HUMIDITY, HUDDLING & THE HIBERNATION ENERGETICS OF BIG BROWN BATS (*EPTESICUS FUSCUS)*

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Abstract

During winter, many mammals hibernate and lower their body temperature and metabolic rate (MR) in prolonged periods of torpor. Hibernators will use energetically expensive arousals (i.e., restore body temperature and MR) presumably to re-establish water balance. Some hibernating mammals however will huddle in groups, possibly to decrease energetic costs and total evaporative water loss (EWL), although the benefit is not fully understood. Research on the relationship between behaviour, physiology, water loss, and energy expenditure of bats during hibernation is especially important because of a fungal disease called white-nose syndrome (WNS). To date, 12 North American bat species are affected by WNS, however big brown bats (*Eptesicus fuscus*) appear resistant, although the underlying mechanism is poorly understood. The overall objective of my thesis was to understand the influence of humidity and huddling on the behavioural and physiological responses of hibernating big brown bats. To test my hypotheses, I used a captive colony of hibernating big brown bats (n = 20). Specifically, for Chapter 2, I first tested the hypothesis that big brown bats adjust huddling and drinking behaviour depending on humidity, to maintain a consistent pattern of periodic arousals, and therefore energy balance during hibernation. I found that bats hibernating in a dry environment did not differ in arousal/torpor bout frequency, or torpor bout duration throughout hibernation but drank at twice the rate as bats in a humid environment. Bats in the dry treatment also had shorter arousals, and huddled in a denser huddle, potentially to reduce rates of total EWL. During late hibernation, for Chapter 3, I used open-flow respirometry to test two additional hypotheses, first that phenotypic flexibility in total EWL helps explain the tolerance of hibernating big brown bats for a wide range of

humidity relative to other bat species. I found that dry-acclimated bats had lower rates of total EWL, compared to bats acclimated to humid conditions. I then tested the second hypothesis that big brown bats can use huddling to mitigate the challenge of dry conditions. I found that, for humid-acclimated bats, rates of total EWL were reduced with huddling bats but there was no effect of huddling on EWL for bats acclimated to dry conditions. These results suggest that the ability of big brown bats to reduce rates of total EWL through acclimation may reduce the need to huddle with conspecifics to avoid water loss and thus dehydration. Overall, my thesis suggests that big brown bats use both behavioural and physiological mechanisms to reduce water loss which could allow them to exploit habitats for hibernation that are unavailable to other bat species and could also help explain their apparent resistance to WNS.

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Dedication

This thesis is dedicated to all the negative, stressful situations during my M.Sc.

Thank you for providing me the opportunity to learn

that the pursuit of science is not grounded on a path of success, prestige and positivity,

but rather one of tenacity, curiosity, and above all, resilience.

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List of Abbreviations

CO_2 – carbon dioxide

EWL – evaporative water loss

GLM – generalized linear model

HSI – huddle size index

H_2O - water

IR – infrared

MR – metabolic rate

O_2 – oxygen

Pd – *Pseudogymnoascus destructans*

RH – relative humidity

T_a – ambient temperature

T_b – body temperature

T_{sk} – skin temperature

WNS – white-nose syndrome

WVP – water vapour pressure

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CHAPTER 1: GENERAL INTRODUCTION

Hibernation In Mammals

During winter, many mammals enter long bouts of torpor (i.e., periods of extreme reduction in metabolic rate (MR) and body temperature (T_b)). Throughout torpor, T_b can fall to within 1– 2°C of the surrounding ambient temperature (T_a) (Geiser, 2004). This decrease in MR and T_b can reduce energy expenditure by up to 99% relative to remaining at normal T_b (Lyman et al. 1982, Geiser 2004). Mammals, however, cannot stay torpid through the entire hibernation period as there are associated costs with remaining at a lower T_b. During torpor, mammals cannot drink and urinate, thereby leading to a build-up of metabolic wastes (Thomas and Cloutier, 1992; Thomas and Geiser, 1997). Additionally, the immune system of hibernators becomes depressed which can cause some species to become susceptible to pathogens (Luis and Hudson, 2006; Prendergast et al., 2002). During torpor bouts, hibernators cannot grow or heal as metabolic processes that allow for cell division, protein synthesis, and gluconeogenesis are downregulated (Frerichs et al., 1998; Kruman et al., 1988; Staples and Hochanchka, 1998).

To ameliorate the costs of prolonged periods of torpor, hibernators will periodically arouse (i.e., restore MR and T_b to normothermic levels) at regular intervals. These periodic arousals are energetically expensive and can account for over 90% of a hibernator's winter energy budget, however they represent only ~1% of a hibernator's time (Karpovich et al., 2009; Thomas et al., 1990a). Previous studies have hypothesized that arousals function to alleviate the costs associated with prolonged torpor bouts (Baumber et al., 1971; Ben-Hamo et al., 2013; Daan et al., 1991; Galster and Morrison, 1970; Hope and Jones, 2012; Németh et al., 2010; Prendergast et al., 2002; Thomas and Geiser, 1997). The heat needed to restore T_b during

arousals comes primarily from metabolic heat production (Humphries et al., 2002; Thomas et al., 1990a). To fuel these energetically costly arousals, hibernating animals need to enter hibernation with either large enough food caches or endogenous fat stores (Speakman and Thomas, 2003). For example, little brown bats (*Myotis lucifugus*) have been recorded to increase their body mass prior to hibernation by 2.1 g or 32.9% for males and 2.3 g or 29.6% for females (Kunz et al., 1998).

Despite gaining large fat stores before hibernation, all hibernators must budget these energy stores to survive until spring emergence (Czenze et al., 2017). During an arousal, bats produce metabolic heat needed to rewarm through shivering and non-shivering thermogenesis (Mejsnar and Jansky, 1970). Brown adipose tissue is mainly used to start arousals via non-shivering thermogenesis, where heat is generated under the control of norepinephrine released by the sympathetic nervous system (Mejsnar and Jansky, 1970). Once a hibernators T_b is raised to 10–17°C, shivering thermogenesis can occur whereby heat is generated via contraction of skeletal muscles, under control from the sympathetic and motor systems (for reviews see: Cannon and Nedergaard, 2004 and Nakamura and Morrison, 2011).

Passive rewarming from torpor may also help conserve energy for hibernators, and the behaviour is common in many mammalian species (Geiser et al., 2004). Numerous mechanisms of passive rewarming exist which include raising T_b through social thermoregulation with conspecifics (i.e., huddling). Mammals can also passively raise their T_b either when the T_a increases, or by moving to a location with a warmer T_a. For example, diurnal increases in T_a in a building hibernaculum allowed for partial passive rewarming by rewarming by big brown bats (*Eptesicus fuscus*) (as the T_a never reached levels equal to normothermic T_b; Halsall et al. 2012).

The increase in T_a provided energy savings of approximately 4–47% equivalent to ~96 mg of body fat, which could fuel 16.5 days of steady-state torpor. One bat in the study used passive rewarming for 80% of its arousals, resulting in energetic savings of 267 mg of body fat, or 46 days of steady-state torpor (Halsall et al., 2012). Thus, passive rewarming can result in enormous energy savings for hibernating bats, which could allow for prolonged hibernation if ambient conditions or food availability remain unfavourable.

White-Nose Syndrome Pathophysiology

White-nose syndrome (WNS) is a fungal disease that has led to dramatic population declines of bats across eastern and central North America, and three species are now listed as federally endangered in Canada (Cheng et al., 2021; Frick et al., 2015). The causative agent of the disease, *Pseudogymnoascus destructans* (*Pd*), which grows on the wings and face of bats, is spread through both direct contact, and indirect contact with surfaces, either before or during hibernation (Lorch et al., 2011). In the fall prior to hibernation, the probability of contact with an infected bat is higher because bats participate in swarming behaviour (i.e., congregating at hibernacula and mating promiscuously; Thomas et al. 1979). Additionally, in the winter, some species of bats will huddle with conspecifics, presumably to decrease energy expenditure or reduce evaporative water loss (EWL; Boratyński et al., 2015), which in turn could provide a direct transmission route for *Pd* spores (Hoyt et al., 2018). Indirect transmission can also occur during the overwintering months because transmission of *Pd* spores between solitary bats is still possible through “cryptic connections” (i.e., exposure through roosting substrate, or

infrequent bat-to-bat contact during arousals, Hoyt et al., 2018). Therefore, the potential for bats to transmit or contract *Pd* spores is high during the both the autumn and winter months.

Pd is a psychrophilic fungus and grows at temperatures as low as 3°C and as high as approximately 20°C (Verant et al. 2012). Some WNS-susceptible bat species, such as the little brown bat, are known to hibernate in temperature conditions that are optimal for the growth of *Pd*. Mycelia growth of *Pd* is inhibited at a T_a of 13°C and relative humidity (RH) below 70% (Marroquin et al. 2017). However, a RH lower than 70% does not restrict production of conidia (i.e. spores) which serve in transmission of the fungus. Thus, while a lower RH could lower disease severity, it would not confer any benefit in disease transmission. As such, bats that roost in dryer environments could potentially benefit from the decrease in the growth of *Pd* (Haase et al., 2019a).

A multi-stage disease progression model of WNS has been proposed in hibernating bats (Verant et al. 2014). In the early stages of the disease, as fungal colonization of the wing membrane occurs, there is an increase in carbon dioxide (CO_2) levels in the blood, resulting in acidemia (low blood pH) and hyperkalemia (elevated levels of potassium in blood). Once blood CO_2 levels increase beyond a threshold, bats induce hyperventilation leading to an increased frequency of arousals to remove excess CO_2 and return blood pH back to normal levels. This increased frequency of arousals (and thus energy expenditure) then leads to depletion of fat reserves (Warnecke et al., 2012; Warnecke et al., 2013). Additionally, increased respiration rate and increased difference in water vapour pressure between the animal and the air, due to the increased T_b during arousals, further contribute to an increased rate of total EWL and dehydration (for review see Verant et al. 2014). Overall, this depletion of energy reserves

before the end of hibernation causes mortality associated with emaciation (Blehert et al., 2009; Cryan et al., 2010). While the exact cause of increased arousals is unknown, the "dehydration hypothesis" (Cryan et al., 2010; Willis et al., 2011) posits that fungal lesions across the skin increase cutaneous EWL, ultimately leading to an increase in the frequency of arousals and energy expenditure. Recent studies have shown that WNS increases MR and EWL during torpor in little brown bats and that low RH exacerbates these effects (McGuire et al. 2017). Overall, this suggests a role for water balance in WNS pathophysiology.

Study Species

Big brown bats are an insectivorous species found throughout most of North America and in northern South America (Kurta and Baker, 1990). In the northern range, they hibernate during winter (Kurta and Baker, 1990) and use shorter-term torpor during summer (Lausen and Barclay 2003). Adult big brown bats show high site fidelity roosting grounds and individuals are thought to move, on average, 11 kilometers between summer and winter roosts (Beer, 1955; Mills et al., 1975). They also use a variety of roosts that can vary between seasons. In the summer, big brown bats roost in tree hollows (e.g., *Populus tremuloides*; Kalcounis and Brigham 1998), rock crevices (Lausen and Barclay, 2002), and buildings (Barbour and Davis 1969), while in winter, they may roost in rock crevices (Lausen and Barclay, 2006a), buildings (Halsall et al., 2012; Whitaker Jr. and Gummer, 1992), and caves or abandoned mines (Mills et al., 1975; Reimer et al., 2014). One possible explanation for the wide variety of roosting habitats used by this species during hibernation is their apparent tolerance for a much lower RH compared to other hibernating bats (Klüg-Baerwald and Brigham, 2017; Kurta and Baker, 1990). Big brown

bats are an ideal species to study water balance mechanisms during hibernation. They adjust well to captivity, and thus, physiological and behavioural mechanisms can be studied while replicating natural conditions in which bats hibernate in (i.e., controlling T_a and withholding food).

WNS is known to affect 12 hibernating bat species in North America, however, some appear to have suffered little to no impact. Big brown bats appear to be resistant to the disease, although mechanisms underlying their reduced susceptibility are still not fully understood. Estimates suggest their populations have declined by ~35% (credible interval 13 – 54%) across 32% of their geographic range (Cheng et al., 2021). However, declines in populations of big brown bats are variable and overall were lower, compared to WNS-susceptible species (Cheng et al., 2021). Previous research on a population of big brown bats from a WNS-positive hibernaculum indicated that bats showed no visible signs of the disease (i.e., no fungal growth on face on wings) and histological analyses indicated no apparent cutaneous infection (Frank et al., 2014). Additionally, torpor bout duration of big brown bats from the hibernaculum did not differ from the normal range of 7-25 days reported by Brack and Twente (1985). The mean body fat content of the hibernating big brown bats in a WNS-positive hibernaculum was also significantly above the summer minimum of 5.5% body fat reported by Hood et al. (2006). Moreover, captive big brown bats exposed to *Pd* exhibited longer torpor bouts than control bats suggesting that big brown bats may adjust their thermoregulatory response when inoculated with *Pd* (Moore et al. 2018). While the exact resistance mechanism in big brown bats is unknown, their potential to acclimatize to dry environments during hibernation could provide additional protection from *Pd* via mechanisms that reduce water loss

and by allowing them to select relatively dry habitat unfavourable for *Pd* growth (Klüg-Baerwald and Brigham, 2017; Langwig et al., 2012). Big brown bats are also ubiquitous across North America and roost in environments of varying RH and temperatures, and thus they provide an ideal model to explore resistance mechanisms in WNS.

