpor were decidedly different between the daily heterotherm and hibernator. At all T_a tested, *N. gouldi* maintained a lower HR during torpor than *S. australis*. However, rates of arousal were not significantly different between the bats. Regardless of the pattern used, it is clear that torpor plays a crucial role in the survival of many bat species and has likely been a key factor enabling their habitation across the world.

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STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

	Author's Name (please print clearly)	% of contribution
Candidate	Shannon E Currie	80%
Other Authors	Gerhard Körtner	10%
	Fritz Geiser	10%

Name of Candidate: Shannon E Currie

Name/title of Principal Supervisor: Professor Fritz Geiser

[redacted]

Candidate

1/3/15

Date

[redacted]

Principal Supervisor

1/3/15

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Figures	121,126,127,128,133,134,135,136,137,138, 139,140,146,149

Name of Candidate: Shannon E Currie

Name/title of Principal Supervisor: Professor Fritz Geiser

[redacted]

Candidate — Date: 1/3/15

[redacted]

Principal Supervisor — Date: 1/3/15

Chapter 6

The effects of temperature on cardiac electrophysiology and respiratory function of hibernating long-eared bats (*Nyctophilus gouldi*)

ABSTRACT

Heterothermic animals regularly undergo profound alterations of cardiorespiratory function associated with torpor. These animals have specialised tissues capable of withstanding fluctuations in body temperature >20°C without adverse effects. Many small bats use torpor year round and are able to transition between torpor and flight within a matter of minutes, making investigation of cardiorespiratory function in these animals very interesting. In order to determine the effect of temperature on cardiac conduction and ventilation frequency during steady-state torpor in bats, electrocardiograms, ventilatory movements, and subcutaneous temperature (T_{sub}) were recorded simultaneously at a range of ambient temperatures (T_a) for hibernating long-eared bats (*Nyctophilus gouldi*). During torpor, cardiac function and ventilation were substantially reduced and animals began breathing intermittently with periods of apnoea up to 51min. Cardiac conduction slowed in a temperature dependent manner, primarily via prolongation along the atrioventricular pathway (PR interval). There was little change in the time taken for ventricular repolarisation (JT interval) with decreasing temperature, or between rest and torpor at mild T_a and there was no isoelectric ST segment until animals reached a T_{sub} ~5°C. There was no significant ventilatory tachycardia during torpor, however both heart rate and ventilation rates during breathing bouts increased with increasing T_a. Throughout all recordings there were no manifestations of significant conduction blocks or ventricular tachyarrhythmias indicating the capacity of bat hearts to withstand extreme fluctuations in rate and T_b without arrhythmogenesis.

INTRODUCTION

Mammalian hibernators regularly undergo a profound shift in behaviour and physiology associated with torpor. These animals are able to withstand extreme reductions in cardiorespiratory function and return to a normothermic state without detrimental consequences. These changes, associated with a reduction in body temperature (T_b), are poorly tolerated in strict homeotherms and result in death at T_b well above the minimum T_b typically experienced in torpor (Milsom et al., 2001)

Therefore amongst mammals, the hearts of heterotherms are unique in their ability to resist complications associated with low T_bs indicative of torpor, and show innate specialisations. For example, the electrical conduction system of the heart works to minimize propagation time while optimizing cardiac output through the sequence of mechanical contraction and in heterotherms the speed of ventricular conduction has been shown to be comparatively more rapid than that of non-hibernating species (Duker et al., 1987; Johansson, 1996). In addition there is a close association between ventricular depolarisation and repolarisation in heterothermic mammals. This is indicated by the lack of a distinct isoelectric segment separating the QRS complex (ventricular depolarisation) and T wave (ventricular repolarisation) and thus a relatively short QT interval compared to other species incapable of torpor. When exposed to low T_b either through hypothermia or natural hibernation conduction is prolonged, but to a lesser extent in these animals than in non-hibernators (Duker et al., 1987). The ability to maintain rapid conduction across the heart against the slowing effects of low T_b and thus heart temperature is important for maintaining adequate cardiac function during torpor and likely constitutes the reason for resistance to the detrimental arrhythmias experienced by non-hibernating animals in hypothermia.

Although the maintenance of excitation and conduction is important for hibernators, the ability of excitation to elicit adequate contraction is of arguably greater importance. Contractility of ventricular muscle is maintained in hibernating species down to very low temperatures approaching 0°C or even below (Lyman and Blinks, 1959; Barnes, 1989;

Geiser et al., 1989). In addition, the optimal temperatures for maximal tension production is lower for hibernators investigated in comparison to homeotherms (Smith and Katzung, 1966; South and Jacobs, 1973), with peak performance often at T_b well below normothermy.

During torpor, energy metabolism and the frequency of ventilation are substantially reduced, with most species breathing intermittently either with cycles of single breaths and short apnoeas (<10min) or in short rapid ventilatory bouts interspersed by long apnoeic periods (up to >1h)(Milsom, 1991). Heterothermic animals are therefore also able to tolerate protracted periods of hypoxia which would likely be detrimental to other species. During torpor, it has been shown that ventilatory pattern is influenced by both conditions of hypercapnia and hypoxia (McArthur and Milsom, 1991). However there is a disproportionate change in relative sensitivity to each state with many animals showing little or no change in breathing frequency or apnoeic pattern down to 3% oxygen, while there is a significant increase in the relative sensitivity to hypercapnia (Harris and Milsom, 1994). In addition, these animals are resistant to ischaemia-reperfusion injury (Carey et al., 2003; Kurtz et al., 2006; Drew et al., 2007). During torpor, there is a reduction in circulatory blood volume associated with splenic storage of red blood cells, blood flow is shunted away from the extremities and overall perfusion is reduced. The differential restriction of blood flow results in the proportionately greater perfusion of essential organs such as the brain, lungs and heart with diminished, almost immeasurable blood flow in the gut and kidneys (Rauch, 1973). The reversible nature of heterothermy means that these animals must regularly undergo drastic alterations in blood and oxygen supply to an array of tissues without significant damage, and as such antioxidant defences are substantially higher in hibernators (Morin and Storey, 2007).

Bats, in their ability to fly, have developed relatively large and highly efficient cardiorespiratory systems compared to terrestrial mammals of a similar size (Canals et al., 2011). During flight, bats maintain a metabolic rate 2.5 to 3 times the maximum rates of their non-flying counterparts (Thomas, 1975) with HRs >1000bpm (Studier and Howell,

1969). At the other extreme, many temperate zone bat species enter torpor throughout the year, which can occur almost daily (Stawski et al., 2014). During torpor, metabolism is reduced to minimal levels around 0.05ml O_2 $g^{-1}h^{-1}$, with HRs <40bpm (Kulzer, 1967). Therefore these animals regularly expose themselves to intense fluctuations in cardiorespiratory function greater than an order of magnitude. Previous studies of cardiovascular and respiratory function during hibernation have mainly been undertaken on large seasonal hibernators that retreat to burrows or caves over winter and do not emerge again until the favourable conditions of spring (Lyman and O'Brien, 1960; Burlington et al., 1971; Zimmer et al., 2000). Prior to the hibernation season, these animals often undergo a preparatory period during which time gene expression and protein composition are altered (for review see Carey et al., 2003). In these seasonal hibernators, there are often seasonal differences in cold resistance of the heart (Smith and Katzung, 1966; Burlington et al., 1972). On the contrary, some small bats show little seasonal variability in the function of their cardiac tissues with relation to temperature (Michael and Menaker, 1963) and many species can enter hibernation or multiday torpor regardless of season (Stawski et al., 2014) leaving little scope for significant preparatory alterations to cardiac function.

The aim of this chapter therefore, was to investigate the effects of temperature on cardiorespiratory function in hibernating insectivorous long-eared bats (*Nyctophilus gouldi*) at different times of year across the same array of ambient temperatures (T_a). I aimed to quantify the relationship between heart rate (HR) and ventilation rate (VR) as well as assess the effect of T_a/T_b on cardiac electrical conduction throughout a torpor bout. *N. gouldi* have been shown to enter torpor frequently in the wild, regardless of season and although there have been a number of studies on thermal energetics in this species, data for cardiac function do not exist. Moreover, previous investigations of cardiac electrophysiology in hibernating bats have used invasive methods to measure electrocardiograms (ECG) or resorted to restraining animals. It is likely that these methods had an effect on torpor patterns as the animals were probably stressed. I

therefore aimed to attain ECGs using a far less invasive and restrictive method (externally adhesive electrodes) to assess the patterns of cardiac conduction and HR during torpor.

METHODS

Nyctophilus gouldi individuals (12 male, 9 female) were trapped in mist nets around the University of New England, Imbota Nature Reserve and Newholme Station all close to Armidale, NSW. Animals were caught in spring and autumn between 2011 and 2013 and housed at the University of New England in outdoor flight cages for a maximum of 5 months. Bats were fed meal worms *ad libitum* from commercially available bird dishes hung from the sides of the cages and water was constantly available from troughs within the enclosure.

ECG and ventilation measurements

Measurements of ECGs and ventilatory movements were conducted throughout the year, encompassing all seasons. Single lead ECGs were recorded using externally adhesive electrodes arranged in a Lead I arrangement. Details regarding equipment and ECG lead attachment are available in Chapter 3. Ventilatory movements were recorded using piezoelectric MLT1010 Pulse Transducers (ADInstruments, Bella Vista, Australia) that lay flush with the bat's chest within respirometry chambers and was sensitive enough to also detect cardiac contractions during apnoeic periods (Figure 1) which was occasionally used as an additional measure of HR. Ventilatory traces were measured simultaneous with ECGs and recorded using LabChart data acquisition software (v7.3 ADInstruments, Bella Vista, Australia) and sampled at a minimum rate of 4kHz/s. Measurements of subcutaneous temperature (T_{sub}) were made using implanted temperature sensitive PIT tags. Details regarding transponder implantation are available in Chapter 2. T_{sub} was measured once every minute while ECG and ventilation were recorded. These variables were recorded continuously where possible following successful attachment of ECG leads, throughout a torpor bout.

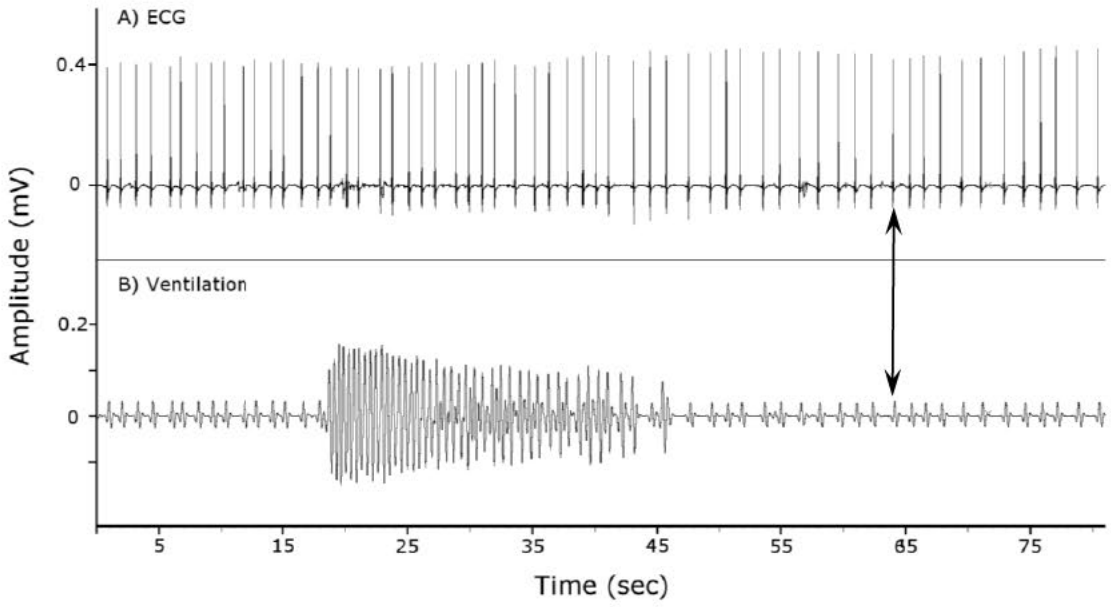

Figure 1. Simultaneous recording of ECG (A) and Ventilation (B) illustrating the sensitivity of pulse transducers in their ability to detect cardiac contractions during apnoeic periods (indicated by arrow).