Objectives and Hypotheses

The broad objective of my thesis was to understand the influence of huddling and humidity on the behavioural and physiological responses of hibernating big brown bats. I used a captive colony of hibernating big brown bats (n = 20) to test my hypotheses for both data chapters. For my first data chapter (Chapter 2), I tested the hypothesis that big brown bats adjust huddling and drinking behaviour depending on humidity, to maintain a consistent pattern of periodic arousals, and therefore energy balance, during hibernation. I predicted that bats hibernating in a dry environment would drink more often during arousals, and form more dense, compact huddles during torpor show no difference in the frequency or duration of torpor bouts or arousals and, thus no difference in loss of mass, compared to bats in a humid environment. To test my hypothesis, I housed bats in two temperature-controlled incubators set at 8°C, with one set to ~50% RH and the second at 98% RH for 110-days. I then quantified arousal and torpor frequency/duration, body mass loss, huddle density, and drinking behaviour for bats in both experimental treatments.

For my second data chapter (Chapter 3), my objective was to understand the potential for phenotypic flexibility in total EWL and torpid MR. I first tested the hypothesis that phenotypic flexibility in total EWL helps explain the tolerance of hibernating big brown bats for

a wide range of humidity. I predicted that total EWL would be lower for solitary bats acclimated to dry conditions compared to bats acclimated to humid conditions. I also tested the second hypothesis that the primary benefit of huddling for big brown bats is to mitigate the hygric challenge of dry conditions rather than providing a direct energetic benefit. I predicted that huddling bats would exhibit lower total EWL but no difference in torpid MR compared to solitary individuals, regardless of whether they had been bats acclimated to humid or dry conditions. To test both hypotheses, I used open-flow respirometry to measure rates of torpid MR and total EWL in solitary individuals and groups of five huddling bats from the same experimental treatments incubators (i.e., high and low RH) used for Chapter 2.

CHAPTER 2: BEHAVIOURAL RESPONSES OF HIBERNATING BIG BROWN BATS (*EPTESICUS FUSCUS*) TO VARIABLE HUMIDITY

Introduction

During winter, or other seasonal periods of low ambient temperature (T_a) and food availability, many mammals employ hibernation which is characterized by long multi-day bouts of torpor, a state of reduced body temperature (T_b) and metabolic rate (MR) (Geiser, 2004). The duration of torpor bouts during hibernation can vary widely between species and between individuals of the same species. For example, maximum torpor bout duration across mammalian hibernators ranges from 39 hours (Eastern rock elephant shrew, *Elephantus myurus*; McKechnie and Mzilikazi, 2011) up to a maximum of over 60 days (little brown bats, *Myotis lucifugus*; Czenze et al., 2017) and some evidence suggests bouts could exceed 80 days (Menaker, 1964; for review see Ruf and Geiser, 2015). Throughout torpor, mammals have a controlled-reduction of the T_b set-point, often to within 1–2°C of the surrounding T_a (Geiser, 2004). This decrease in T_b coupled with a dramatic reduction in MR can reduce energy expenditure by up to 99% relative to remaining at normal T_b (Lyman et al. 1982, Geiser 2004).

Torpor results in enormous energy savings but mammals cannot stay torpid through the entire hibernation period and will periodically arouse, restoring MR and T_b to normothermic levels. Periodic arousals are energetically expensive and can account for over 90% of a hibernator's total winter energy budget despite representing only ~1% of a hibernator's time (Thomas et al., 1990a). Previous studies have hypothesized that arousals may help to restore immune function (Prendergast et al., 2002), repay a sleep deficit (Daan et al., 1991), provide opportunities to forage (Hope and Jones, 2012), restore balance of metabolites such as ketone bodies or carbohydrates (Baumber et al., 1971; Galster and Morrison, 1970), provide an

opportunity to urinate and excrete metabolic wastes (Németh et al., 2010), or drink to replenish water lost during torpor (Ben-Hamo et al., 2013; Fisher and Manery, 1967; Thomas and Geiser, 1997).

Hibernators can lose water to the environment either through the respiratory tract (respiratory or pulmonary evaporative water loss (EWL)) or across the skin (cutaneous EWL), with the sum of these losses equal to total EWL. During prolonged torpor, respiratory rate is dramatically reduced and can include long periods of apnea (e.g. 13–128 minutes; Thomas et al., 1990b) and thus rates of respiratory EWL may be relatively low. For example, in hibernating little brown bats, due to low ventilation rates and lung volumes, respiratory EWL accounted for only 0.3% of total EWL (Thomas and Cloutier, 1992) while the highly vascularized wings with large surface area appear to result in relatively high rates of cutaneous EWL (Hosken and Withers 1997, 1999).

To test the hypothesis that dehydration induces arousals, Ben-Hamo et al. (2013) measured torpor bout duration and total EWL of singly hibernating Kuhl's pipistrelle (*Pipistrellus kuhlii*). Bats were tested at the same T_a, but under varying humidity (6% and 65% relative humidity (RH)). Bats with increased total EWL had shorter torpor bouts and torpor bout duration did not differ for hibernating bats in the two environments. These results suggest that torpor bout duration (and thus the frequency of arousals from torpor) is more dependent on rates of EWL for individual bats rather than the ambient humidity in the local environment.

Some hibernators use social thermoregulation, or huddling, to regulate T_b, EWL, and energy conservation during periods of low T_a. Bats often huddle or cluster in large groups and this behaviour may also help regulate EWL by reducing exposed surface area (for review see

Gilbert et al., 2010). Boratyński et al. (2015) measured torpid MR and total EWL in solitary individuals and groups of four or five hibernating Natterer's bats (*Myotis nattereri*) and found that huddling reduced total EWL by almost 30% with no significant change in torpid MR. The reduction in total EWL was attributed to the decrease in exposed skin for each huddling bat, which provided evidence that the main function of huddling during hibernation was to reduce total EWL (Boratyński et al. 2015). However, a reduction in total EWL could also provide an indirect energetic benefit, and reduce the loss of body fat during hibernation, by reducing the frequency of periodic arousals (Boratyński et al., 2015). Although there was no effect of huddling on energy expenditure for torpid bats, a reduction in exposed surface area from huddling could still reduce an individual's cost of thermoregulation during rewarming (Gilbert et al., 2010). For example, huddling and hibernating Indiana bats (*Myotis sodalis*) reduce energy expenditure by minimizing heat loss through a reduction in exposed surface area when individuals rewarmed to normothermic T_b (Boyles et al. 2008).

Understanding the relationship between behaviour, total EWL, and energy expenditure for hibernating bats has become an increasing concern because of white-nose syndrome (WNS), a fungal disease caused by the pathogen *Pseudogymnoascus destructans* (*Pd*). Currently, WNS is causing mass mortality in North American hibernating bats (Frick et al., 2015). WNS is known to affect 12 hibernating North American bat species but population impacts vary widely among species with some showing rapid and severe declines and others virtually unaffected (Langwig et al., 2016). Previous studies have shown that the disease increases arousal frequency, and thus energy expenditure during hibernation (Reeder et al., 2012a; Warnecke et al., 2012; Warnecke et al., 2013). The exact cause for increased arousals is still not

fully understood but the “dehydration hypothesis” (Cryan et al., 2010; Willis et al., 2011) posits that damage to the skin of flight membranes by fungal lesions increases EWL which, in turn, causes bats to increase arousal frequency and energy expenditure. Consistent with the dehydration hypothesis, little brown bats inoculated with *Pd* showed higher levels of EWL, along with higher rates of torpid MR, both of which could lead to increased arousal frequency and greater overwinter energy expenditure (McGuire et al. 2017).

The impact of WNS on big brown bat (*Eptesicus fuscus*) populations has been considered moderate to severe and this species appears to be resistant to WNS (Cheng et al., 2021). However, the exact mechanism underlying resistance is not fully understood (Frank et al., 2014). Captive big brown bats inoculated with *Pd*, responded by expressing longer torpor bouts compared to sham-inoculated controls, which could translate to greater energy savings (Moore et al. 2018). Big brown bats infected with *Pd* thus will adjust their thermoregulatory behaviour during hibernation in response to WNS (Moore et al. 2018). However, behaviour of big brown bats during hibernation could also explain their apparent resistance to *Pd* infection or an ability to avoid becoming infected with *Pd*. Big brown bats roost primarily alone during hibernation and are not as gregarious as some WNS-affected species, although they have been observed huddling in groups of up to ~20 individuals (Brack Jr. and Twente, 1985; Moosman et al., 2017; Phillips, 2014). Additionally, big brown bats roost across a wider range of humidity as compared to species heavily impacted by WNS, and appear capable of acclimatizing to dry environments during hibernation which could play a role in their resistance to WNS (Klüg-Baerwald and Brigham, 2017).

My objective was to understand the ability of big brown bats to tolerate variation in environmental humidity and to understand the influence of humidity on hibernation behaviour in this WNS-resistant species. I tested the hypothesis that big brown bats adjust huddling and drinking behaviour depending on humidity, to maintain a consistent pattern of periodic arousals, and therefore energy balance, during hibernation. I predicted that bats hibernating in a dry environment (at the low end of the humidity range experienced by this species in nature) would drink more often during arousals, and form more dense, compact huddles during torpor, but show no difference in the frequency or duration of torpor bouts or arousals and, thus no difference in loss of body mass, compared to bats in a humid environment (near the upper limit of humidity for hibernation in this species).

Methods

Adjustment Period of Study Animals

All animal handling procedures were approved by the University of Winnipeg Animal Care Committee (protocol AE12193). I used a captive colony of 20 non-reproductive adult, female, big brown bats housed at the University of Winnipeg (Winnipeg, Canada). The bats were originally caught in June 2017 from two netting sites 328 km apart: Bismarck, North Dakota (46.76°N, 100.76°W), and Ada, Minnesota (47.30°N, 96.51°W), and were housed together at the University of North Dakota for 28 months prior to my study. In summer, bats were housed in 2.5 x 2.5 x 2.5 m outdoor flight cages (as described by Boyer et al., 2020) and in winter, the bats were moved to modified incubators for hibernation (Erin Gillam, University of North Dakota, personal communication). At capture, each bat was outfitted with up to two coloured, plastic forearm bands on either the right or left forearm (Boyer et al., 2020) and I used colour combinations, numbers of bands, and positions of bands (i.e., right, left or both forearms) to identify individuals throughout my study.

The colony was transported by car, to the University of Winnipeg on 19 October 2018 and then divided randomly in two groups of 10 and housed in temperature-controlled incubators set at 8°C (see **Figure S.1** for timeline). The incubators were humidified by saturated sponges, which were rewet during periodic health checks (see below). The bats were adjusted to the facilities from 19 October 2018 to 18 December 2018 to ensure they were in good body condition (≥ 18 g) and had recovered from transport before hibernation. Both incubators were equipped with infrared (IR) cameras (Hawk Eye Nature Camera, Songbird Garden, Cape Fair, Missouri, U.S.A.) attached to the ceiling to give an overhead view of the mesh cage. The IR

cameras were connected to a video monitoring system (VMAX480 DW-VMAX-16, Digital Watchdog, Cerritos, CA, U.S.A.) that continuously recorded activity and allowed me to monitor the bats without disturbing them. Inside each incubator, bats were housed in custom-built nylon mesh cages (modified from Exo-terra Flexarium/Flextray© PT2556, Hagen Inc., Montreal, QC, Canada; 49.5 x 20.3 x 38.8 cm and 43.2 x 26.7 x 57.2 cm). The mesh was removed from the top of each cage, and a layer of plastic sheeting was connected from the top of the cage frame to the ceiling of the incubator to ensure that bats could not crawl higher than the field of view of the IR cameras.

To record skin temperatures (T_{sk}) of individual bats, I used temperature sensitive dataloggers (DS2422 iButton; Maxim Integrated products, Sunnyvale CA USA, modified as per Reeder et al., 2012a). For small bats, T_{sk} gives a good approximation of T_b during torpor at stable T_a above freezing (Willis and Brigham, 2003) and has been used in multiple studies of captive hibernating bats (Mayberry et al., 2018; Turner et al., 2014; Warnecke et al., 2012). Dataloggers were coated in a layer of black synthetic rubber (Plasti Dip®, Plastic Dip International, Blaine, Minnesota) to protect the circuit board and battery from humidity (Reeder et al., 2012b) and attenuate ultrasound which can be emitted by iButtons and potentially disturb bats (Willis et al., 2009). I calibrated each datalogger after the experiment in a water/ethylene glycol mixture inside a temperature-controlled cabinet. Temperature of the water/ethylene glycol mixture was measured with a thermocouple thermometer (Model TC-2000, Sable Systems, Las Vegas, USA) calibrated to a NIST-traceable mercury thermometer. I set iButtons to record temperature once per minute and recorded 20 readings per temperature at four temperatures between 0°C to 40°C. I then fit a linear calibration curve for each iButton and

used the equations for these curves to correct data recorded for each bat during hibernation. I painted each individual datalogger with a unique symbol to provide an additional means of identifying individual bats in IR videos. On 20 October 2018 I attached the iButtons to each bat directly on the skin, after trimming the fur between the scapula with surgical adhesive (Osto-Bon, Montreal Ostomy, Vaudreuil, QC, Canada).

From 19 October to 18 December 2018, I performed health checks every second day for the bats' first week at the University of Winnipeg, once every 3–5 days for the next two weeks, and once every 7–11 days for the final six weeks of adjustment. During each health check, I measured body mass (± 0.1 g) with an electronic balance (Ohaus Corporation, CS200, Pine Brook, New Jersey, U.S.A.) and hand fed each bat up to 40 mealworms (larval *Tenebrio molitor*) supplemented with vitamin/mineral supplement (following Barnard et al. 2013). On the same day as health checks, an additional 400–450 mealworms and water were provided *ad libitum* to each cage.

Hibernation - Experimental Treatments

On 27 November 2018, for hibernation, bats were moved into a second set of larger temperature and humidity-controlled incubators (Environmental Chamber, Model 6041, Caron, Marietta, OH, U.S.A.) set at 8°C and 98% RH. The incubators were humidified via a single condensate recirculating system (Condensate Recirculating System CRSY102, Caron, Marietta, OH, U.S.A). I housed bats in the same groups of 10 as during the adjustment period to minimize stress from disruption of social dynamics. Bats were housed in a second set of custom-built nylon mesh cages (Exo-terra Flexarium/Flextray© PT2556, Hagen Inc., Montreal, QC, Canada;

91.4 x 43.2 x 43.2 cm). These mesh cages were identical to the cages during the adjustment period, except larger in size (See **Figure S.2**). The mesh cages were situated on a shelf located in the middle of each incubator. There was no mesh on the top of the cage, and, again, a layer of plastic was attached from the top of each cage to the ceiling of the incubator to ensure bats could not crawl out of view of IR cameras.

Two temperature and RH sensors were placed inside each incubator (HOBO Onset, Model S-THB-M008), one at the top and one halfway down to record data from areas in the cages where bats roosted most often. In each incubator, IR cameras (Intense IR Dome Camera, HD5941T, Speco Technologies, Amityville, NY, U.S.A) were installed to continuously monitor the bats. One camera was situated on the ceiling of each cage, 70.5 cm above the edge of the mesh, to monitor arousals and huddling. The second was mounted flush with the cage mesh at the bottom to provide a view of bats visiting the water dish on the cage floor. Water was provided *ad libitum* via tubing that passed into the chamber, allowing me to refill water dishes without disrupting hibernating bats. Aquarium rocks were placed inside the water dish to allow bats to climb out if they fell in. On 7 December 2018, I removed iButtons using a medical grade adhesive remover (Uni-solve, Smith & Nephew Inc, Mississauga, ON, Canada) and recorded body mass. I confirmed that bats did not differ in body mass between the two experimental treatment (Welches t-test; $t = -0.15$, $df = 11.22$, $p = 0.88$). I re-attached iButtons after the body mass measurements as described above. iButtons were set to record once every 15 min (± 0.5°C) beginning on 7 December 2018.

On 14 December 2018, I weighed and hand fed all bats then decreased RH in one incubator to 50% (i.e., the *dry treatment*, water vapour pressure = 0.57 ± 0.08 kPa at 8.37 ±

0.14°C). The other incubator remained at 98% RH and 8°C (i.e., the *humid treatment*, water vapour pressure = 1.03 ± 0.08 kPa at 8.57 ± 0.12°C). Due to animal and equipment limitations, I was not able to replicate each of the two experimental treatments. On 18 December 2018, I weighed and handfed the bats, then returned them to their respective incubator for undisturbed hibernation. Food was withheld throughout the experiment to match natural conditions in the wild and encourage normal hibernation. Hibernation proceeded normally for 73 days but on 1 March 2019, bats in the *dry treatment* chewed a small hole through the mesh of their cage. On two occasions, two individuals escaped through the hole during arousals, but they returned to the huddle of bats inside the cage and did not need to be re-captured. On 21 March 2019 (study day 93) at 14:43, I opened the incubator in short intervals to repair the mesh taking care to minimize disturbance to the bats. The repair took less than five minutes, and I replicated this disturbance in the *humid treatment* incubator.

On 08 April 2019 starting at 07:30, I removed all bats from hibernation, removed the iButtons, and measured body mass. Overall, six bats had shed their iButtons during hibernation, two iButtons malfunctioned (**ID15** and **ID20** from the *dry treatment*) and did not record, and, due to a programming malfunction, all remaining iButtons stopped recording on 19 January 2019 (see **Table S.1** for details). This reduced T_{sk} recording to 31 days from 10 bats in the *dry treatment* and 31 days from 8 bats in the *humid treatment*. However, I was able to use these T_{sk} data to confirm that the continuously recorded video provided a good approximation of torpor and arousal (see below). Upon removal of the bats on 8 April 2019, it was discovered that one bat in the *dry treatment* (**ID13**) had gotten caught in the tape lining the mesh cage while obscured from view by the huddle of bats. Based on video observations of its arousals this

individual died sometime after 11 February 2019 and I removed hibernation data from this individual from my analysis.

Arousal Definition

Most studies that depend on measurements of T_{sk} to quantify torpor bouts during hibernation rely on an arbitrary T_{sk} threshold to define the start and end of torpor and arousal bouts (e.g., when T_{sk} was 10°C or more below the highest measured T_{sk}, Reeder et al., 2012b, or when T_b varied passively with T_a, Audet and Fenton, 1988). I attempted to refine this approach, using an iterative, systematic method to define a T_{sk} threshold. I first quantified both the number of arousals and arousal durations for each individual bat using T_{sk} thresholds ranging from 13–27°C in 1°C increments. I considered bats normothermic if T_{sk} increased above the specified T_{sk} threshold for at least two datalogger readings (i.e., 30 minutes) and torpid if T_{sk} fell below the T_{sk} threshold for at least two readings. I then used linear regression to test for an effect of T_{sk} threshold on arousal duration (square root transformed to achieve normality) (**Figure 2.1A**) and breakpoint regression to test for an effect on the number of arousals detected (**Figure 2.1B**). Not surprisingly, as the T_{sk} threshold increased, arousal duration declined and this relationship was linear (**Figure 2.1A**, $F_{15,1432} = 11.29$, $p < 0.001$, range of n = 112 for 13°C and n = 76 for 27°C). However, for the total number of arousals, there was a clear breakpoint in the effect of T_{sk} threshold at 17.4°C (**Figure 2.1B**). Below 17.4°C there was an obvious increase in the numbers of arousals detected as the T_{sk} threshold fell

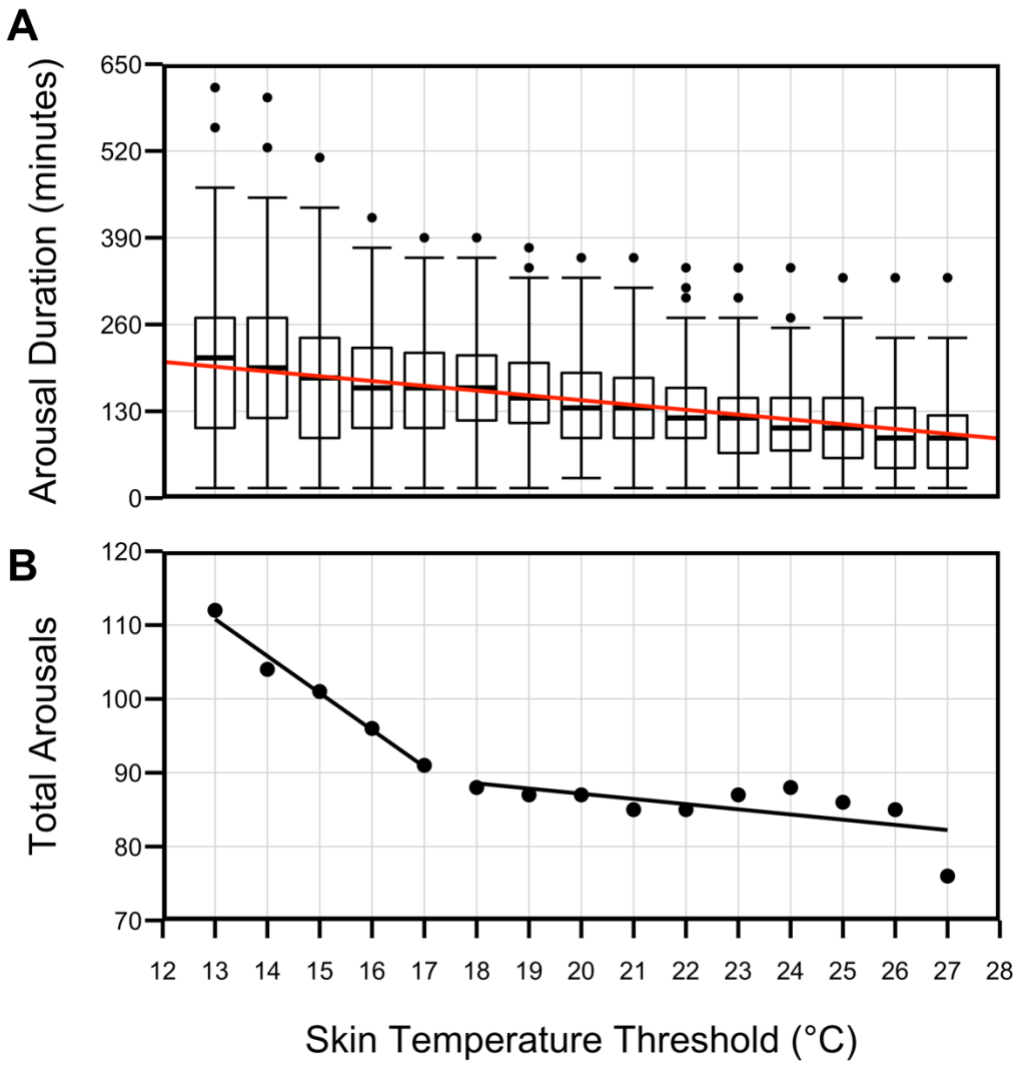

Figure 2.1: **A** Boxplots of arousal bout duration (minute) for each skin temperature threshold (°C) I examined from 13°C to 27°C from hibernating big brown bats (*Eptesicus fuscus*, n = 18). The median is represented by the solid horizontal middle line, and the top and bottom of each box represent the 25th and 75th quartiles, respectively. Whiskers represent maximum and minimum values and outliers are indicated by solid black dots. The red line represents a negative relationship between arousal bout duration and temperature threshold. **B** Breakpoint regression showing relationship between T_{sk} threshold and total number of arousals with a break in the slope occurring at a threshold of 17.4°C.

(b = -5.00, t = -6.08, $p < 0.001$), but above 17.4°C there was no effect of T_{sk} threshold (b = -0.70, t = -2.46, p = 0.06). Thus, a T_{sk} threshold from 18°C –27°C provides a reasonable approximation of torpor expression without a significant effect of the threshold itself on the results. I then used a repeated measures analysis of variance (with Bat ID included as a random effect) to test for an effect of T_{sk} threshold and behaviour observed in the IR video (see below) on arousal duration (square-root transformed to achieve normality). I used Levene's test for equality of variances among groups. The overall model was significant ($F_{1,1429} = 37.45$, $p < 0.001$) so I followed with a Dunnett's post hoc test to compare arousal duration based on behaviour against arousal duration based on T_{sk} at each T_{sk} threshold. There was a significant difference in arousal duration based on behaviour versus T_{sk} for thresholds from 13–19°C (all $p < 0.05$) but there was no difference for T_{sk} thresholds from 20–27 °C (all $p > 0.05$). Therefore, since a T_{sk} threshold of 18–27 °C reliably predicted arousal frequency, and a T_{sk} threshold 20-27°C reliably predicted arousal duration, I chose a T_{sk} threshold of 20°C for subsequent analysis.

The approach above allowed me to quantify arousal frequency and duration but, because iButtons failed to record throughout the entire hibernation period, my T_{sk} dataset was limited. I therefore relied on behaviour recorded by the IR camera system throughout the 110-day hibernation period to quantify arousal frequency and duration after using T_{sk} data from the start of the experiment to confirm that video reliably recorded the start and end of arousals. I defined an arousal for a given bat based on the start and end of activity recorded in the video. Defining the beginning of arousals was straightforward because virtually every time a bat was observed being active, its T_{sk} rose above my T_{sk} threshold of 20°C for at least two consecutive readings of the T_{sk} datalogger. On five occasions (out of 87 total arousals), T_{sk} increased above

the 20°C threshold for more than two readings of the iButton with no movement by these bats. In all of these five instances, almost all other bats in the huddle of 10 were active so the increase in the bats' T_{sk} was likely an artifact of heat produced by surrounding normothermic bats. On another five occasions, bats showed activity without an increase of T_{sk} above the 20°C threshold for two or more readings. There was still an increase in T_{sk} (e.g., increase to 18.6°C for one reading of the iButton). However, for a total of 85 arousals by 18 bats, activity reliably predicted an increase in T_{sk} above 20°C for at least two readings confirming the reliability of video observations as a way to identify arousal onset.

Defining the end of arousals was more difficult because sometimes bats in the midst of arousals were inactive in the video before re-entering torpor despite remaining at a high T_{sk}. To systematically define an activity threshold marking the end of arousals, I visually compared T_{sk} data to the behavioural observations. If bats remained inactive for more than 1.5 hours, their T_{sk} always fell by at least 7°C from the maximum arousal T_{sk} and, on all but one occasion, fell by more than 10°C (indicating that bats were re-entering torpor). If bats remained inactive for less than 1.5 hours after activity was first detected, their T_{sk} always remained higher than my T_{sk} threshold of 20°C. On two occasions, there was <1.5 hours between bouts of activity by bats but T_{sk} was continually declining during these events as the bats were returning to torpor so I considered this as one arousal. Based on these observations, I defined the end of an arousal as the time at which a previously active bat had not been moving for >1.5 h. If a bat exhibited activity after this 1.5 h period, I counted that bout of activity as a new arousal.

Overall, behavioural analysis of bats reliably predicted the frequency and duration of arousals that were also identified based on a T_{sk} threshold of 20°C. Mean duration of arousals

based on behavioural thresholds (120.6 ± 76.8 min) was approximately 25 min shorter than arousal duration based on a T_{sk} threshold of 20°C (145.2 ± 73.8 min) but this difference was not statistically significant ($p = 0.17$) and the total number of arousals based on behaviour ($n = 90$) was almost identical to that based on my T_{sk} threshold ($n = 87$).