Although the electrocardiogram acquisition system enabled recording of HRs greater than 800bpm, it was very sensitive to external disturbances making analysis of some ECG waveforms difficult in some recordings. For example, the solenoid valves switching channels in the respirometry or electrical interplay of computing and recording equipment resulted in ECG interference (for example of impedance associated with PIT tag scanner see Figure 2). Furthermore, full complement ECG waveforms (i.e. PQRST- Figure 3) were often not distinguishable above a HR of 200bpm. This was most often related to muscle artifact associated with shivering and/or movement of the limbs due to rapid ventilation rates, increasing the sampling rate to 10kHz/s resulted in little improvement of the signal.

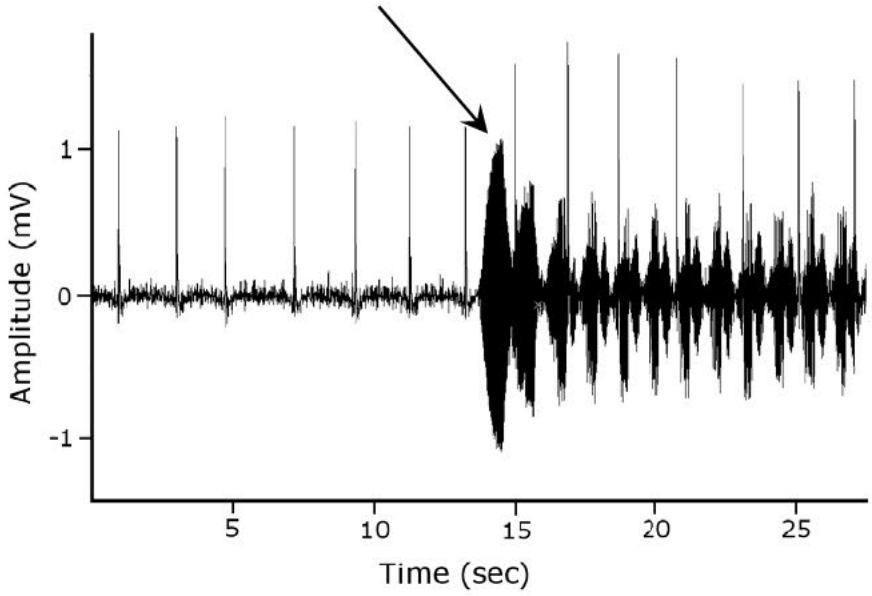

Figure 2. ECG after filtration was applied showing electrical interference from PIT scanner (arrow indicates when scanner turned on) which continued for ~30 sec if the animal was out of range.

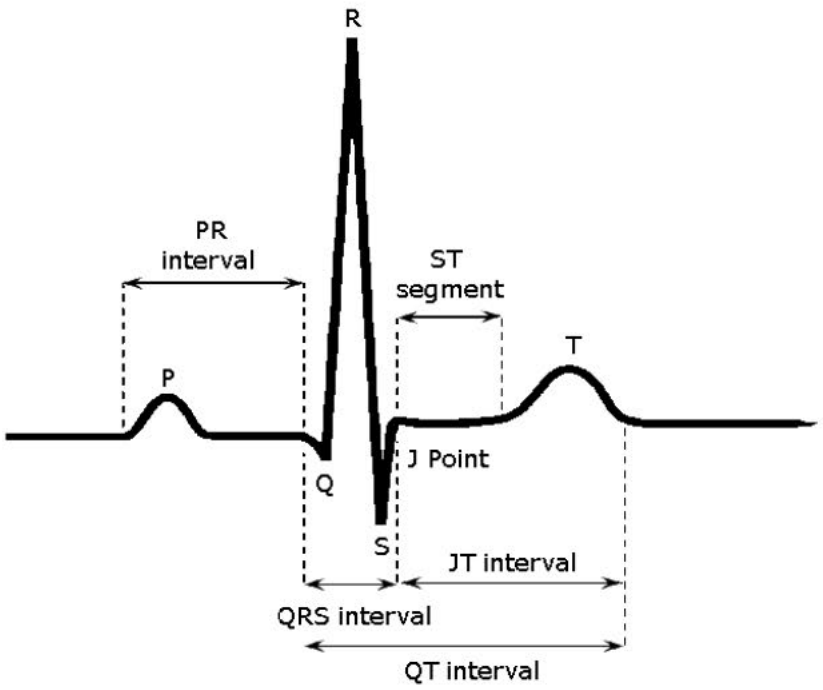

Figure 3. Schematic of a typical mammalian ECG signal showing P wave, PR interval, QRS complex, QT interval, ST segment, and T wave.

Statistical analysis

Instantaneous HR and VR were calculated from the distance between peaks on recorded traces using LabChart v7.3 software and then averaged for each second over the entire

torpor bout. These values were then exported to Microsoft Excel (Microsoft Corporation) and averaged to values per minute for further analyses. To ensure that data for torpor bouts were comparable across different T_a only data from torpor bouts <24h were included in analyses.

Resting ventilation rate (RVR) was calculated over a 5min period following arousal from torpor, corresponding to measurements for resting heart rate (RHR). Steady-state ventilation rate during torpor was averaged over a 30min period when HR was at its minimum. During torpor at low T_a, animals were often apnoeic for longer than 30min and as such the period of time over which VR and HR were averaged was lengthened to 45min to ensure a representative rate. Additional values for steady-state VR during torpor were also taken from torpor bouts overnight or in the early morning, as animals occasionally moved away from pulse transducers during the day.

Oxygen pulse (OP) was calculated using oxygen consumption values that were simultaneously recorded to measurements of HR at rest and during torpor (for details see Chapter 3). The percentage contribution of RHR to increased oxygen transport needs associated with thermoregulation of normothermic individuals at decreasing T_a, was calculated using the equation of Bartholomew and Tucker (1963);

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

using HR and OP ($OP = \dot{V}O_2 \div HR$) at T_a 1 and T_a 2. HR contribution was also calculated between torpor (HR/OP_1) and rest (HR/OP_2) at the same T_a.

To assess cardiorespiratory association during torpor, HR was measured per second over torpor bouts less than 12hrs during either periods of apnoea or eupnoea as designated from ventilatory traces. The values for each apnoeic or eupnoeic period were then averaged over the time animals were considered to be thermoconforming, i.e. from the time at which T_{sub} reached its minimum average until the point at which T_{sub} began to rise (>1°C) associated with arousal. ECG waveform analysis was undertaken using the ECG

Analysis Module v2.3.2 for LabChart Pro v7.3 (ADInstruments, Bella Vista, Australia). Common mammalian ECG intervals (Figure 3) were assessed where possible (ie P, PR, QRS, QT, TP) and were averaged for the same 30-45 min period as minimum HR and VR in torpor or over the 5 min period of RHR in normothermic bats. This meant that the number of beats incorporated into the analysis varied with HR from around 200 to 3200 beats. Due to the lack of isoelectric ST segment in all recorded ECGs for *N. gouldi* JT interval was calculated; this was measured from the end of the QRS complex to the end of the T wave (Figure 3).

All statistical tests were carried out in R v3.1.0. Paired t-tests were used to assess the difference between HR during apnoeic periods versus HR during breathing bouts at T_as where enough samples were permitting. Linear mixed effects models were used to determine the relationship between T_a and RVR, VR during breathing episodes, length of apnoeic periods, and length of breathing bouts, with animal as a random effect to account for the influence of repeated measures. Standardised major axis regression was used to assess the relationship between average apnoeic length and the length of breathing bouts. Degrees of freedom were taken from linear mixed effects model, to account for animals as a repeated measure, and used to calculate significance of the regression.

RESULTS

Electrocardiograms

When bats were normothermic (T_{sub}=34.2 ± 1.1°C *n=13*) HR increased linearly with decreasing T_a (Figure 4). Instantaneous RHR tended to be quite variable often without clear cardiorespiratory association (Figure 5). At HRs above 400bpm, P and T waves often became indistinguishable and only the QRS complex was clear. Average QRS duration in resting bats was 0.016 ± 0.001s (*n*=8) and showed little variation over a range of T_a (5.4-27.9°C). Similarly PR interval did not vary greatly with changing T_a over a RHR range of 300bpm (228-527bpm), with an average PR interval of 0.03 ± 0.005s (*n*=8). The

QT interval in normothermic bats was relatively rapid at 0.04 ± 0.01s (*n*=8) and did not show a clear relationship with RHR.

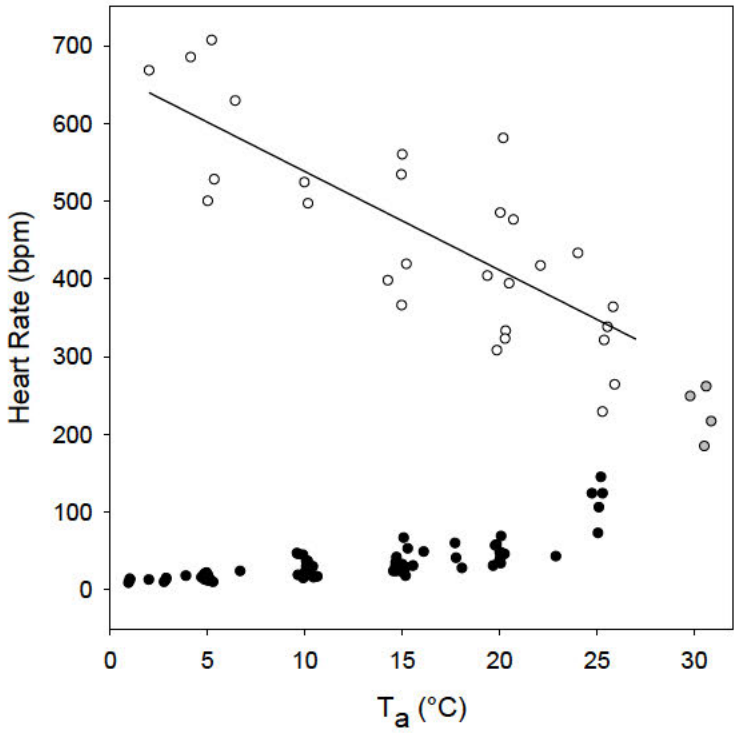

Figure 4. Average HR as a function of T_a for resting (circles) and torpid (black circles) *N. gouldi* as well as BHR in the TNZ (grey circles). RHR (bpm) = 581.8-7.24(Ta), r2=0.50, p<0.01.

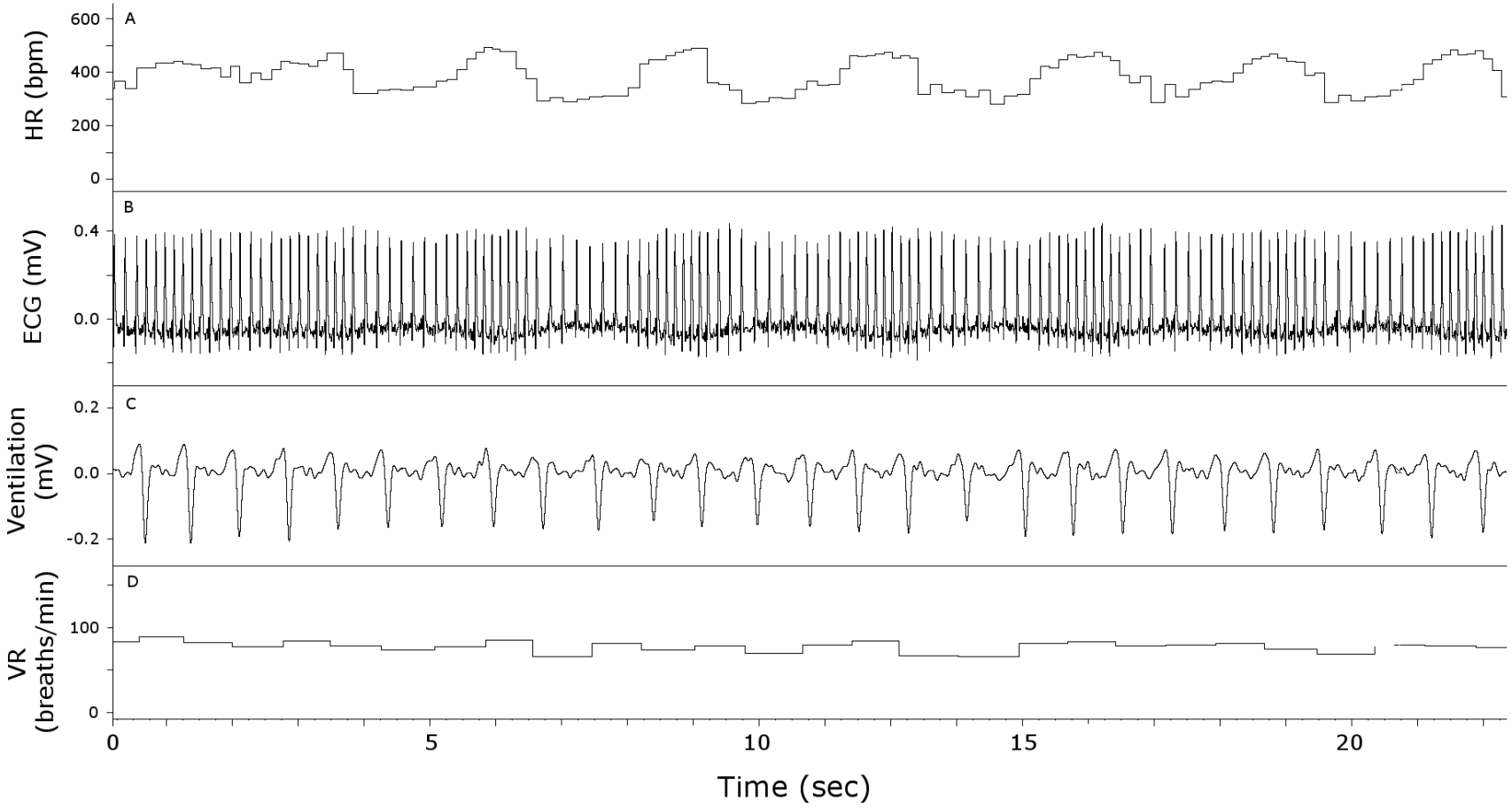

Figure 5. Trace of resting ECG and ventilation at a T_a of 27°C with average HR of 385bpm and VR 77 breaths min^{-1} showing the variability of HR with regular ventilatory rhythm.

As animals entered torpor, HR declined in a variable pattern with occasional periods of cardio-acceleration and bradycardia, but an overall slowing as the animal progressed into torpor. The heart slowed primarily from an extension of the period between beats (TP interval) and as animals entered into torpor the amplitude of the QRS complex increased (Figure 6).

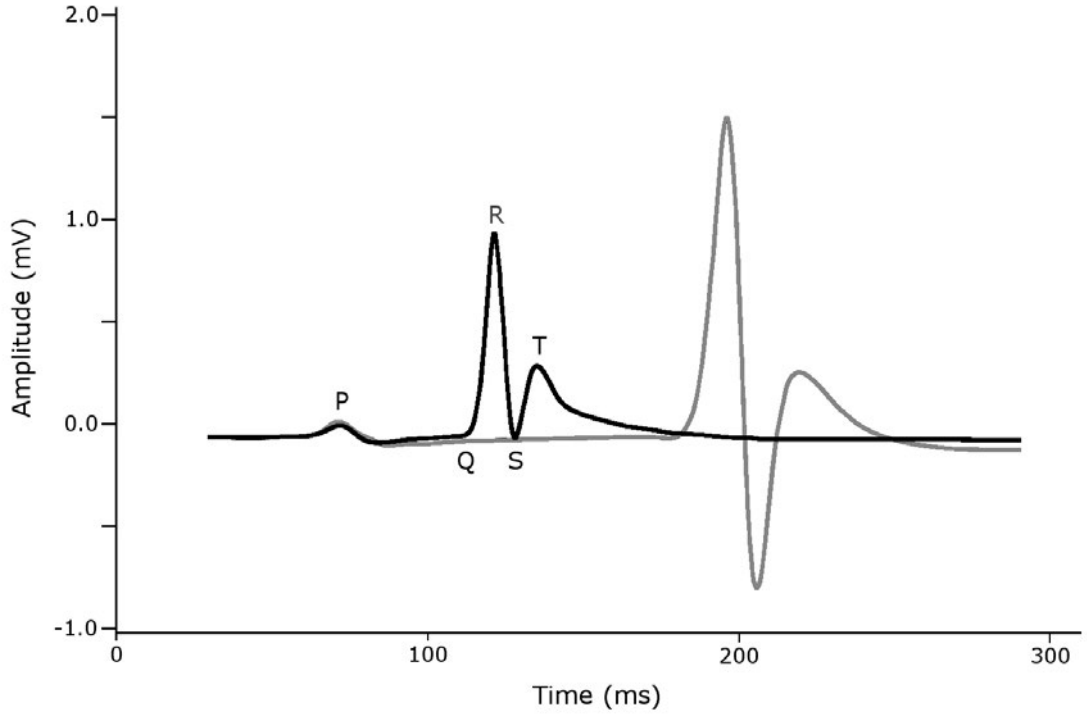

Figure 6. Representative ECG of an individual *N. gouldi* at HR ~150bpm (T_{sub} 26.4°C) during entry into torpor (black trace) and in steady-state torpor (grey trace) with HR ~17bpm (T_{sub} 15.7°C) at T_a 15°C, aligned at the P-wave. Note the increase in amplitude of the QRS complex when animals were torpid.

There was an overall lengthening of cardiac conduction intervals when bats were in torpor and this was a curvilinear function of T_a. At all T_a tested, PR interval showed the greatest prolongation of conduction between rest and torpor (Figure 7). There was no isoelectric ST segment present on the ECG of resting *N. gouldi* and this did not change when animals entered torpor. Interestingly, it wasn't until T_a fell below 15°C that ventricular repolarisation in torpid bats, measured from the end of the QRS to the end of the T wave (JT interval), showed any considerable prolongation from resting values (Figures 7 & 8).

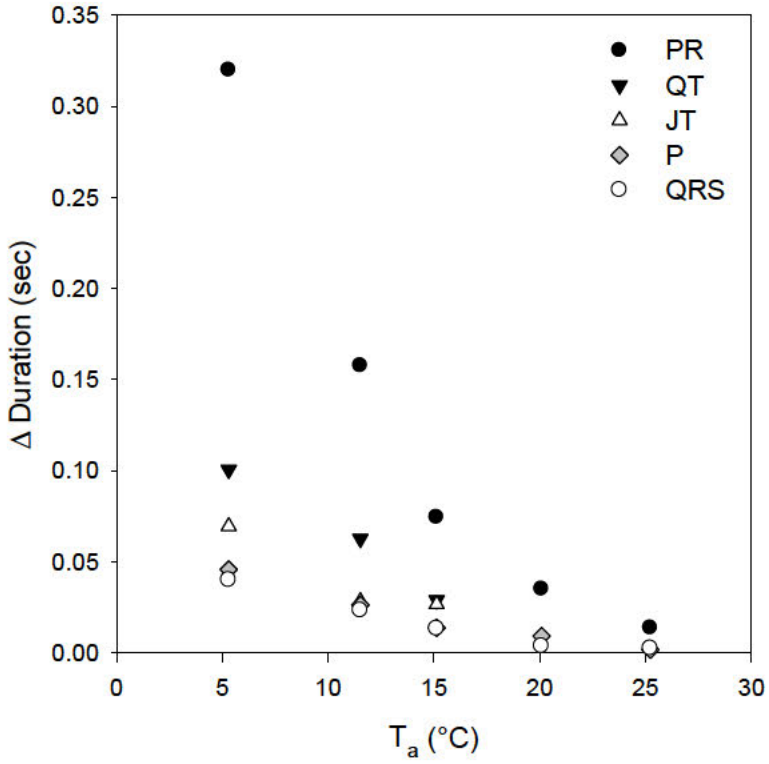

Figure 7. Prolongation of conduction intervals measured as the difference in each ECG interval between resting and torpid *N.gouldi* presented as a function of T_a.

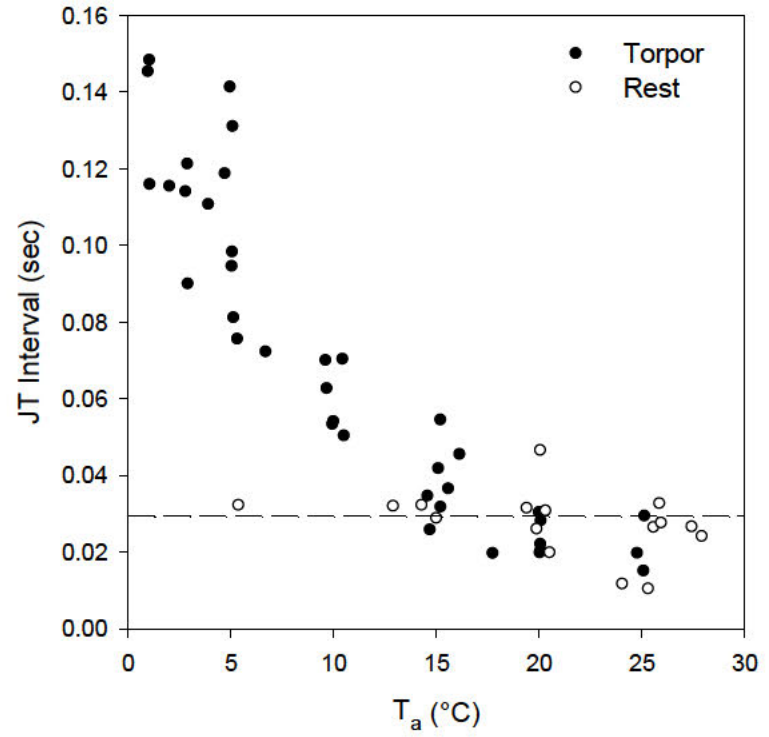

Figure 8. Ventricular repolarisation as measured by JT interval presented as a function of T_a in torpid (black circles) and resting (circles) *N. gouldi*. Dashed line represents the mean JT interval of resting bats across all T_a.

When animals were in steady-state torpor, there was a curvilinear relationship between ECG intervals and T_{sub} (Figure 9). As T_{sub} decreased below 20°C, conduction velocity decreased (Figure 10). Again, PR interval showed the greatest stretch with decreasing T_{sub} (following TP), with QRS interval the shortest duration at all T_{sub} (Figure 9).