Statistical Analyses

I used two-sample t-tests to compare the total number of arousals per bat, and the proportions of arousals per bat that included at least one bout of drinking, between experimental treatments. I recorded the total drinking frequency per bat as the number of visits to the water dish during which I could observe drinking and then used Welch's two-sample t-test (because of unequal variances between treatments) to test for a difference in drinking frequency between experimental treatments. I tested for an effect of humidity treatment using a linear mixed model (R package 'nlme', Pinheiro et al. 2021) with arousal bout duration (square-root transformed to achieve normality) as the response variable, experimental treatment and initial body mass as predictor variables and Bat ID included as a random effect. I used a second linear mixed model with torpor bout duration (square-root transformed to achieve normality) as the response variable, and similarly, experimental treatment and initial body mass as predictors. Bat ID was included as a random effect.

Bats in both treatments huddled in one large cluster per cage throughout hibernation, and all bats aroused either singly or simultaneously in groups (i.e., they never staggered arousals at different points in a day). Therefore, I was able quantify the density of huddles of bats between each treatment using screenshots from the IR video recordings in each cage. Both

cages were identical in size with overhead cameras positioned the same distance from the top of mesh cages where bats roosted allowing a direct comparison between experimental treatments. For each image I ensured that all bats had been inactive for at least one hour (for example, see **Figure 2.2**) to ensure that huddle size reflected groups of bats that were torpid or returning to steady-state torpor. All images were the same resolution of 352 x 238 pixels.

I quantified huddle size using ImageJ (version 1.51, National Institute of Health, Bethesda, MD, U.S.A.). Huddle photos were randomized, and files were renamed to ensure I was blind to both study date and experimental treatment when analysing huddle size. I outlined huddles in each photo using ImageJ and then calculated a huddle size index (HSI) as the percentage of total image area in pixels, occupied by the huddle of bats. To confirm that assessment of HSI was repeatable, a second observer, who was also blind to both study date and experimental treatment, repeated measurements of the same images and there was no difference in our assessments (Welch's two-sample t-test, $t = -0.38$, $df = 91.3$, $p = 0.71$). I then used my measurements to compare the HSI between experimental treatments with an analysis of covariance. I included date and the two way interaction between date and experimental treatment as covariates. I fit the most complicated model first, then removed non-significate covariates, until only significant effects remained.

I calculated effect size as Cohen's d using the R package "effsize" (Torchiano, 2020) to compare the magnitude of treatment effects for total arousals per bat, drinking frequency per bat, proportion of arousals per bat that contained a drinking event, and HSI between the two experimental treatments.

Figure 2.2: View from the top of the cage inside an incubator during hibernation. **A** indicates the huddle of big brown bats (*Eptesicus fuscus*; n = 10), **B** indicates the water dish (with rocks) and **C** indicates tubing that exits the mesh enclosure to the outside of the incubator for refilling of the water dish. All bats roosted in one huddle throughout hibernation.

I calculated the change in body mass for individual bats from the start to the end of the experiment and used a generalized linear model (GLM) in the 'MASS' package (Venables and Ripley, 2002) in R to test for an effect of experimental treatment on loss of body mass. These data were right skewed, so I used a gamma error distribution with initial body mass, experimental treatment, and total number of arousals as predictor variables and two-way interactions between all predictors. I fit the most complicated model first, then used model reduction to remove predictors with the highest p-values until only significant predictor remained.

All statistical analyses were conducted in R Version 4.0.4 (R Core Team, 2021) using RStudio (Version 1.4.1106) with graphs produced using 'ggplot2' (Wickham, 2016) in RStudio. For all statistical tests, significance was assessed at $p < 0.05$, and values are reported as means ± SD and samples as n = number of measurements.

Results

I recorded 129 arousals and 119 torpor bouts from 10 big brown bats in the *humid treatment* and 124 arousals and 115 torpor bouts from nine bats in the *dry treatment* (**Table 2.1**). There was no effect of experimental treatment on the number of arousals per bat (**Figure 2.3;** t = -0.84, df = 17, p = 0.41, Cohen's d = 0.39) but bats in the *dry treatment* had shorter arousal durations (duration = 106 ± 72 min; p = 0.01; **Table 2.2**) compared to bats in the *humid treatment* (duration = 134 ± 82 min). Bats in the *humid treatment* had torpor bout durations of 8.3 ± 4.1 days versus 7.9 ± 4.9 days for bats in the *dry treatment,* but there was no effect of experimental treatment or initial body mass (**Table 2.3**). The number of drinking bouts per bat was 52% higher in the *dry treatment* compared to the bats in the *humid treatment* (**Table 2.1**; **Figure 2.4;** t = -3.51, df = 10.95, p = 0.005, Cohen's d = 1.67). Additionally, bats in the *dry treatment* had a higher proportion of arousals that contained drinking events, as compared to bats in the *humid treatment* (**Figure 2.5**; t = -2.88, df = 16.95, p = 0.01, Cohens' d = 1.32). During the first 31 days when iButtons were recording T_{sk}, drinking behaviour always occurred at relatively warm T_{sk} for all bats. Bats in the *humid treatmen*t drank at T_{sk} = 29.1 ± 2.6°C and bats in the *dry treatment* drank at T_{sk} = 29.5 ± 2.0°C (see **Figure S.3** and **Figure S.4** for T_{sk} data)

The HSI was smaller (Cohen's d = 2.12) for bats in the *dry treatment* compared to bats in the *humid treatment* (**Figure 2.6**;p < 0.001) indicating a more compact huddle for bats in the *dry treatment*. Additionally, there was no effect of date (p = 0.28) or the two-way interaction between date and experimental treatment (p = 0.10) on the HSI.

There was no effect of the number of arousals, experimental treatment, or any of the two-way interactions on body mass loss throughout hibernation (all p > 0.05). There was a

significant effect of initial body mass on the loss of body mass loss (**Figure 2.7**, GLM: $p < 0.001$), with the heaviest bats losing the most body mass over the 110-day study period.

Table 2.1: Summary of initial, final and change in body mass, individual hibernation patterns and drinking behaviour for hibernating big brown bats (*Eptesicus fuscus;* n = 20) from 18 December 2018 to 08 April 2019.

Bat ID	Experimental treatment	Initial body mass (g)	Final body mass (g)	Change in body mass (g)	No. arousals	Mean arousal duration (min)	No. torpor bouts	Mean torpor bout duration (days)	No. drinking bouts
ID01	*Humid*	23.6	19.5	- 4.1	12	103.1 ± 105.1	11	8.9 ± 4.6	8
ID02	*Humid*	26.7	20.0	- 6.7	16	165.6 ± 85.2	15	6.7 ± 4.7	5
ID03	*Humid*	23.4	20.0	- 3.4	11	86.2 ± 62.3	10	9.8 ± 2.3	11
ID04	*Humid*	24.7	20.1	- 4.6	14	113.4 ± 57.0	13	7.5 ± 4.9	12
ID05	*Humid*	24.0	19.8	- 4.2	13	168.7 ± 95.1	12	8.0 ± 3.4	4
ID06	*Humid*	22.4	19.4	- 3.0	12	121.9 ± 81.1	11	9.2 ± 3.3	6
ID07	*Humid*	24.7	20.3	- 4.4	10	165.0 ± 87.6	9	10.8 ± 4.6	12
ID08	*Humid*	23.8	19.9	- 3.9	14	164.3 ± 79.2	13	7.7 ± 4.0	9
ID09	*Humid*	22.6	18.6	- 4.0	12	119.3 ± 66.8	11	8.9 ± 2.8	7
ID10	*Humid*	25.6	21.2	- 4.4	15	120.0 ± 66.7	14	7.0 ± 4.7	6
ID11	*Dry*	22.6	17.7	- 4.9	13	106.0 ± 55.6	12	8.6 ± 5.2	24
ID12	*Dry*	24.5	19.8	- 4.7	8	122.4 ± 99.7	7	14.0 ± 5.9	10
ID13	*Dry*	23.6	-	-	10	294.8 ± 405.0	9	5.5 ± 1.2	18
ID14	*Dry*	28.0	19.9	- 8.1	17	115.1 ± 63.3	16	6.4 ± 3.5	28
ID15	*Dry*	24.2	19.7	- 4.5	15	104.3 ± 105.0	14	7.2 ± 4.7	11
ID16	*Dry*	21.1	18.0	- 3.1	15	107.53 ± 80.8	14	6.9 ± 4.8	12
ID17	*Dry*	22.0	18.9	- 3.1	16	93.8 ± 85.3	15	6.6 ± 4.8	14
ID18	*Dry*	23.4	19.6	- 3.8	12	96.5 ± 67.3	11	8.9 ± 6.0	11
ID19	*Dry*	29.5	19.9	- 9.6	15	101.7 ± 63.9	14	7.3 ± 3.3	19
ID20	*Dry*	23.4	19.9	- 3.5	13	120.1 ± 67.0	12	8.4 ± 5.2	16

Table 2.2: Results of a linear mixed model testing for an effect of experimental treatment on arousal bout duration by hibernating big brown bats (*Eptesicus fuscus*; n = 19) with initial body mass (g) as a fixed factor and Bat ID as a random factor. Significant effect in bold.

Variable	Coefficient*	t-value	p-value
Experimental treatment (Humid treatment)	**39.32**	**- 2.75**	**0.01**
Initial body mass (g)	57.62	1.23	0.22

*Coefficients are based on back-transformed data and t-values and p-values are based on transformed data.

Table 2.3: Results of a linear mixed model testing for an effect of experimental treatment on torpor bout duration by hibernating big brown bats (*Eptesicus fuscus*; n = 19) with initial body mass (g) included as a fixed factor and Bat ID as a random factor.

Variable	Coefficient*	t-value	p-value
Experimental treatment (Humid treatment)	10.41	- 0.77	0.45
Initial body mass (g)	10.91	- 0.82	0.43

*Coefficients are based on back-transformed data and t-values and p-values are based on transformed data.

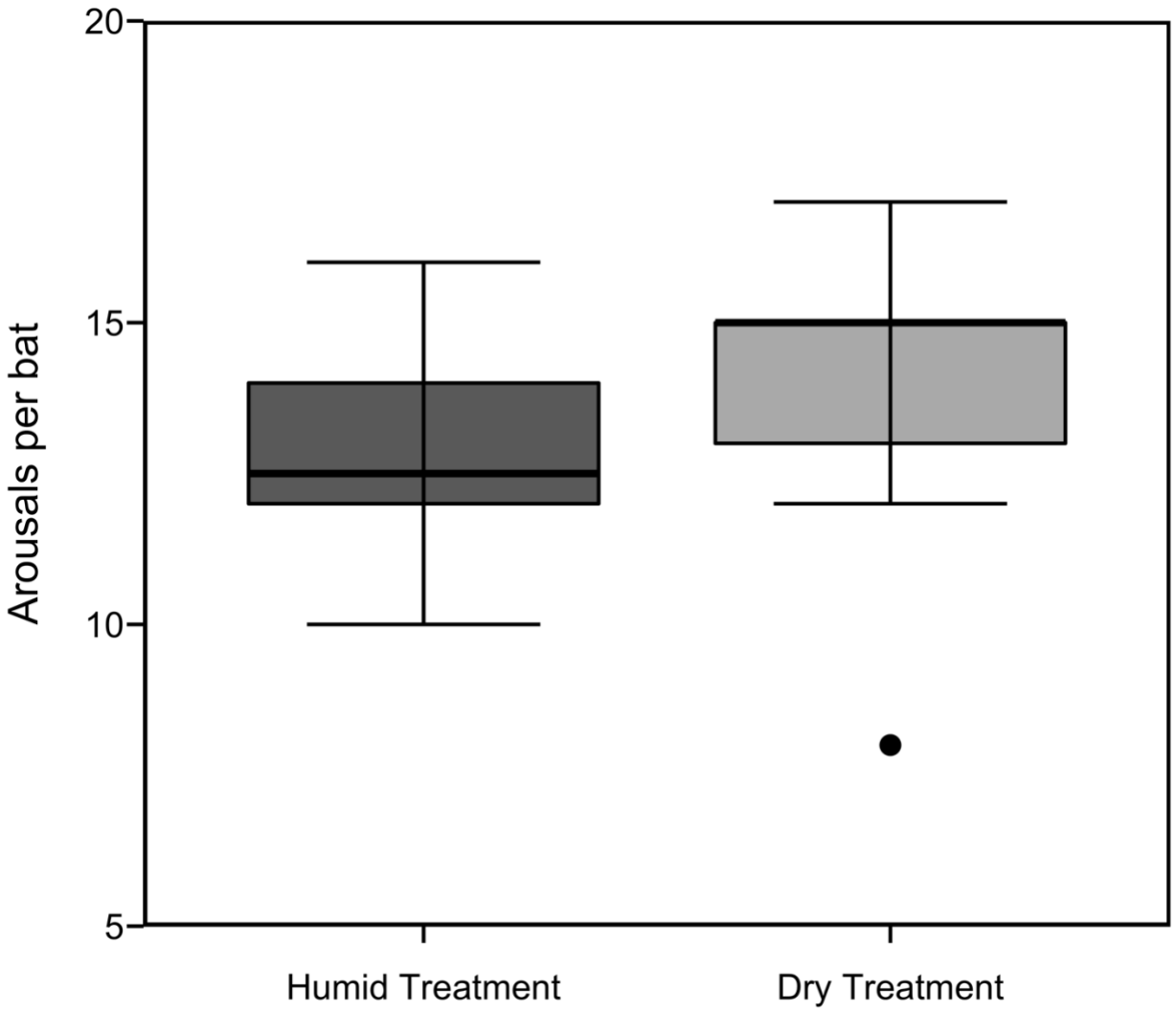

Figure 2.3: Boxplots of the total arousals per big brown bat (*Eptesicus fuscus*) for *humid treatment* (n = 10) and *dry treatment* (n = 9) bats over the 110-day study period. The median is represented by a solid horizontal line, the top and bottom of each box represents the 25th and 75th percentile, respectively. Whiskers represent maximum and minimum values. Outliers are indicated by solid black dots.

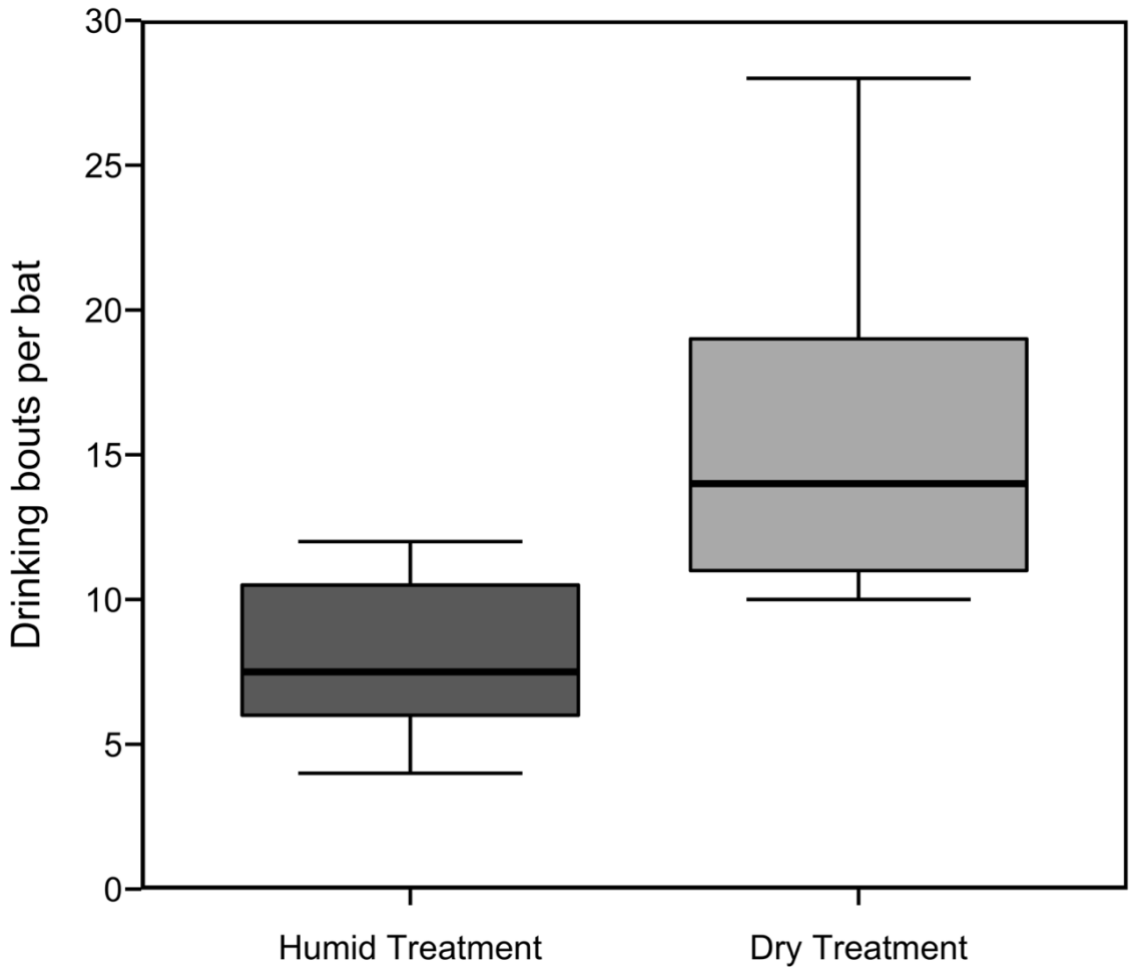

Figure 2.4: Boxplots of the total drinking bouts per big brown bat (*Eptesicus fuscus*) from the *humid treatment* (n = 10) and *dry treatment* (n = 9) bats over the 110-day study period. The median is represented by a solid horizontal line, the top and bottom of each box represents the 25th and 75th percentile, respectively. Whiskers represent maximum and minimum values.

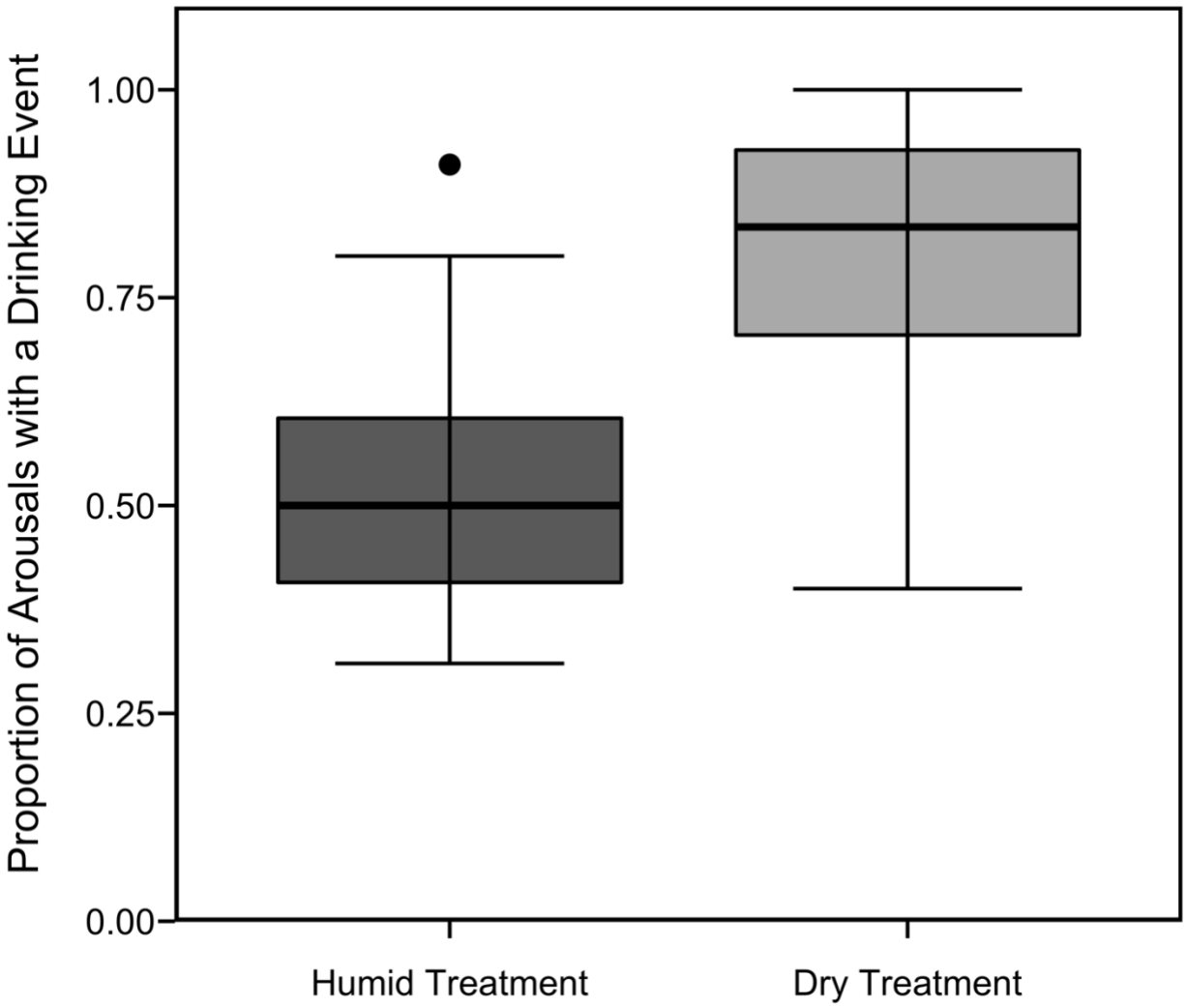

Figure 2.5: Boxplots of the proportions of arousals per bat with drinking events for big brown bats (*Eptesicus fuscus*) from the *humid treatment* (n = 10) and the *dry treatment* (n = 9) over the 110-day study. The median is represented by a solid horizontal line, the top and bottom of each box represents the 25th and 75th percentile, respectively. Whiskers represent maximum and minimum values. Outliers are indicated by solid black dots.

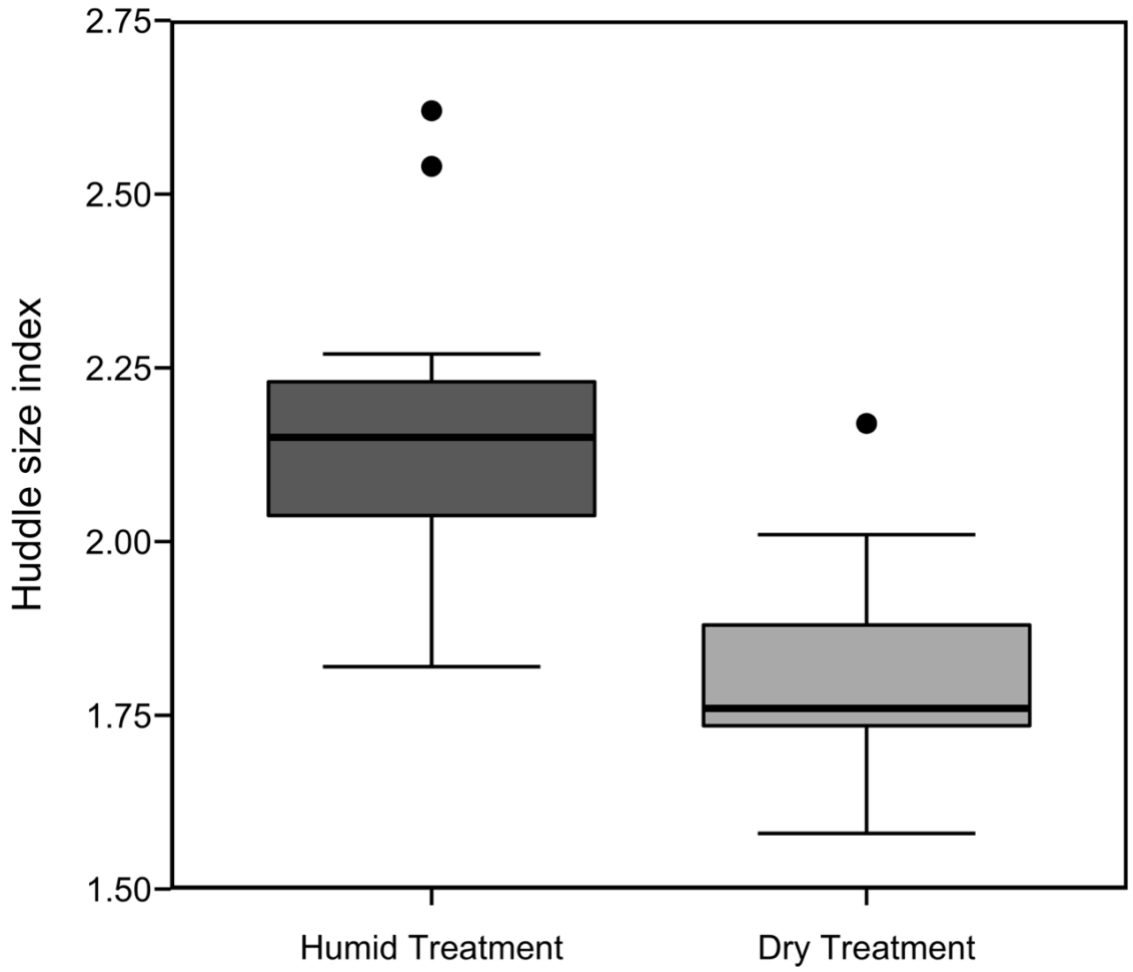

Figure 2.6: Boxplots for the Huddle Size Index (i.e., percentage of the total image area) of huddling big brown bats (*Eptesicus fuscus*) from the *humid treatment* (n = 24) and *dry treatment* (n = 27) over the 110-day study. The median is represented by a solid horizontal line, the top and bottom of each box represents the 25th and 75th percentile, respectively. Whiskers represent maximum and minimum values. Outliers are indicated by solid black dots.