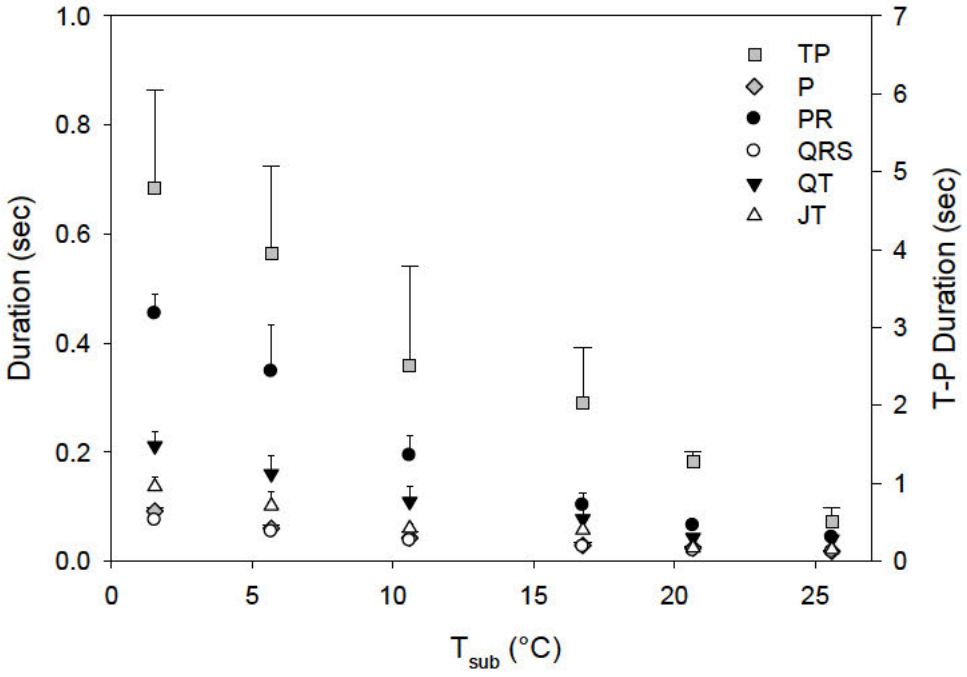

Figure 9. Average duration of ECG intervals (plus standard deviation) as a function of T_{sub} in hibernating bats. TP duration was substantially longer than the other ECG intervals hence the separate axis.

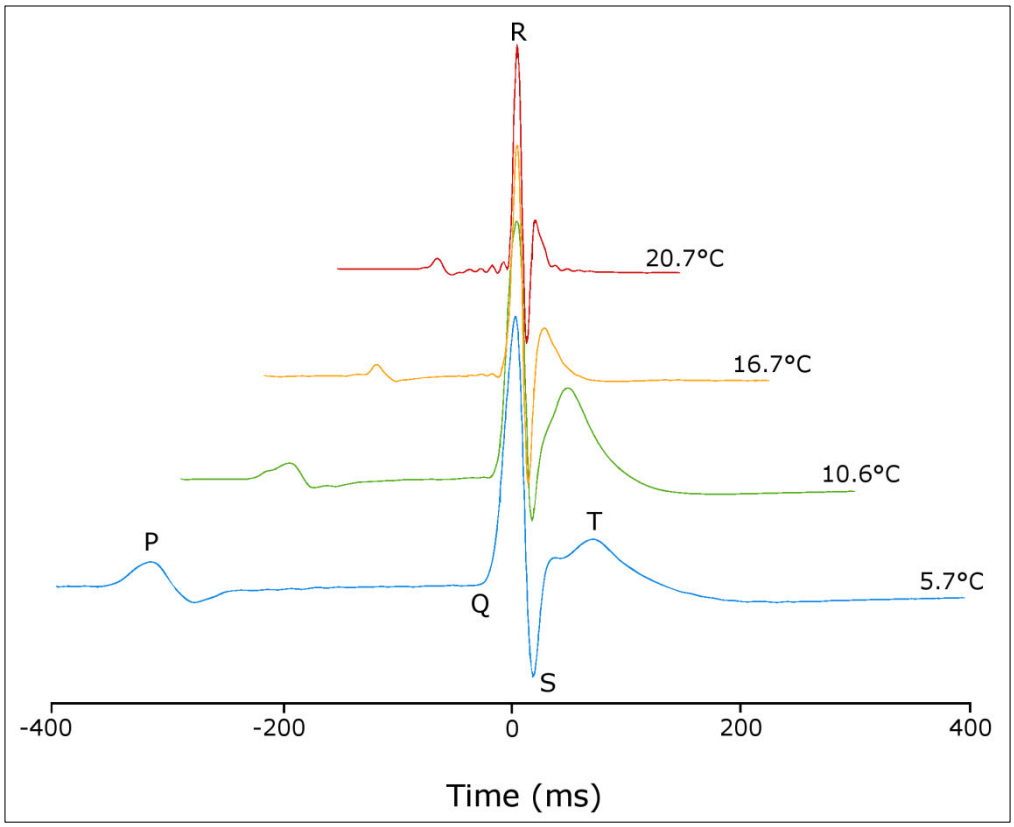

Figure 10. Average ECG waveforms from torpid bats at different T_{sub} aligned at the R wave. Note the prolongation of both atrial and ventricular conduction and the significant lengthening of P-R interval with decreasing temperature, and the lack of clear S-T segment until 5.7°C.

Ventilation rate

In normothermic bats at rest (T_{sub}=34.4 ± 1.2°C, *n*=13), VR increased linearly with decreasing T_a outside of the TNZ (Figure 11). VR was basal at T_a between 29.5 and 35°C with an average rate of 88 ± 13 breaths min^{-1} (*n*=5).

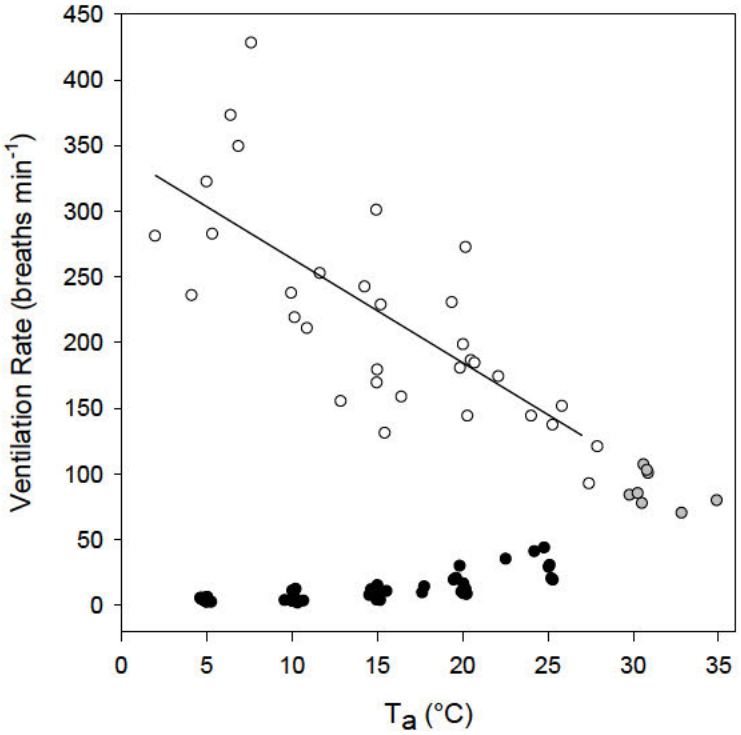

Figure 11. Ventilation rate (VR) as a function of T_a in normothermic resting individuals (circles), torpid individuals (black circles) and individuals within the thermoneutral zone (grey circles). VR = 343.23-7.92(T_a), r^2= 0.67, p<0.001.

As bats entered into torpor, VR slowed simultaneous with the drop in HR and this occurred before any substantial fall in T_{sub} (Figure 12). Following the initial slowing of VR, bats began to breathe in an episodic pattern, oscillating between short bouts of rapid ventilation interspersed with periods of apnoea. Below T_a of 15°C bats were apnoeic on average 88.4 ± 6.9% of the time, and bats at the lowest T_a of 4.8°C were apnoeic for a maximum 93.7% during steady-state torpor. The longest apnoeic period recorded in a torpor bout <24h was 51 min at T_a of 9.9°C. As T_a declined both the average apnoeic period (p<0.001) and length of breathing during torpor increased significantly (p<0.001) (Figure 13).

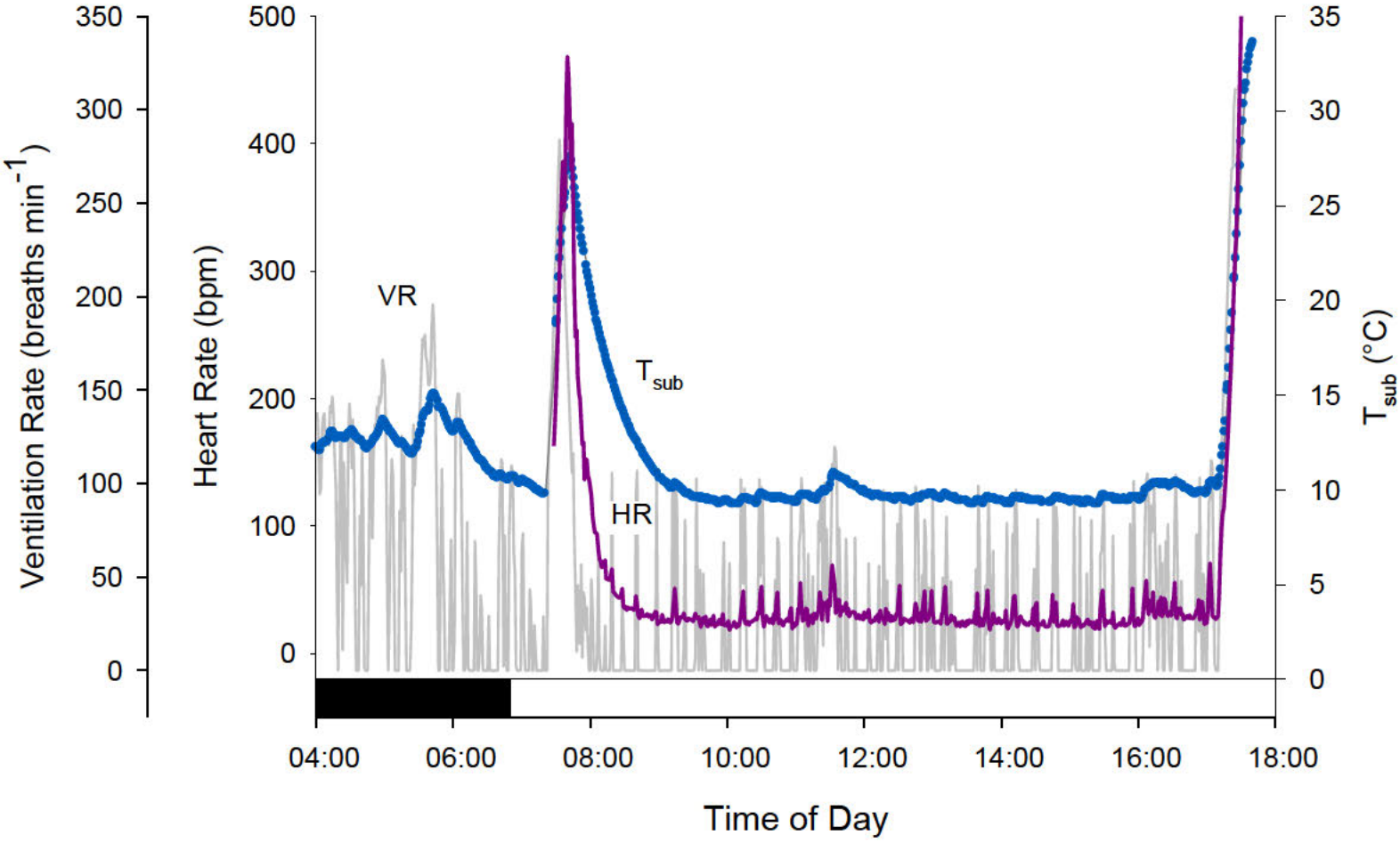

Figure 12. Representative trace of HR (purple line), VR (grey line), and T_{sub} (blue circles) over time throughout a torpor bout for an individual *N. gouldi* at a T_a of 10°C. Black bars indicate scotophase.

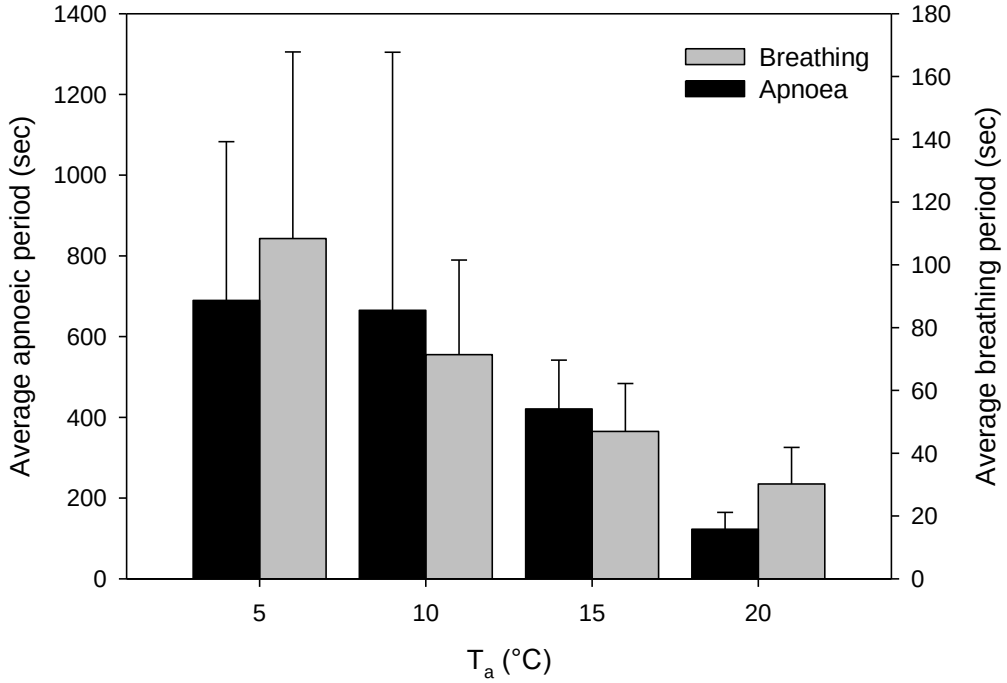

Figure 13. Average length of apnoeic period (black bars) and average length of breathing bouts (grey bars) plus standard deviation for *N. gouldi* in steady-state torpor at a range of T_a.

During steady-state torpor, the relationship between minimum average VR (calculated over 30-45 mins) and T_a was curvilinear (Figure 11). However when VR was calculated within individual breathing bouts the relationship with T_a was positive and linear (r^2= 0.54, $p<0.01$) (Figure 14). There was also a positive linear correlation between average apnoeic period and average length of breathing bouts, although with poor predictive power (r^2= 0.21, $p<0.001$)(Figure 15).

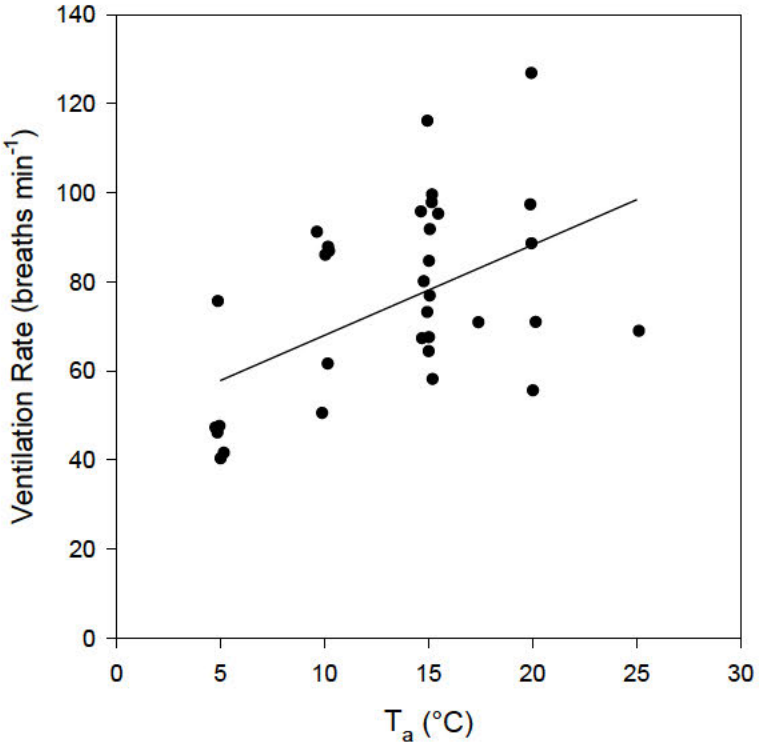

Figure 14. Average VR during breathing bouts in torpid *N. gouldi* increased linearly with increasing T_a. VR = 2.03(T_a) + 47.7, r^2=0.54, p<0.01.

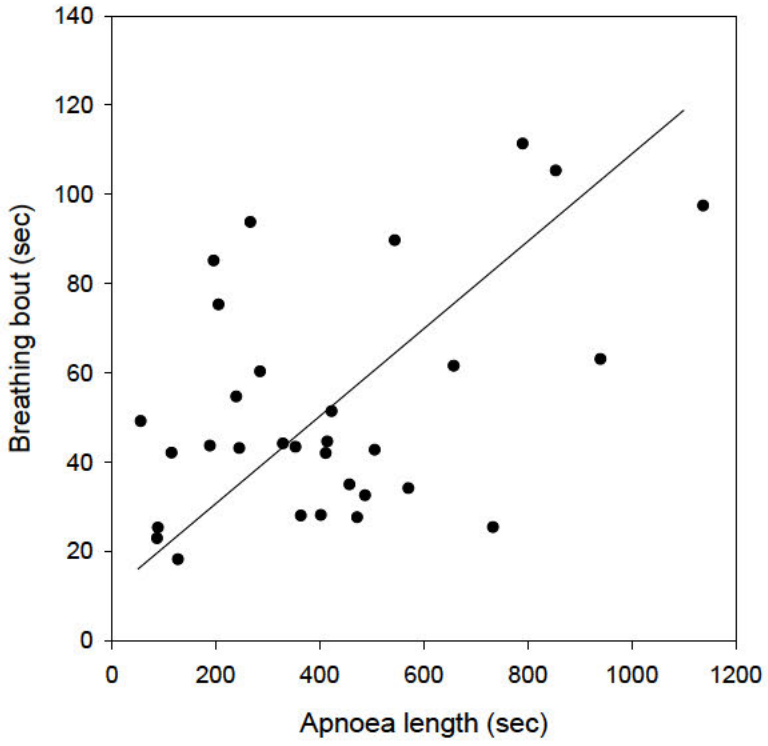

Figure 15. Average length of breathing bouts (BR) as a function of average apnoeic period (AP) for *N. gouldi* in torpor at T_a between 4.8 and 25.1°C. There was a positive correlation between apnoeic period and breathing length; BR = 0.098(AP) + 11.037, r^2=0.21, p<0.001.

When bats were thermoconforming in steady-state torpor, there was often cardio-acceleration associated with periods of ventilation (Figure 16A). However this was not always the case and some ventilatory bouts had accompanied asystoles on ECG recording (Figure 16B). When the average HR during periods of apnoea was compared to HR averaged during a breathing bout, there was no significant difference at either T_a of 5°C (paired t-test, t=-0.92, df=3, p=0.43) or 15°C (paired t-test, t=-2.49, df=4, p=0.07) (Figure 17). Unfortunately, there were not data from enough animals for statistical analyses at any other T_a.

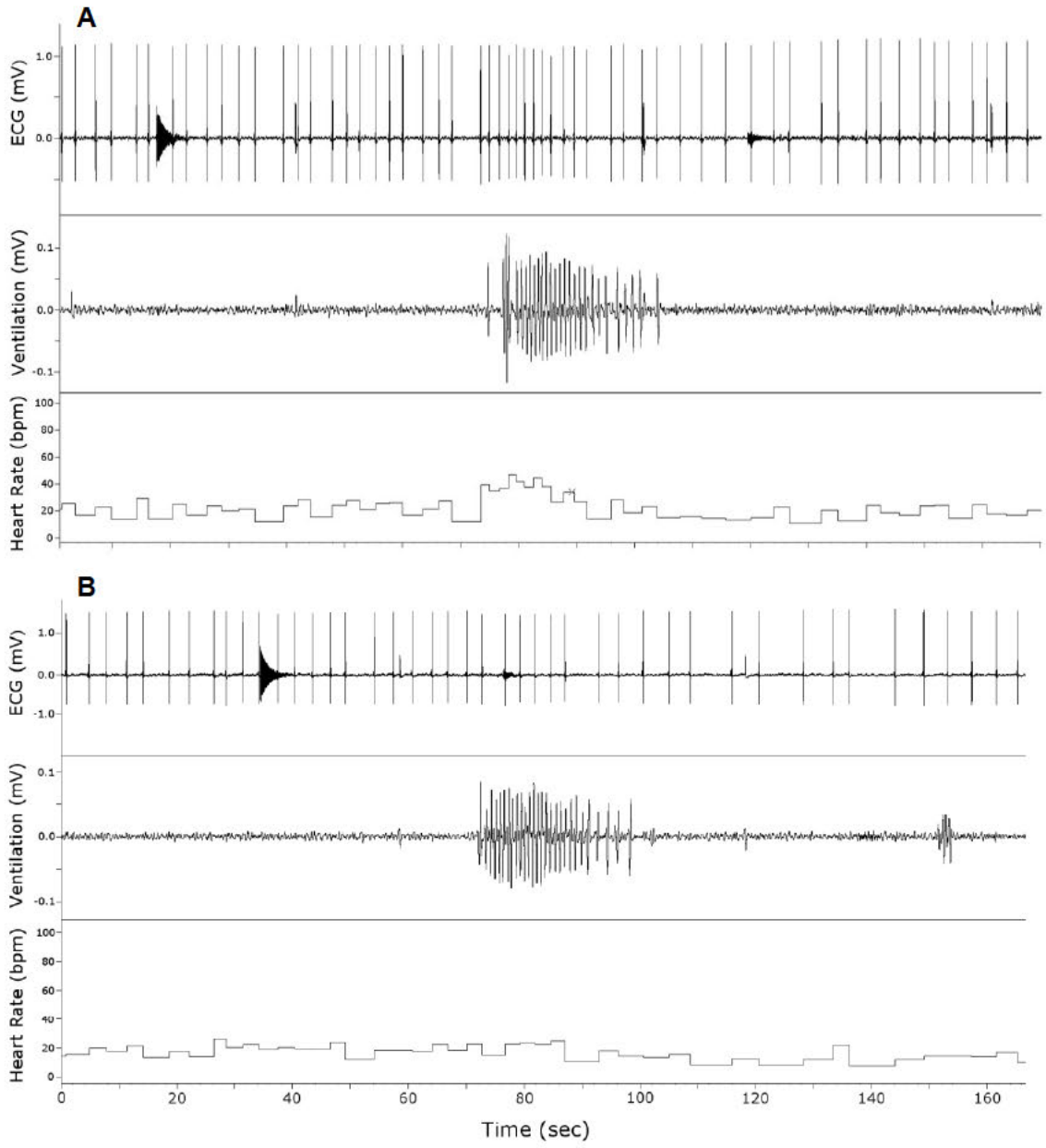

Figure 16. ECG and ventilation trace with calculated HR at two different breathing periods during a single torpor bout for an individual *N. gouldi* at T_a of 15°C. **A)** Shows cardio-acceleration associated with breathing. **B)** Breathing bout without substantial increase in HR.