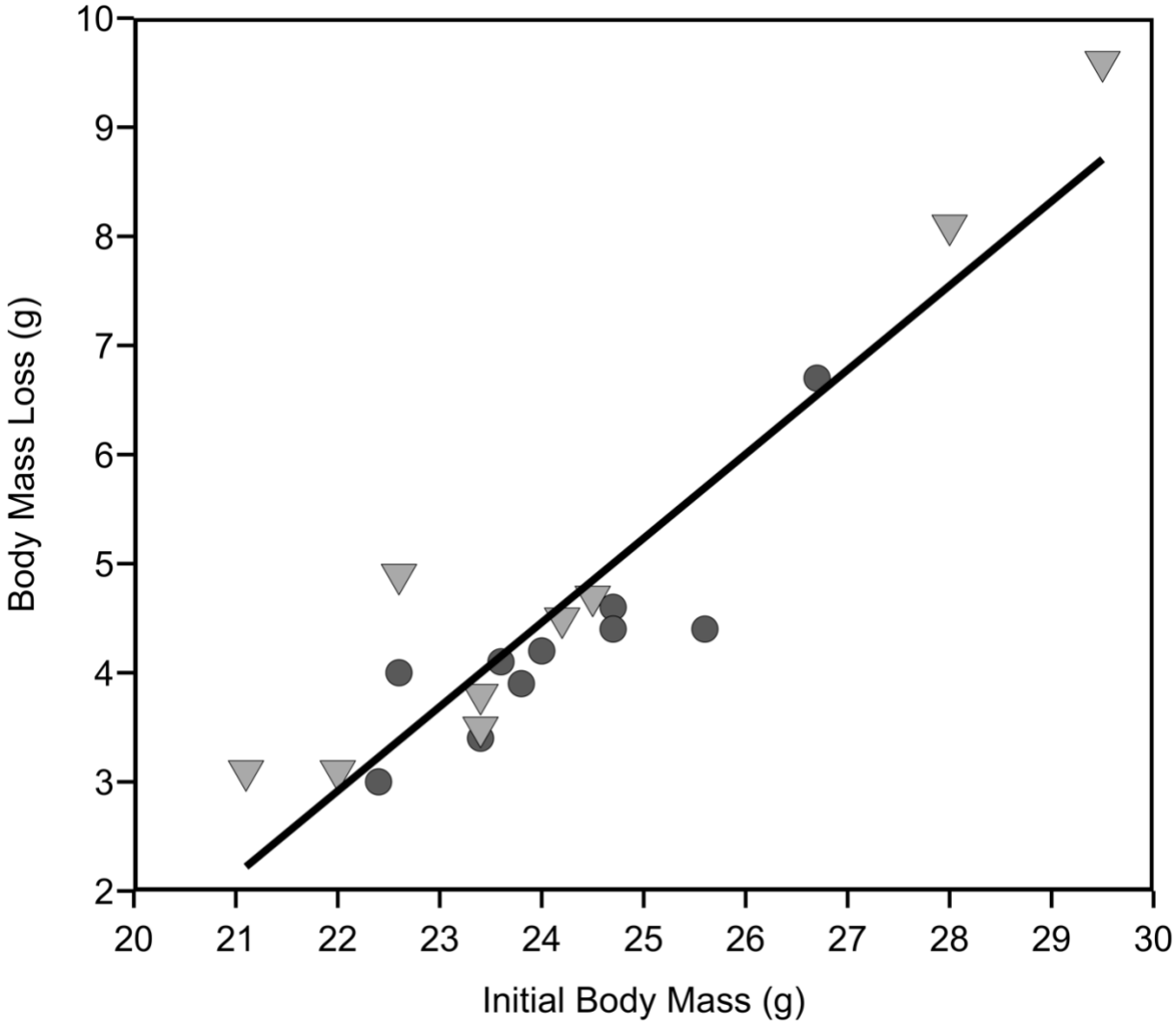

Figure 2.7: Positive relationships between the initial body mass (g) and body mass loss (g) of the big brown bats (*Eptesicus fuscus*) over the 110-day study. Dark grey circles represent bats from the *humid treatment* whereas light grey triangles represent bats from the *dry treatment*. The solid black line represents the relationship between the two variables (R^2 = 0.85; n = 19).

Discussion

My results support the hypothesis that big brown bats adjust huddling and drinking behaviour to maintain consistent patterns of arousals, and therefore energy balance in conditions of varying humidity. In my study, bats hibernated in either humid (98% RH at 8°C) or dry (50% RH at 8°C) conditions for 110 days, which represents the range of humidity that big brown bats experience in the wild (Klüg-Baerwald and Brigham, 2017). Consistent with my prediction, there was no difference in either the frequency of arousals and torpor bouts, or the duration of torpor bouts, between bats from the two treatments. Previous studies have proposed that dehydration induces arousals, and hibernators will return to a normothermic T_b to drink and restore normal water balance (Ben-Hamo et al., 2013; Fisher and Manery, 1967; Thomas and Geiser, 1997). Arousals, however, are energetically expensive, and bats need to budget the high energetic costs of returning to a normothermic T_b against physiological costs of remaining torpid (e.g. dehydration) (Boyles et al., 2020; Humphries et al., 2003). In my study, bats in the *dry treatment* did not differ in arousal frequency (and torpor frequency/duration) likely due to their ability to conserve water via huddling and their increased water intake during arousals (see below). A previous study hypothesized that EWL equivalent to approximately 5% body mass may be a critical threshold for arousal in some species (Kallen, 1964). Thus, bats in the *dry treatment* were likely able to reduce EWL below this threshold to prevent the need for more frequent arousals (or shorter torpor bout durations).

Consistent with my prediction, bats in the *dry treatment* drank at a greater rate and used a greater proportion of their arousals for drinking compared to bats in the *humid treatment*. For inactive hibernators some water can be produced endogenously through

metabolic processes during torpor bouts but this does not fully compensate for EWL over hibernation (Thomas and Cloutier, 1992; Thomas and Geiser, 1997). Thus bats in the *humid treatment* still needed to arouse and drink, but at a lower rate than bats in the *dry treatment*. Conversely, bats in the *dry treatment* likely experienced higher rates of EWL and were required to drink more to restore water balance, however not at a rate that affected arousal frequency, possibly because of their ability to reduce EWL by huddling in a group.

In the beginning of the experiment, bats in both experimental treatments drank only at a normothermic T_{sk} (i.e., ~29°C). This suggests that in the beginning of hibernation, bats need to use a large amount of energy to rewarm to normothermia and drink as opposed to using "cold arousals" (i.e., bats exhibiting activity despite no increase in T_b to normothermic levels) which have been observed in other bat species (e.g., greater mouse-eared bat, *Myotis myotis* Blažek et al., 2019; little brown bats, Mayberry et al., 2018). In the wild, bats roosting in humid conditions may be able to drink condensation that has collected on the walls of caves or on their fur (Davis, 1970). In the *humid treatment* for my study, condensation did not form on the mesh cages so bats in both treatments, which all roosted at the top corner of the cage opposite to the water dish, needed to crawl down to drink. Thus, torpid bats in both treatments would need to rewarm to normothermia (i.e., expend energy) and crawl to the water dish to restore water balance. For big brown bats roosting in dry conditions in the wild, these energetic costs are likely higher as bats might need to rewarm to normothermia and fly to a water source, potentially outside the hibernaculum (Lausen and Barclay, 2006a). Snow or frozen water near a cave would require energy would be needed to melt the snow/ice and warm it to normothermic T_b (Cooper and Withers, 2014). Thus, snow or frozen water would require higher

energetic inputs, particularly for small-bodied mammals like bats, compared to ingesting water (Cooper and Withers, 2014). Flight is energetically expensive with MRs 15-16 times higher than at rest (Speakman and Thomas, 2003) and rates of EWL are also elevated during flight (Studier, 1970). Combined with the added costs of thermoregulation, the energetic cost of acquiring water for bats in the wild might be very high which could explain why big brown bats appear capable of using huddling to reduce the need to drink (see below).

Consistent with my prediction, big brown bats in the *dry treatment* consistently huddled in a more compact huddle during torpor than bats in the *humid treatment* throughout hibernation. This may have allowed individual bats to reduce their surface area exposed to the dehydrating environment. A reduction of exposed surface area has been quantified in huddling mammals including western harvest mice (*Reithrodontomys megalotis*; 28% surface area reduction, Pearson 1960), golden mice (*Ochrotomys nuttalli*, 23% surface area reduction, Springer et al. 1981), and white-footed mice (*Peromyscus leucopus noveboracensis,* 31% surface area reduction, Glaser and Lustick 1975). Overall, a decrease in cutaneous EWL (through an increase in huddle density/decrease in exposed surface area) coupled with a decrease in cutaneous and respiratory EWL (through a decrease in arousal bout duration; see below) could contribute to an overall decrease in total EWL. Therefore, huddling big brown bats in dry conditions appear to make adaptive behavioural adjustments that reduce EWL which will, in turn, reduce arousal frequency and, thus, energy expenditure.

Although humidity did not affect loss of body mass throughout hibernation, bats that started with the highest initial body mass experienced the greatest decline over the study. Body mass loss ranged from a decrease of 3.0 g (initial body mass = 22.4 g) to a decrease of 9.6 g

(initial body mass = 29.5 g). In a previous study, Kuhl's pipistrelle that hibernated under dry conditions showed higher arousal frequencies and greater mass loss (Ben-Hamo et al. 2013) which indicates that, for some bats, dry conditions cause increased energy expenditure. In my study, I did not find an effect of body mass loss on torpor bout duration, or conversely, an effect of the total number of arousals per bat on body mass loss. Therefore, the relationship between initial body mass and loss of body mass of hibernating bats cannot be attributed to changes in arousal or torpor bouts. Generally, hibernating mammals that have larger fat stores are not constrained by energy availability and have more flexibility in arousal expression (e.g. edible doormice; *Glis glis*, Bieber et al., 2014). Mammals that have smaller fat stores express longer and deeper torpor bouts and would need to balance energetically expensive arousals (for review see: Humphries et al., 2003). In this study, bats with larger fat stores did not express an increase in arousal frequency (or decrease in torpor bout duration). This indicates that the variation of body mass loss cannot be attributed to balancing the energetically expensive arousals with prolonged torpor.

While social thermoregulation and huddling can be beneficial it may also come with costs for individuals, which may have contributed to the variations in loss of body mass that I observed. In both experimental treatments, bats remained in a single huddle throughout hibernation and aroused from torpor in groups of two to ten, infrequently arousing on their own. One possibility is that bats with larger initial body mass were the first to rewarm during these shared arousals and, therefore, experienced higher energetic costs. Conversely, bats in poorer body condition could benefit from passive arousals (i.e., "arousal cascades"; Turner et al., 2014). The effect of group synchrony and passive rewarming on energy expenditure has

been quantified in other mammalian species. Alpine marmots (*Marmota marmota*) have a well-defined synchronization of arousals from hibernation, with adults arousing first, followed by juveniles of the group (Ruf and Arnold, 2000). Adults experienced a greater energetic expense while juveniles had a net energetic benefit through passive rewarming. For my study, bats with the highest loss of body mass may be individuals who initiated arousal cascades, and thus experienced a greater energy expenditure while bats with the lowest loss of body mass, may have aroused last within the cascade, and experienced a net energetic benefit. This phenomenon may be explained by the social dynamics and sex composition of the groups. In this study, all bats were female, and housed together for 28 months prior to the beginning of the study. Little is known about social dynamics of big brown bats during hibernation but, during the active season, female big brown bats aggregate in maternity colonies to give birth to their pups and conform to a "fusion-fission" colony structure in trees, where individual bats switch between roosts, but remain loyal to colony mates (Willis and Brigham, 2004). Female bats that roost communally and give birth to pups can benefit from mutual warming of pups (e.g., Pallid bat, *Antrozous pallidusi*; Trune and Slobodchikoff, 1978) and, thus, overall shared energetic costs. Some evidence indicates that female big brown bats exhibit natal philopatry (i.e., return to site of birth; Brenner, 1968) which can result in colonies of related individuals. Kerth (2006) suggested that aggregation of female bats with relatives, could facilitate the evolution of cooperation among members, resulting in cohesiveness and persistency of social groups. Future studies could analyze the colony structure of big brown bats at hibernacula and determine if social dynamics affect social thermoregulation patterns.

In contrast to my prediction, bats in the *dry treatment* had shorter arousal bouts than bats in the *humid treatment*. Shorter arousals may function to decrease the time spent normothermic, and thus decrease respiratory and cutaneous EWL. In Kuhl's pipistrelle, cutaneous EWL was lower during shallow torpor than normothermia, and respiratory EWL declined as MR decreased (i.e., as bats were becoming torpid) (Muñoz-Garcia et al., 2012a). In my study, bats in the *dry treatment* may have returned to torpor quickly, potentially to decrease both cutaneous and respiratory EWL and preserve more of the water acquired by drinking. During hibernation, behaviours that contribute to a decrease in energy expenditure may also allow for a decrease in EWL. Energetic costs are high to defend a normothermic T_b in cold T_a, and by returning to torpor quickly, hibernators can reduce their energy requirement to as low as ~1% as those needed to defend normothermia (Geiser, 2004). However, in this study, body mass loss did not differ between bats in the two experimental treatments, and if shorter arousal durations contributed to a decrease in energy expenditure, the effect was likely minimal. Therefore, a decrease in arousal bout duration for bats in the *dry treatment* may have been more important for decreasing total EWL rather than energy expenditure.

My results provide evidence that big brown bats use behavioural mechanisms to conserve water loss in conditions of variable humidity. Given the associated link between dehydration and WNS, these behaviours in big brown bats may contribute to their resistance to WNS. However, to further explore mechanisms of WNS resistance in big brown bats, future studies could analyse behaviour of big brown bats infected with *Pd*, in dry conditions. A field study by Frank et al. (2014) analysed hibernation patterns, body condition, and wing damage of WNS-affected big brown bats; however, no record of the ambient humidity of the mine was

reported. Moore et al. (2018) analysed thermoregulatory patterns of captive big brown bats exposed to *Pd*, however bats remained in conditions of 85–95% RH (at 4°C). Thus, any conclusions of underlying resistance mechanisms can only be considered for big brown bats that hibernate in environments with humidity levels that allow for growth of *Pd*. Big brown bats that have the ability to roost in and conserve water loss in dry environments may benefit from an overall decrease in *Pd* growth due to the low ambient humidity, which may further contribute to underlying resistance mechanisms to WNS.