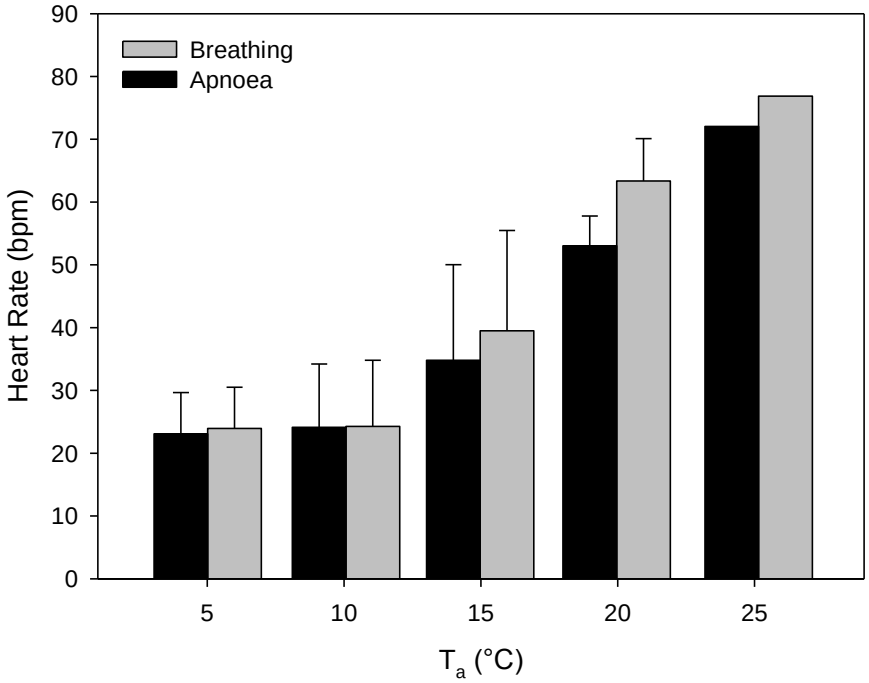

Figure 17. Average HR plus standard deviation during periods of eupnoea versus apnoea in thermoconforming torpid *N. gouldi* as a function of T_a, data at 25°C is from a single individual.

Oxygen Pulse

When bats were normothermic at rest, oxygen pulse (OP) increased linearly with decreasing T_a (Table 1, Figure 16). When T_a decreased from 30 to 20°C a rise in HR contributed 44.8% to increased OP in resting individuals and an additional fall in T_a from 15 to 5°C only resulted in an increase in the contribution of RHR to OP to 52.3%. When animals were in torpor, there was a substantial drop in OP and this declined with decreasing T_a in a curvilinear manner (Figure 16). At the lowest T_a (5.6°C) average OP in torpid bats was reduced to 17% of resting values while average HR was reduced to only 2% of resting rates. When animals were in torpor HR changed to a considerably greater degree than OP with changes in T_a. Between 25 and 15°C a change in HR contributed 91% to the slight increase in OP, and from T_a 15 to 5°C this only changed to 84%.

Table 1. Average oxygen pulse and corresponding HR at rest or during torpor at T_a between 5 and 30°C.

T_a (°C)	RHR	OP Rest (ml O_2 g^{-1} $beat^{-1}$ $\times10^{-4}$)	THR	OP Torpor (ml O_2 g^{-1} $beat^{-1}$ $\times10^{-4}$)
5.6	675 ± 33	2.75 ± 0.67	15 ± 4	0.46 ± 0.11
10.8	459 ± 92	2.31 ± 0.45	27 ± 11	0.41 ± 0.16
15.2	437 ± 95	1.82 ± 0.69	32 ± 13	0.56 ± 0.14
20.1	401 ± 63	1.96 ± 0.68	45 ± 12	0.70 ± 0.16
25.2	324 ± 72	1.49 ± 0.41	101 ± 38	0.68 ± 0.20
30.5	227 ± 34	1.01 ± 0.23		

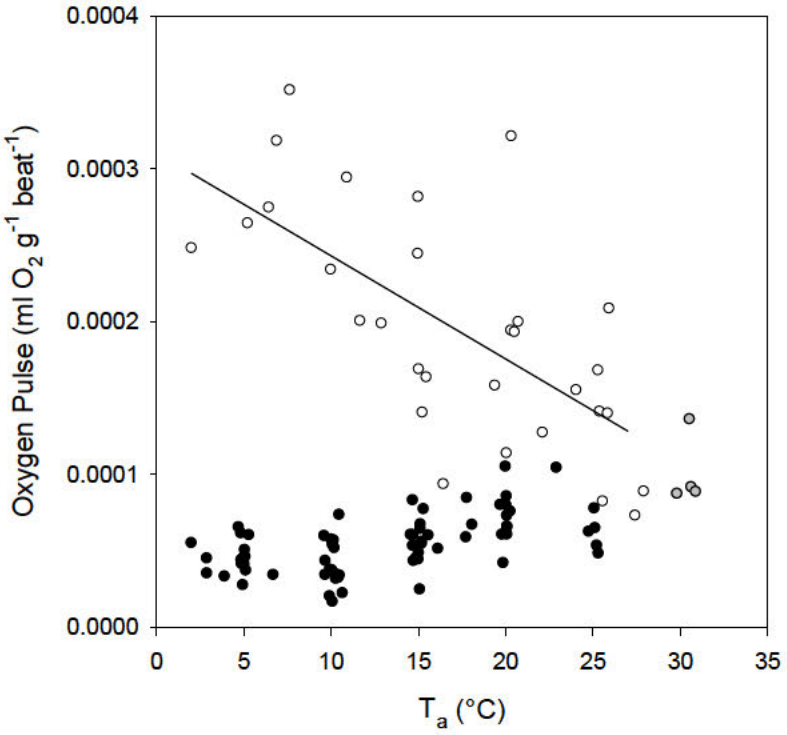

Figure 18. Oxygen pulse (OP) as a function of T_a for resting (circles), and torpid (black circles) *N. gouldi* as well as individuals in the TNZ (grey circles). OP ($\times10^{-4}$) =3.104×10^{-4}-0.068×10^{-4}(T_a), r^2=0.75, p<0.001.

DISCUSSION

Regardless of physiological state the cardiovascular and respiratory systems work in concert to deliver adequate oxygen to tissues throughout the body. When animals enter torpor these systems respond in a different functional pattern to decreases in T_b. I show that, although both the HR and VR decline drastically in response to low T_b and reduced oxygen requirements of tissues, the manner by which this reduction is achieved differs. In *N. gouldi* the heart is slowed to a steady low rate as torpor progresses while ventilation pattern slows only slightly before an abrupt alteration in breathing pattern, where cycles of extended apnoeas and short breathing bouts result in overall reduced rates.

ECG dynamics

My study shows that over a HR range of greater than 500bpm and T_{sub} range up to 30°C, the hearts of *N. gouldi* continued to beat in a coordinated manner with no significant deformation of ECG morphology. When bats entered into torpor, there was an increase in the amplitudes of all of the ECG waves as T_b declined, which was reversed with arousal. It is possible that this represents a temporal overlap in ventricular depolarisation and repolarisation when animals were normothermic, such that repolarisation began before the complete depolarisation of the entire heart. Therefore, as animals cool and there is a prolongation of the start of repolarisation, the amplitude of the QRS complex increases as repolarisation shifts away.

At all T_a tested, the period between beats, measured as the TP interval, showed the greatest extension when animals entered torpor. Following this, the prolongation of AV node conduction, indicated by the length of the PR interval, showed the next greatest change and increased in a curvilinear pattern with decreasing T_a. In bats, the degree of stretch of PR interval between rest and torpor was greatest at T_a 5°C increasing by 12.5 fold and was similar to previously reported values for much larger hibernators at the same T_a; approximately 13 fold increase in ~500g Arctic ground squirrels (*Spermophilus parryii*) and around 12 fold increase in ~600g Franklin's ground squirrels (*S. franklinii*) (Dawe and

Morrison, 1955). As heart mass influences the speed of cardiac conduction and heart mass and PR interval both scale in a log-linear manner with body mass in resting bats (Noujaim et al., 2004; Canals et al., 2005), it is interesting that PR interval prolongation should be similar between these hibernators. The speed of cardiac conduction is slowed in hibernators, not only by the effects of temperature on the heart, but also by reductions in metabolism. In small hibernators, metabolic inhibition has been suggested to play a significant role in maintaining very low MR during torpor and therefore it is probable that this is contributing to the prolongation of PR intervals seen in *N. gouldi*. In addition, increased vagal tone may also be acting on the atrioventricular node to slow conduction prior to ventricular depolarisation in these animals.

Interestingly however, there was limited change in ventricular depolarisation in *N. gouldi* with QRS width only broadening by 3.8 fold at its maximum, compared to the 7-9 fold increase found for QRS complexes in hibernating *S. parryii* and *S. franklinii* (Dawe and Morrison, 1955) and 5.3 fold extension in torpid hedgehogs (*Erinaceus europaeus*) (Sarajas, 1954). The retention of proportionately rapid ventricular propagation (QRS interval) in bats reflects an innate precision of ventricular conduction capacity, necessary to support the rapid HRs experienced during flight. There was no effect of season on the speed of ventricular conduction in torpor, further indicating the ability of bats to enter low T_b torpor year round. This was further supported by the proportionately shorter increase in QT interval in bats of 2.7 fold compared to 4-6 fold in *S. parryii, S. franklinii and E. europaeus* (Sarajas, 1954; Dawe and Morrison, 1955). Following arousal from torpor, many bats can begin to fly immediately, even before T_b has reached normothermic levels. This has been suggested to be a mechanism to speed up arousal, whereby the heat generated by flight muscles is used to raise T_b the final few degrees to normothermy (Willis and Brigham, 2003). The ability to move during torpor is not unique to bats (Rojas et al., 2012), however locomotion in other heterothermic animals at T_bs below normothermy can be sluggish and uncoordinated. Bats on the other hand, are capable of functional flight well before the completion of arousal which is likely advantageous in

relation to predator avoidance as well. Therefore the retention of rapid ventricular conduction capacity and repolarisation would facilitate the often swift transition from torpor to flight in these animals.

An interesting phenomenon common to the ECGs for all heterothermic mammals studied to date, is the lack of a clear isoelectric ST segment. The ST segment represents completed ventricular depolarization and is a consistent feature of the ECG of large homeothermic mammals, such as humans. Although the lack of an ST segment appears to be a trait of heterothermy, most heterothermic animals are small (<1kg) (Ruf and Geiser, 2014) and the close proximity of the T wave to the QRS complex may also represent the need for rapid repolarisation of the ventricles associated with high HRs at rest. This is illustrated by small non-hibernating species such as rats (*Rattus norvegicus*), which also lack an ST segment, but whose hearts are unable to function at T_b below 15°C. When bats entered torpor, there was little or no seperation of the QRS complex and T wave. This has been reported in a number of hibernating animals such as ground squirrels (Dawe and Morrison, 1955; Steffen and Riedesel, 1982), hedgehogs (Sarajas, 1954; Dawe and Morrison, 1955), eastern pygmy possums (*Cercartetus nanus*; Bartholomew and Hudson, 1962) and bats (*Myotis myotis, Nyctalus noctula, Nyctalus leisleri* and *Plecotus auritus*; Kulzer, 1967), as well as during daily torpor in Djungarian hamsters (*Phodopus sungorous*; Mertens et al., 2008) and a few species of shrew (*Crocidura russula, C. leucodens, C. suaveolens, Neomys fodiens* and *Sorex araneus*; Nagel, 1986). Concurrent to this, is a relatively short QT interval in both resting and torpid individuals. In *N. gouldi*, the QT interval showed greater prolongation than QRS or P intervals, however this was generally due to substantial contribution of T wave prolongation. When animals are in torpor, the initial phase of repolarisation remains relatively rapid as demonstrated by the minimal dispersion of T_{peak} from the QRS complex; hence the extension of the T wave is primarily driven by prolongation of the period from T_{peak}-T_{end}. This has also been reported in hibernating ground squirrels and hedgehogs where measurements of RST segment were virtually unchanged at low T_b (Dawe and

Morrison, 1955), and in torpid Djungarian hamsters where QT_{peak} was measured and again showed little change from normothermy to torpor (Mertens et al., 2008). The T wave encompasses repolarisation across different regions of the ventricle where T_{peak} corresponds to repolarisation of the epicardial action potential, while T_{end} coincides with the completion of mid-myocardial repolarisation (Mertens et al., 2008). In hedgehogs (*E. europaeus*), the ventricular epicardial action potential is more rapid than that of non-hibernating guinea pigs, both at normothermic temperatures and under hypothermic conditions, and lacks a significant plateau phase (Duker et al., 1987).

It is widely considered that the retention of the close association between ventricular depolarisation and repolarisation in heterothermic mammals works as a protective mechanism against the generation of disruptive arrhythmias at low T_b. The significance of this cardiac reserve, represented by QT intervals, may even be independent of BM and thus the influence of HR. Relatively short QT intervals have been found in the largest hibernators (≥100kg) the bears (grizzly, *Ursus arctos* and polar, *U. maritimus*) (Folk et al., 2008). These animals are interesting hibernators as they do not undergo typical periodic arousals and show only a slight reduction in T_b during torpor (Tøien et al., 2011), thus indicating that retention of a rapid conduction/repolarisation cycle is important even at high hibernation T_bs.

The majority of previous investigations into cardiovascular physiology of hibernation have taken place on relatively large and strictly seasonal hibernators that have been shown to undergo a preparatory period prior to the hibernation season. During this time, there is often considerable fattening, as well as a substantial change in the composition of proteins and lipids, and therefore the functional capacity of many tissues and organs (Andrews et al., 1998). The hearts of some seasonal hibernators have been shown to vary considerably in their responses to hypothermic conditions induced in summer compared to induced hypothermia or natural hibernation in winter (Caprette and Senturia, 1984; Johansson, 1996). Bats, on the other hand, cannot store large quantities of body fat and many will enter torpor year round, showing little variation in cardiac function with

season. ECGs in my study were collected from bats throughout the year and there was no evidence of seasonal difference in torpor pattern or conduction capacity at any of the T_as animals were exposed to. This suggests that it is unlikely that these animals experience a preparatory period before the 'hibernation season' and that the heart is always capable of adequate performance at low T_b in torpor regardless of season.

The remarkable capacity of the hearts of hibernators to withstand fluctuating T_bs without generating significant arrhythmias is well documented (Duker et al., 1983; Johansson, 1996; van Veen et al., 2008). However there have been occasions where conduction arrhythmias have been noted (Lyman et al., 1982; Eagles et al., 1988; Elvert and Heldmaier, 2005). In these cases, it is possible that experimental protocol and disturbance of the animals may have generated the reported ectopy; however it is also possible that these arrhythmias have been mislabelled due to the quality of recording equipment. The presence of any considerable conduction arrhythmias in hibernators seems highly unlikely, particularly in bats where torpor use is common year round and the heart plays an acutely important role during the often frequent arousals. Moreover, the need for precise electrophysiological coordination at the extreme HRs experienced during flight would be further counterintuitive to torpor induced arrhythmogenesis in bats. In over 2500h of ECG recording across 21 individuals only one bat in my study exhibited two instances of conduction arrhythmia in the form of an atrial premature beat (Figure 19). In hibernating thirteen-lined ground squirrels it has been noted that electrical depolarisations occasionally occurred without substantial changes in pulse pressure (Lyman, 1982a). Unfortunately, in the case of the bat, this animal was not laying flush with the pulse transducer so it cannot be assessed whether depolarisation was followed by contraction in this instance. Otherwise, there were no significant manifestations of conduction block, tachyarrhythmia or ventricular ectopy recorded during any phase of torpor.

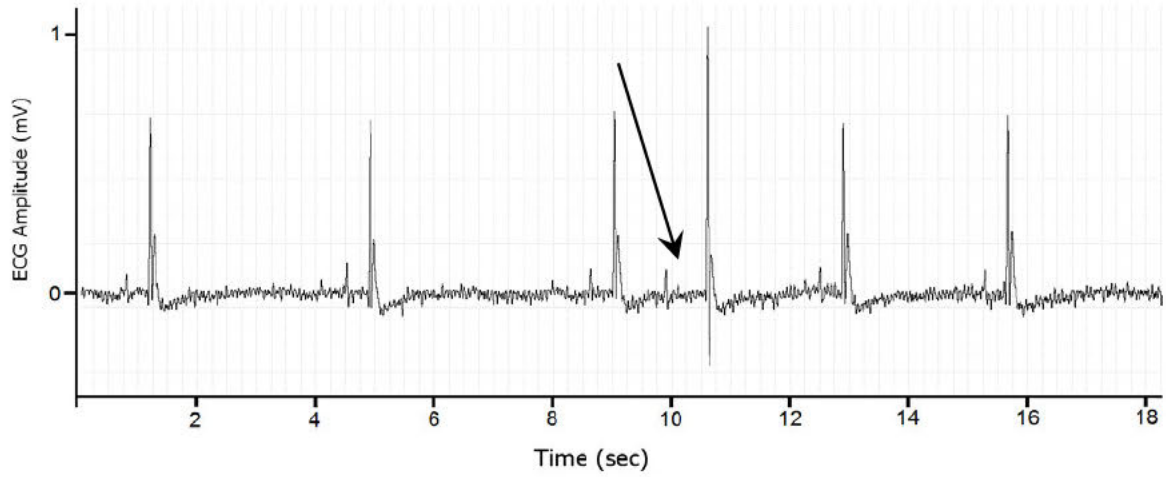

Figure 19. ECG trace from a torpid *N. gouldi* individual illustrating one of only two incidences of conduction arrhythmia detected in all experiments. The arrow indicates the premature atrial pacing with a prolonged PR interval.

Ventilation

During hibernation, bats breathed intermittently in a pattern quantitatively similar to that expressed in many other hibernating species (for review see; Zimmer et al., 2000). At all T_as tested, bats spent a significant proportion of torpor bouts apnoeic (54-94% of the time). The longest apnoeic period recorded for *N. gouldi* during torpor over one day was 51min and significant periods of apnoea lasting >2h have been reported in other species of hibernating bats at similar low T_b ~5°C (Thomas et al., 1990; Szewczak and Jackson, 1992b; Szewczak and Jackson, 1992c). Previous studies have indicated a great deal of variability in the length and frequency of breathing bouts and periods of apnoea have been shown to increase and become more stable as torpor bouts progress into multiday torpor (McArthur and Milsom, 1991). For relevant comparison between T_as, analyses were restricted to torpor bouts <24h in my study. However when analyses were extended to one animal that hibernated for four days, the maximum apnoeic period recorded was 74min. Across all T_a, there was a great deal of variability in the length of breathing bouts as well as the ventilation frequency within breathing bouts, however both variables were a clear function of T_a. This was contrary to the findings of Zimmer and Milsom (2001) in larger hibernators, the golden-mantled ground squirrel (*Spermophilus lateralis*), where

changes in temperature did not have a significant effect on the frequency of breathing bouts or ventilation rate during hibernation. In *N. gouldi*, although the frequency of ventilation within a breathing bout increased with increasing T_a the average length of the breathing bouts decreased, although this was not reflected by total compensation of oxygen consumption. The increased VR is likely a response to the effect of increased T_b on metabolism and the functional capacity of ventilatory muscles. There is also an effect of increased temperature on the rate of oxygen diffusion and carbon-dioxide expulsion from the lungs as well as oxygen affinity of the blood which in turn reduces the time needed for ventilation.

There has been much research conducted on episodic breathing patterns in hibernating mammals, however a clear understanding of the mechanisms controlling the length of apnoeic periods remains elusive. In bats, extended apnoeas are thought to be facilitated by oxygen uptake during this period which has been suggested to be substantial (Szewczak, 1997). Moreover, the increased acidosis during apnoeic periods has been suggested as a mechanism of metabolic suppression in torpor (Malan, 1982; Szewczak and Jackson, 1992a). Previous studies have suggested that during this period in many hibernators, including bats, the glottis may remain open to enable passive diffusion of oxygen down the trachea (Szewczak and Jackson, 1992b).

Although there was an association between periods of ventilation and cardio-acceleration in my study, the frequency of this respiratory tachycardia and its degree was less prominent in *N. gouldi* than has been described in previous studies on other hibernating species (Zimmer and Milsom, 2001; Zosky and Larcombe, 2003). It is believed that during hibernation, control of cardiorespiratory function is related to parasympathetic action or the alternation between parasympathetic and sympathetic outflow, which may contribute to generating the ventilatory tachycardia shown in many hibernating individuals. In *N. gouldi*, although cardio-acceleration was not always associated with breathing, there were often periods of asystole during ventilation episodes. This may indicate the action of the parasympathetic nervous system working to maintain a slow HR against the drive for

increased HR at the onset of breathing. Bats have an extremely high capacity gas exchange system necessary to support the needs of powered flight. When these animals enter torpor, this system is working to drive a high capacity organ at severely depressed rates. Clearly, coupling of ventilation with increased HR ensures inspired oxygen is efficiently transported around the body. However when the respiratory exchange system is adapted for maximum capacity workloads, as is the case in bats, it may be more advantageous to slow HR early during this period so as to maintain low energy consumption.

Oxygen Pulse

Changes in oxygen pulse, the amount of oxygen transported per heart beat, provides a representation of changes in the circulatory system at different physiological states. When *N. gouldi* are normothermic the rate of oxygen consumption increases linearly with decreasing T_a associated with thermoregulation (Currie et al., 2014) and this was also the case for OP. In these bats, adjustments in HR to compensate for the decline in T_a are nearly equivalent to changes in OP, contributing ~50%. My results suggest therefore, that there is a proportionate change in HR with both stroke volume and arterio-venous difference in resting bats as T_a changes. Moreover, the average calculated values for OP at rest in *N. gouldi* and rate of linear increase with decreasing T_a were similar to that found for a similar sized heterotherm, the European white toothed shrew, at rest (~11.5g, *Crocidura russula*) (Nagel, 1986).

My study is the first to present OP as a function of T_a during torpor in hibernating bats. I show that there is a substantial reduction in OP during torpor compared to resting rates, and that OP increases with increasing T_a in a curvilinear pattern. Although there have been no direct measurements of stroke volume in hibernating bats the increased peripheral resistance and drop in HR and cardiac output measured in other hibernators suggested that stroke volume declines as well (Lyman, 1982b). During torpor in *N. gouldi* HR increased to a greater extent than OP with increased T_a. This suggests that there is minimal change in stroke volume or oxygen extraction rates during torpor regardless of

temperature and that alterations in HR contribute proportionately more to the increased oxygen transport at mild T_a. Previously presented data on OP in bats by Studier and O'Farrell (1976) showed that HR contributed <50% to oxygen transport at most T_a that bats were exposed to (15-30°C). Unfortunately it is not clear whether these results were taken from normothermic bats only or if torpid individuals were included in the calculations as associated T_bs are not presented.