Overall, my results show that big brown bats adjust huddling and drinking behaviours while maintaining consistent patterns of arousals in conditions of variable humidity. Big brown bats in a dry environment increased huddle density and drinking behaviour which could allow for the conservation and restoration of water balance. These results suggest a level of behavioural flexibility for big brown bats that are not typical for other hibernating bat species. The ability for big brown bats to hibernate, and conserve water in, dry environments could play a key role in their resistance to WNS.

CHAPTER 3: PHYSIOLOGICAL RESPONSES OF HIBERNATING BIG BROWN BATS (*EPTESICUS FUSCUS*) TO VARIABLE HUMIDITY

Introduction

Some heterothermic mammals can employ hibernation (i.e., long multi-day torpor bouts) to conserve energy during seasonal periods of low ambient temperatures (T_a) and food availability (Speakman and Rowland, 1999). Hibernation can be divided into four distinct phases: 1) reduced, steady-state body temperature (T_b) and metabolic rate (MR) (i.e., torpor); 2) rapid warming to normothermia; 3) brief periods of normothermia; and 4) a slow cooling phase to return back to torpor (Jonasson and Willis, 2012). During the first phase of steady-state torpor, a hibernator's T_b may be reduced to approximately 1–2°C of the surrounding T_a which is coupled with a marked reduction in MR, heart rate and respiratory rate (Geiser, 2004). Torpid MR is less than 1% of that during normothermia, resulting in enormous energetic savings throughout hibernation (Geiser, 2004). Hibernators, however, cannot stay torpid indefinitely, and periodically return to normothermic T_b. The second phase (i.e., rapid warming) is the most energetically expensive portion of the torpor-arousal cycle because it requires large amounts of metabolic heat production (Geiser, 2004; Thomas et al., 1990a).

The function of periodic arousals to normothermia is not fully understood, but a leading hypothesis is that they allow hibernators to drink and restore water lost during torpor bouts (Ben-Hamo et al., 2013; Fisher and Manery, 1967; Thomas and Geiser, 1997). Other studies have hypothesized that arousals help restore immune function (Prendergast et al., 2002), provide opportunities to forage (Hope and Jones, 2012), allow for sleep which cannot occur during deep torpor (Daan et al., 1991), allow for urination and nitrogenous waste excretion (Németh et al., 2010), or restore balance of metabolites such as ketone bodies or

carbohydrates (Baumber et al., 1971; Galster and Morrison, 1970). Regardless of the exact function, the time spent normothermic can represent as little as ~1% of a hibernator's time, but account for 75–90% of the total winter energy budget (Thomas et al., 1990a). Following the period of normothermia, hibernators adjust their thermoregulatory set-point and re-enter torpor. Cooling rates and associated energetic costs are variable both between and within species (Haase et al., 2019b).

Although water loss is dramatically reduced during prolonged torpor, hibernators continue to lose water to the environment through the respiratory tract (respiratory evaporative water loss; EWL) or across the skin (cutaneous EWL), with the sum of the two representing total EWL. Reduction in total EWL during torpor is variable between species and can range from a decrease of 24 – 42% in stripe-faced dunnarts (*Sminthopsis macroura*; Cooper et al., 2005), to ~40% in cactus mice (*Peromyscus eremicus*; Macmillen, 1965), to over 90% in Gould's long-eared bats (*Nyctophilus gouldi*; Morris et al. 1994). Despite this reduction, total EWL can account for up to 85% of total water loss during hibernation, with the remainder from urination (Studier et al., 1970). For hibernators, some water can be produced endogenously through metabolic processes but this does not entirely compensate for water loss (Thomas and Cloutier, 1992). Thus, EWL could be a potential driver of periodic arousals (Thomas and Cloutier, 1992).

Some hibernating mammals huddle in groups, which can influence the physiology of individuals in a huddle. The main benefit to huddling is thought to be a reduction in MR, which has been quantified in several mammals (for review see Gilbert et al., 2010). Overall, in group sizes ranging from two to nine animals, huddling can allow for metabolic savings to individuals

of approximately 26 ± 11 % (range 8 – 53%) (Gilbert et al., 2010). However, in addition to energy savings, huddling can also reduce total EWL for individuals. The effect of huddling on total EWL has been quantified in many mammals during normothermia, such as the Australian hopping mouse (*Notomys alexis*), with cutaneous EWL reduced by 25% in huddles of four animals versus solitary mice (Baudinette, 1972). In naked mole rats (*Heterocephalus glaber*) increasing the group size from two to eight individuals reduced EWL by 30.5% (Yahav and Buffenstein, 1991). The effects of huddling on total EWL or MR have not been well-studied in hibernating animals, especially during torpor bouts.

Hibernating bats are good model organisms to study patterns of total EWL during prolonged torpor because many species are highly gregarious, and often hibernate in large huddles, and some species exhibit some of the longest torpor bouts of any hibernating mammal. For example, torpor bout durations for little brown bats (*Myotis lucifugus*) can last over 60 days (Czenze et al., 2017) and some evidence suggests bouts could exceed 80 days (Menaker, 1964; for review see Ruf and Geiser, 2015). These long torpor bouts could increase the potential of bats to lose water and become dehydrated relative to other hibernators. Hibernating bats have small lung volumes and dramatically reduced respiratory rates during torpor, so they experience relatively low rates of respiratory EWL (Thomas and Cloutier, 1992). However, due to their highly vascularized flight membranes with large surface areas, bats can experience high rates of cutaneous EWL (Phillips, 1984). The rate of cutaneous EWL is proportional to the difference in water vapour pressure (WVP) between the skin surface and the surrounding ambient air (Schmidt-Nielsen 1997). Any increase in this skin-air differential, or decrease in the boundary layer on the skin, will increase cutaneous EWL (Schmidt-Nielsen

1997). Additionally, because the T_b of hibernating bats is often similar to T_a of hibernacula (Schmidt-Nielsen 1997, Geiser 2004) this reduced T_b will reduce the skin-air differential, thus lowering cutaneous EWL.

Huddling could be important for helping hibernating bats reduce water loss during torpor bouts. Boratyński et al. (2015) measured total EWL and torpid MR in natterer's bats (*Myotis nattereri*) roosting either solitarily or in groups of five or six huddling bats. Huddling allowed bats to reduce total EWL by almost 30% compared to roosting solitarily, however torpid MR did not vary for bats roosting solitarily or huddling in groups (Boratyński et al., 2015). These different results for total EWL and torpid MR suggests that huddling bats benefit from reduced cutaneous, as opposed to respiratory EWL, likely due to a reduction in exposed skin. Thus, the direct function of huddling for bats may be to reduce EWL through a reduction in exposed surface area, as opposed to a direct reduction in energy expenditure although, over the long-term, reduced EWL could provide indirect energetic benefits by allowing bats to arouse from torpor less often throughout hibernation (Boratyński et al., 2015).

Research on the relationship between physiology, water loss, and energy expenditure of bats during hibernation has become especially important because of a recently emerged disease called white-nose syndrome (WNS). WNS continues to cause mass mortality in multiple North American bat species (Frick et al., 2015) and three species have been listed as endangered in Canada as a result. The causative agent, *Pseudogymnoascus destructans* (*Pd*), is a psychrophilic fungus that grows at temperatures of 3°–20°C (Verant et al. 2012). The fungus grows on the face, and into the wing and tail membranes of bats (Verant et al., 2014; Warnecke et al., 2013). As the disease progresses, bats exhibit increased arousal frequency and overall

energy expenditure which prematurely depletes their hibernation fat reserves (Warnecke et al., 2012; Warnecke et al., 2013). The precise cause of increased arousal frequency is not fully understood but the "dehydration hypothesis" suggests that fungal lesions across the skin increase cutaneous EWL (Cryan et al., 2010; Willis et al., 2011). Consistent with this hypothesis, McGuire et al. (2017) showed that bats with WNS exhibit increased total EWL suggesting that WNS pathophysiology plays a role in the water balance of hibernating bats.

So far 12 hibernating bat species have been diagnosed with WNS in North America but some do not appear heavily impacted by the disease. Big brown bats (*Eptesicus fuscus)* appear resistant although the underlying mechanisms are not fully understood (Frank et al., 2014; Moore et al., 2018). One possibility is that big brown bats are relatively flexible in their habitat requirements for hibernation, using rock crevices (Lausen and Barclay, 2006a), buildings (Halsall et al., 2012; Whitaker Jr. and Gummer, 1992), and caves (Mills et al., 1975; Reimer et al., 2014) that vary widely in humidity. Big brown bats can tolerate a much lower relative humidity (RH) compared to other hibernating bats, and can roost in RH as low as 52% at 0.6 ± 0.91°C (Klüg-Baerwald and Brigham, 2017; Kurta and Baker, 1990). Big brown bats are insectivorous and occur throughout most of North America, and into northern South America (Kurta and Baker, 1990). In their northern range, they hibernate throughout winter (French, 1985; Kurta and Baker, 1990) and use short-term torpor during summer (e.g. Lausen and Barclay 2003). Their geographic range spans a large latitudinal gradient and measurements of MR can vary among populations. Torpid MR of big brown bats was measured from three populations across this gradient in a range of T_a (Dunbar and Brigham 2010). Bats from the southern population maintained higher torpid MR at cooler T_a, but lower torpid MR at warmer T_a compared to

northern bats with the mid-latitude population exhibiting intermediate values of torpid MR (Dunbar and Brigham 2010). These results highlight the intraspecific variation in thermoregulatory responses of this species although whether they reflect phenotypic plasticity or genetic differences is unknown.

Understanding factors influencing the water balance and energetics of hibernating big brown bats could be important for understanding their resistance to WNS. Overall, big brown bats adjust well to captivity, and physiological mechanisms such as torpid MR and total EWL can be studied while controlling for confounding variables. Thus, big brown bats are an ideal species to study water balance mechanisms during hibernation. My objectives were, therefore, to understand the influence of huddling on the total EWL of hibernating big brown bats and to understand their potential for phenotypic flexibility in total EWL and torpid MR following acclimation to dry conditions. I used open-flow respirometry to first test the hypothesis that phenotypic flexibility in total EWL helps explain the tolerance of hibernating big brown bats for a wide range of humidity. I predicted that total EWL would be lower for bats acclimated to dry conditions compared to bats acclimated to humid conditions. I then tested a second hypothesis that the primary benefit of huddling for big brown bats is to mitigate the hygric challenge of dry conditions rather than providing a direct energetic benefit. I predicted that huddling bats would exhibit lower total EWL but no difference in torpid MR compared to solitary individuals regardless of whether they were acclimated to humid or dry conditions.

Methods

Adjustment Period of Study Animals and Hibernation

All animal handling procedures were approved by the University of Winnipeg Animal Care Committee (protocol AE12193). To test my hypotheses, I used a captive colony of 20 non-reproductive female adult big brown bats housed at the University of Winnipeg (Winnipeg, Canada). The bats were originally caught in June 2017 at two netting sites approximately 328 km from one another: Bismarck, North Dakota (46.77°N, 100.76°W), and Ada, Minnesota (47.30°N, 96.51°W) and then housed at the University of North Dakota for 28 months for behavioural studies. In the summer, bats were housed in outdoor flight cages (2.5 x 2.5 x 2.5 m, as described by Boyer et al., 2020), and in the winter, bats were housed in modified incubators for hibernation (Erin Gillam, University of North Dakota, personal communication). At capture, bats were outfitted with up to two coloured, plastic forearm bands for individual identification (i.e., left, right or both forearms) (Boyer et al., 2020).

The colony was transported by car, to the University of Winnipeg on 19 October 2018 (see **Figure S.1** for timeline). Upon arrival bats were divided randomly into two groups of 10 and housed in two temperature-controlled incubators set at 8°C. Inside each incubator, bats were housed together in a single custom-built nylon mesh enclosure (modified from Exo-terra Flexarium/Flextray© PT2556, Hagen Inc., Montreal, QC, Canada; 49.5 x 20.3 x 38.8 cm and 43.2 x 26.7 x 57.2 cm). These incubators were humidified with saturated sponges, which were rewet during periodic health checks (see below). To record the skin temperatures (T_{sk}) of bats, I affixed temperature-sensitive dataloggers (DS2422 iButton; Maxim Integrated, Sunnyvale CA USA, modified as per Reeder et al., 2012). I marked each datalogger with a unique symbol for

identification of individuals. For small bats, T_{sk} gives a good approximation of T_b when T_a is stable and well below normothermic T_b (Willis and Brigham, 2003), and has been used in multiple studies of captive hibernating bats (Mayberry et al., 2018; Turner et al., 2014; Warnecke et al., 2012). Dataloggers were coated in a layer of black synthetic rubber (Plasti Dip®, Plastic Dip International, Blaine, Minnesota) to protect the circuit board and battery from humidity and attenuate ultrasound which can be emitted by iButtons (Willis et al., 2009). I calibrated iButtons after the experiment by placing them in a sealed bag and immersing them in a water/ethylene glycol mixture inside a temperature incubator. Temperature of the water/ethylene glycol was measured with a thermocouple thermometer (Model TC-2000, Sable Systems) calibrated to a NIST-traceable mercury thermometer. I set iButtons to record temperature once per minute with 20 readings per temperature at four temperatures between 0°C and 40°C. I then fit a calibration curve for each iButton and used the equations for these curves to correct data recorded for each bat during hibernation and for respirometry trials.

Bats were adjusted to the facility at the University of Winnipeg from 19 October 2018 to 18 December 2018 and provided water and mealworms (larval *Tenebrio molitor*) supplemented with vitamins and minerals (following Barnard et al. 2013) *ad libitum*. Body mass was measured with an electronic scale (± 0.1 g; Ohaus Corporation, CS200, Pine Brook, New Jersey, U.S.A.) and bats were hand fed a maximum of 40 mealworms every second day for one week, then once every 3–5 days for the next two weeks, and once every 7–11 days for the final six weeks of adjustment. This nine-week adjustment period allowed me to monitor the body mass of bats closely and ensure that the bats were entering hibernation with a large enough fat store (≥ 18 g).