My study is the first to quantify the cardiac dynamics and ventilatory function of an Australian bat simultaneously during torpor and as a function of T_a. When bats entered into torpor VR and HR declined prior to a drop in T_b, and breathing became episodic. During steady-state torpor, ventilatory frequency was a function of T_a and although there was an association between breathing bouts and changes in HR, there was not a significant respiratory tachycardia. The hearts of hibernating *N. gouldi* retained remarkable integrity of conduction rhythms across a wide range of T_b and HR. There was a rapid repolarisation of the ventricles indicated by the lack of a clear isoelectric ST segment and short QT/JT intervals retained at near normothermic length until T_{sub} below 16°C. The absence of significant arrhythmias exemplifies the important and innate cardiac reserve related to rapid depolarisation and the close coupling of repolarisation, present in all heterothermic mammals for which ECG data have been attained. Moreover, these bats showed no variation in cardiac excitation rhythms or respiratory function across seasons, thus indicating their innate ability to withstand extreme variations in cardiorespiratory function, from torpor to flight, without detriment.

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STATEMENT OF AUTHORS' CONTRIBUTION

(To appear at the end of each thesis chapter submitted as an article/paper)

We, the PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

	Author's Name (please print clearly)	% of contribution
Candidate	Shannon E Currie	80%
Other Authors	Gerhard Körtner	10%
	Fritz Geiser	10%

Name of Candidate: Shannon E Currie

Name/title of Principal Supervisor: Professor Fritz Geiser

[redacted]

Candidate

1/3/15

Date

[redacted]

Principal Supervisor

1/3/15

Date

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We, the PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

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Name of Candidate: Shannon E Currie

Name/title of Principal Supervisor: Professor Fritz Geiser

Candidate — Date: 1/3/15

Principal Supervisor — Date: 1/3/15

Chapter 7

Final Discussion

Bats are unique amongst mammals in their ability to fly and have developed a highly specialised and efficient cardiorespiratory system to support the extreme oxygen demands associated with flight. However these animals are capable of reducing cardiorespiratory output and metabolism to comparatively minuscule levels during torpor. The ability of bats to maintain coordinated cardiac function over this extraordinary range provides insights into the adaptability of the mammalian cardiovascular system. Previous investigation into cardiac function in heterothermic bats has generally been limited to temperate zone species of the northern hemisphere and involved the use of invasive methods due to an unfortunate compromise between signal quality and animal disturbance.

In my PhD I investigated cardiorespiratory function and metabolism during rest and torpor in two Australian species of heterothermic bats (*Nyctophilus gouldi* and *Syconycteris australis*). Although previous data exist for the thermal energetics of these species their small size <20g has previously restricted the use of implantable temperature telemetry for regular measurement of T_b. In my study, however, small, lightweight temperature-sensitive transponders were able to be implanted subcutaneously and were shown to provide reliable measurements of T_{sub} over an array of T_a, both at rest and during torpor. In addition the use of externally adhesive ECG electrodes or modified bat bands meant that the bats were only lightly restrained during respirometry and could easily remove the apparatus if they were uncomfortable- this occurred quite rarely in *N. gouldi* but was more frequent in *S. australis*. Consequently, bats in my study tolerated the experimental protocol well and I have presented some of the lowest HR values for bats, including the first HR data for a small pteropodid bat during torpor.

It is extremely important for a comprehensive understanding of the physiology of torpor, that simultaneous measurements of multiple variables be attempted, as discerning causal relationships cannot be derived from an isolated measurement. Previous studies of $\dot{V}O_2$ in hibernating bats have often reported either extremely low or relatively high average rates of oxygen consumption for hibernators. This is likely the result of time span measurements were averaged over and/or the time at which measurements were taken. In many cases short measurement time would result in an underrepresentation of $\dot{V}O_2$ in torpid bats as the episodic breathing pattern results in lengthy apnoeas, which can make up 94% of a torpor bout as is the case for *N. gouldi*. As I have shown, the combination of traditional open-flow respirometry with piezoelectric measurements of ventilatory movement enabled a more accurate assessment of $\dot{V}O_2$ by ensuring periods corresponding to breathing bouts (i.e. gas exchange) were incorporated into average measurements. Furthermore, the simultaneous measurement of both ventilation and electrocardiography enabled investigation of cardiorespiratory associations during torpor and assessment of previously described ventilatory tachycardia in bats. I show that unlike in larger hibernators, there was not a consistent significant cardioacceleration associated with ventilation, as HR during apnoeas was not considerably different to HR during breathing bouts.

The combination of respirometry and ECG measures with consistent measurements of T_{sub} also resulted in a greater understanding of how disturbance and/or 'discomfort' of the animals impact torpor measurements. This was indicated by some individuals that maintained a large T_{sub}-T_a differential when in torpor even at T_a above the minimum T_{set} for thermoregulation. It is possible therefore that values of HR reported for other hibernating bats (≥40bpm) may not represent minimum values in thermoconforming animals as HR was the only variable consistently measured in these studies and the values reported are similar to my findings in *N. gouldi* that were thermoregulating at the same T_a; ~45bpm at 5°C compared to minimum HR in thermoconforming animals ~8bpm at 5°C.

Previously, measurements of T_b in small heterothermic mammals have often required disturbance and handling for rectal thermometry which results in an interruption of torpor and/or premature arousal. In my study, T_{sub} was recorded regularly (approximately every minute) and remotely via modification of a transponder scanner. The sensitivity of torpid animals to external disturbance has meant that the ability to measure a number of physiological variables during the different phases of torpor has been difficult in the past as long term monitoring is required. Many previous studies investigating arousal physiology have been taken from induced arousals which have, more recently, been shown to not be entirely representative of spontaneous arousal from torpor. Also, as entry into torpor is not a predictable process, and animals encumbered by experimental apparatus have tended not to enter torpor in the past, this phase has been particularly difficult to study, especially in small animals. The use of temperature-sensitive transponders, which require no battery, to measure T_{sub} remotely in my study enabled generally uninterrupted recording from individuals which could be repeated over time. In addition, bats in my study were not overly hampered by the ECG electrodes and frequently re-entered torpor following the initial disturbance of lead attachment. Consequently, I was able to provide new information regarding the relationships between HR, $\dot{V}O_2$ and T_{sub} during transitional periods of entry into torpor and spontaneous rewarming that have previously been unavailable for small heterothermic bats. Furthermore, my study shows how these relationships change with increasing T_a and consequential passive rewarming, and is the first to provide data on cardiac function during this phase.

Many bats and other heterothermic species will exploit increases in T_a after sunrise to facilitate passive rewarming from torpor, and *N. gouldi* has been shown to regularly capitalize on the benefits of passive rewarming in the wild. My data suggest that not only does passive rewarming reduce the energetic cost of active arousal, but it may also reduce demands on the cardiovascular system by warming the body and therefore the heart, to optimal levels prior to the initiation of active arousal. In both bat species

comparisons between entry and arousal show that for HR and $\dot{V}O_2$ the relationship with T_{sub} differs dramatically during these two phases. There was a clear hysteresis of both HR and $\dot{V}O_2$ with both variables being significantly lower during entry than at the same T_{sub} during arousal. In addition for *N. gouldi* I have shown that metabolic inhibition may not be fully applied until the later stages of torpor entry and this is at least partially withdrawn in response to increasing T_a during passive rewarming, likely as a precursor to active arousal.

Essential to the further understanding of overall changes in cardiac physiology during torpor is the underlying alterations to the cardiac conduction system with changing T_b. In hibernating *N. gouldi* there was no considerable deformation of the ECG waveform over a reduction in T_{sub} of up to 30°C. However, when bats entered torpor there was an overall prolongation of the entire cardiac conduction cycle, with PR interval showing the greatest elongation in this sequence. My findings are in line with those of previous studies from heterothermic animals and also indicate the remarkable importance of coupling between ventricular depolarisation and repolarisation cycles. *N. gouldi*, like many larger hibernators, showed no isoelectric ST segment and retained a relatively short QT interval which displayed little prolongation when animals entered torpor.

In the past, the differences between daily torpor and hibernation have been based on comparisons in animals that express the epitome of either trait- larger temperate seasonal hibernators at very low T_a (≤5°C) or small strict daily heterotherms at mild T_a (≥15°C). Moreover data used for comparison was not generally collected within a single study using the same methods, and to date have not incorporated cardiac physiology, which is likely to differ substantially between the two torpor patterns. Therefore I aimed to investigate HR, $\dot{V}O_2$ and T_{sub} on two species of bats that either enter daily torpor, or hibernation, using similar methods. Bats are especially interesting to compare as they will use torpor throughout the year without a clear and significant preparatory period prior to torpor use, and hibernating species will often enter torpor over temporally similar periods to daily heterotherms enabling a more relevant comparison of the two states. My results

show that for two species of heterothermic bat <20g whose habitat ranges overlap in the wild, the physiological patterns during torpor were vastly different at the same T_a. The rate of cooling during entry into torpor was significantly slower in *S. australis* as these animals thermoregulate during this phase more frequently than *N. gouldi*. Although analyses were restricted to torpor bouts <24h for both bats, *N. gouldi* often remained in torpor until the dark stimulus in the evening and occasionally a couple of hours later, while *S. australis* aroused much earlier in the afternoon and never remained in torpor for more than 8h. Minimum HR during steady-state torpor at the same T_a was much lower in *N. gouldi* than in *S. australis* and this was also true for $\dot{V}O_2$. In addition, the minimum T_{set} for thermoregulation in torpor was substantially higher for the daily heterotherm *S. australis* at ~15°C than the hibernator *N. gouldi* ~3°C.

FUTURE DIRECTIONS

Knowledge of the physiological processes involved in coordination and maintenance of torpor is critical for the comprehensive understanding of how heterothermic animals are able to budget their energy and survive in the wild. This is particularly pertinent when referring to taxa such as bats, where torpor use is essential to the survival of many species and of the >1100 species and 19 families distributed almost globally, exhaustive data on torpor use is only available within the Vespertilionidae.

While laboratory studies provide essential information regarding an animal's physiology under specific controlled conditions, animals in their natural habitat experience a much greater array of challenges. For example, torpor use is often underestimated in laboratory settings where animals may be under duress and generally have access to energy *ad libitum*. Therefore the ability to measure physiological variables in free-ranging animals is of great interest to ecophysiologists. As such, the advent of small temperature sensitive transmitters has enabled a greater understanding of the frequency of torpor in animals in their natural habitat, however data regarding metabolism and cardiac function is lacking.

While HR has been shown to be a viable predictor of MR in large homeothermic mammals and birds over a wide variety of physiological states and in the wild, data do not exist for heterothermic animals during torpor. Very recently miniaturised HR transmitters have become available and have been used in small bats and birds, however these studies unfortunately did not specifically investigate the use of torpor, nor did they undertake comprehensive laboratory verification, involving regression validation during the different physiological states of the animals. My study has shown that HR and $\dot{V}O_2$ in *N. gouldi* are strongly correlated during torpor and at rest; however this relationship differed significantly between the states. Therefore my data suggest that HR may provide a reliable estimate of metabolism in heterothermic bats during both torpor and rest in the field. However it is essential that predictions are restricted to the range of activities over which accurate regression analysis has been undertaken, which for bats, must include the important phase of flight. Moreover, I recommend the incorporation of T_{sub}/T_{sk} measurements as well as T_a where possible as I have shown a significant effect of thermoregulation during torpor on both $\dot{V}O_2$ and HR. This has further implications for understanding the depth of torpor and therefore energy use in wild populations, which may also impact conservation efforts. This is pertinent at the moment in the United States where populations of hibernating bats have been rapidly declining since 2006 due to a disease known as white-nose syndrome which is caused by a “cold-loving” fungus (*Pseudogymnoascus destrucans*). Bats inoculated with the fungus in captivity have been shown to have increased MR during torpor with more frequent arousals, which leads to the complete depletion of the small energy reserves in these animals prior to spring foraging resulting in death. The ability to measure HR and T_b in these bats in the wild would therefore provide much needed information regarding energy expenditure in these animals and how this may relate to over-winter survival.