For hibernation, on 27 November 2018, bats were moved into a separate set of two larger temperature and humidity-controlled incubators (Environmental Chamber, Model 6041, Caron, Marietta, OH, U.S.A.) set at 8°C and 98% RH (see **Figure S.2** for schematic diagram). The incubators were humidified via a single condensate recirculating system (Condensate Recirculating System CRSY102, Caron, Marietta, OH, U.S.A). I housed bats in the same groups of 10 to avoid affecting social dynamics of the groups. Bats were housed in their groups of 10, in custom-built nylon mesh cages (modified Exo-terra Flexarium/Flextray© PT2556, Hagen Inc., Montreal, QC, Canada; 91.4 x 43.2 x 43.2 cm) situated on a shelf located in the middle of each incubator. Water was provided *ad libitum* via tubing that passed into the chamber, allowing me to refill water dishes without disrupting hibernating bats. Aquarium rocks were placed inside the water dish to prevent bats from falling in.

On 14 December 2018, one incubator remained at 98% RH and 8°C (i.e., *humid treatment* incubator, WVP = 1.03 ± 0.08 kPa at 8.57 ± 0.12°C) and I decreased humidity in the second incubator to 50% (i.e., *dry treatment* incubator, WVP = 0.57 ± 0.08 kPa at 8.37 ± 0.14°C; hereafter treatments are referred to as 'acclimation treatment'). Due to limitations of both animals and equipment, I was not able to replicate the two experimental treatments. On 18 December 2018, I weighed, and hand fed the bats but food was withheld thereafter throughout hibernation to match natural conditions in the wild. Bats remained in these conditions for a total of 110 days. On 08 April 2019, I removed all bats from hibernation, removed their iButtons, and measured body mass. When the bats were being removed from the incubators, one bat in the *dry treatment* incubator (**ID13**) was found to have gotten caught in the tape lining the mesh cage while in the single huddle of bats and died sometime after 11 February

2019. On 08 April 2019, as part of a separate study, all bats had a sample of blood taken (max = 150 µL) from the interfemoral vein, were hand fed mealworms, and then returned to their respective incubators.

Respirometry

Starting on 15 April 2019, I used open-flow respirometry to measure torpid MR and total EWL of solitary and huddling big brown bats from both the *humid treatment* (i.e., 98% RH) incubator and the *dry treatment* (i.e., 50% RH) incubator. I followed respirometry methods used by McGuire et al. (2017) for solitary bats and Boratyński et al. (2015) for huddling bats. Pressurized laboratory air was first passed through a carbon dioxide (CO_2) and water (H_2O) absorbing system (PCDA series, PureGas, Broomfield, CO) to provide dry, CO_2-free air (**Figure 3.1**). Air was then humidified by passing it through a custom-made water-bubbler system inside a temperature-controlled cabinet, also set at 8°C. A series of valves allowed me to switch between providing dry air (i.e., bypassing the bubbler) or humidified air to the respirometry chambers depending on the respirometry treatment. Using dry air allowed me to quantify total EWL of big brown bats under conditions consistent with most respirometry studies while wet air allowed me to replicate conditions similar to natural hibernacula. A multi-channel flow controller (Model FB8, Sable Systems) regulated air flow to each sealed chamber (between 200 and 400 ml min^{-1} for the solitary chambers and 800 and 1000 ml min^{-1} for the group chamber). Bats were placed in custom-made sealed respirometry chambers (250 ml for solitary chamber and 1000 ml for the group chamber) lined with mesh to allow them to hang comfortably. Each

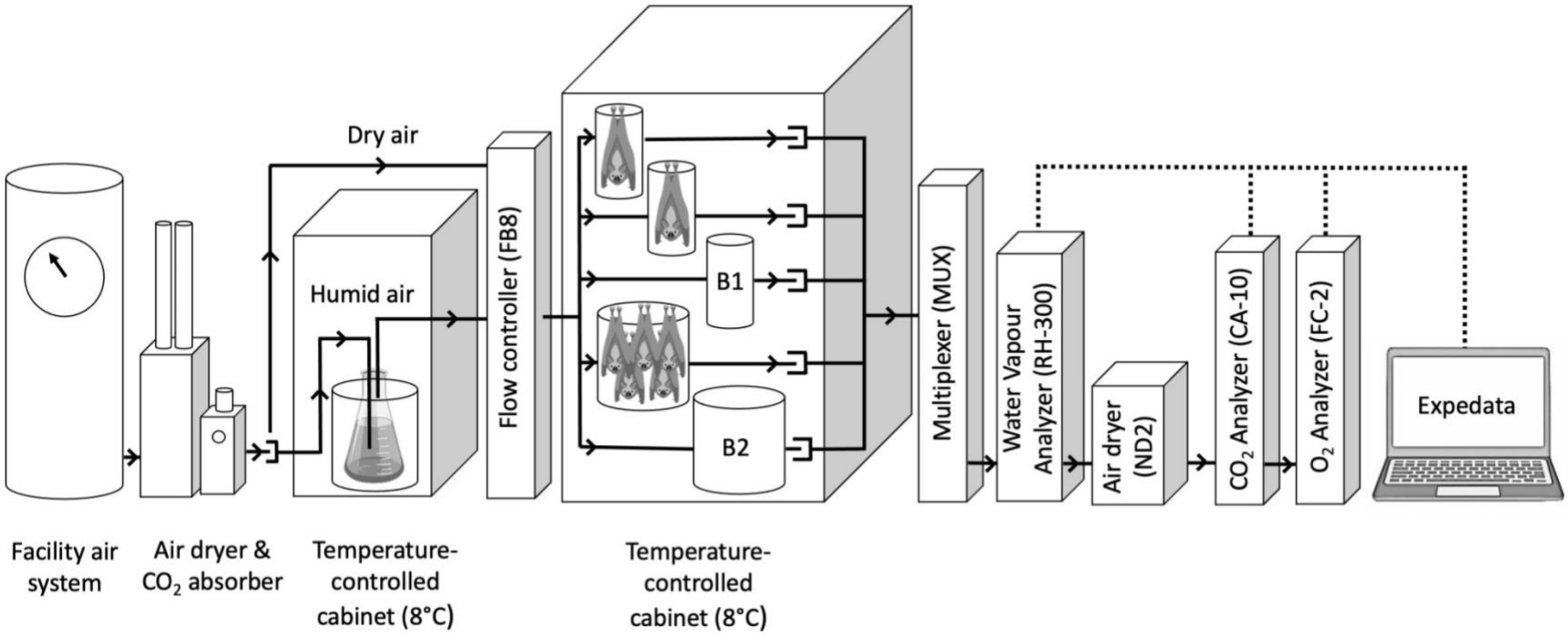

Figure 3.1: Schematic diagram of the open-flow respirometry system used to measure torpid metabolic rate and total evaporative water loss of solitary and huddling big brown bats (*Eptesicus fuscus*) within a temperature-controlled cabinet. Air flow is indicated by solid black lines with arrows and the flow of data is indicated by dotted lines. One valve allowed me to switch between wet and dry air (i.e., bypassing the water flask). B1 refers to the baseline chamber for the solitary trials, and B2 refers to the baseline chamber for the huddling trial. Water vapour, CO_2, and O_2 analysers collected data which was transferred to the computer to be analysed with *Expedata*. Diagram adapted from Boratyński et al. (2015).

chamber contained a layer of mineral oil at the bottom to isolate feces/urine and prevent introduction of humidity and bats were separated from the mineral oil by a steel mesh platform. Respirometry chambers were placed inside another temperature-controlled cabinet set at 8°C. I installed an infrared camera (Hawk Eye Nature Camera, Songbird Garden, Cape Fair, Missouri, U.S.A.) on the inside of this incubator so I could confirm that bats in the group chamber were torpid (i.e., not moving) and huddling during measurements.

Air was continually pumped through each chamber and a multiplexer (MUX, Sable Systems) was programmed to sub-sample at 100 ml/min from each chamber sequentially. The excurrent air was first passed through a water vapour analyser (model RH-300, Sable Systems), and then through an air dryer (model ND2, Sable Systems) to remove water vapour. The airstream was then passed through a gas analyser to record CO_2 (model CA-10, Sable Systems) and oxygen (O_2) (model FC-2, Sable Systems) concentrations. Data for water vapour, O_2 and CO_2 gas concentrations were recorded at 1 Hz using a laptop running *ExpeData* (v 1.3.0, Sable Systems).

Before data collection, I calibrated the humidity analyser using a two-point calibration method (Sable Systems Inc, 2009). First, I pushed dry 100% nitrogen through the analyser for 10 minutes for zero calibration. Second, I ran air through the bubbler set at 8°C to create a nearly saturated air stream which I ran through the humidity analyser for 45 minutes until the recording stabilized. I then used **Equation 3.1** to determine the WVP (Pascals; where T is the air stream temperature in ˚C). I adjusted the analyser so that both the displayed and calculated WVP were equivalent.

$$WVP = 0.61121\ exp\ ((18.678 - \frac{T}{234.5})(T * 257.14 + T))$$ **(3.1)**

I calibrated CO_2 and O_2 analysers using a two-point calibration before the respirometry experiment began and twice, between trials throughout the experiment. I first zeroed the analysers by running pure N_2 through the airstream for 10 minutes. I used 0.5079% CO_2 to span the CO_2 analyser and I used dry air from the experimental setup (i.e., 20.94% oxygen) to span the O_2 analyser.

Respirometry Trials

I conducted respirometry trials within a short 14-day period (15 - 29 April 2019) to ensure that all bats were at a similar stage of hibernation. Before trials began, on 15 April 2019 (*humid treatment* incubator) and 16 April 2019 (*dry treatment* incubator), I reattached temperature dataloggers so I could confirm that bats were in steady-state torpor during measurements. IButtons were set to record T_{sk} once every 10 minutes. One iButton (**ID20**) failed to record but I obtained data from the remaining 18 bats.

Respirometry trials began at 19:00 each day and lasted for 23 hours. Before the experiment began, I used haphazard random sampling to assign bats to a respirometry trial schedule so that each individual underwent a maximum of two solitary respirometry trials and five group trials (i.e., maximum of seven trials per bat). I alternated between measuring bats from the *dry treatment* incubator and the *humid treatment* incubator to ensure that bats did not undergo respirometry trials on consecutive days. I completed a total of seven trials with bats from the *humid treatment* incubator and six trials with bats in the *dry treatment* incubator (**Table 3.1**).

Table 3.1: Respirometry trials for big brown bats (*Eptesicus fuscus*) between 15 – 29 April 2019. Trials alternated between bats from the *humid treatment* and *dry treatment* incubator. For each trial, individual bats were each placed in the solitary chambers whereas five bats were placed in the group chamber.

Date	Trial No.	Experimental Treatment Incubator	Solitary Chamber #1	Solitary Chamber #2	Group Chamber Bat #1	Group Chamber Bat #2	Group Chamber Bat #3	Group Chamber Bat #4	Group Chamber Bat #5	Huddling
15 – 16 April 2019	1	*Humid*	ID10[b]	ID05	ID07	ID08	ID04	ID03	ID02	No
16 – 17 April 2019	2	*Dry*	ID20	ID16	ID14	ID17	ID11	ID15	ID18	Yes
17 – 18 April 2019	3	*Humid*	ID03	ID02	ID06	ID09	ID01	ID07	ID10	No
18 – 19 April 2019	4	*Dry*	ID11	ID15	ID16	ID19	ID12	ID18	ID20	Yes
19 – 20 April 2019	5[a]	*Humid*	ID06	ID07	ID04	ID02	ID05	ID09	ID01	-
20 – 21 April 2019	6	*Dry*	ID18	ID19 [c]	ID20	ID12	ID15	ID17	ID14	Yes
21 – 22 April 2019	7	*Humid*	ID04	ID01	ID10	ID05	ID06	ID03	ID08	Yes
22 – 23 April 2019	8	*Dry*	ID12	ID14[c]	ID19	ID11	ID20	ID16	ID17	Yes
23 – 24 April 2019	9	*Humid*	ID07 [b]	ID08 [b]	ID04	ID10	ID03	ID09	ID02	No
25 – 26 April 2019	10	*Dry*	ID16[d]	ID17[e]	ID12	ID15	ID19	ID14	ID18	Yes
26 – 27 April 2019	11	*Humid*	ID01[d]	ID09	ID10	ID05	ID08	ID06	ID02	Yes
27 – 28 April 2019	12	*Dry*	ID20[d]	ID15[d]	ID17	ID18	ID11	ID16	ID14	Yes
28 – 29 April 2019	13	*Humid*	ID08[d]	ID03[d]	ID01	ID06	ID05	ID04	ID07	Yes

[a] Trial was terminated before metabolic measurements were taken due to condensation in the group chamber and solitary chamber #1.

[b] Bat did not enter steady state torpor under wet air.

[c] Bat did not enter steady state torpor under wet and dry air.

[d] Trials were removed from analyses due to small sample sizes which did not allow for repeated measures.

[e] Measurements under dry and humid air removed due to highly influential outlier

I programmed the multiplexer (MUX, Sable Systems) to sub-sample from each animal chamber for 15 minutes at a time and from the empty baseline chambers for five minutes between each animal chamber. I measured body mass of each bat before and after respirometry trials (± 0.1g, Ohaus Corporation, CS200, Pine Brook, New Jersey, U.S.A). I placed bats in the respirometry chambers under wet air at approximately 19:00 to ensure bats had at least 12 hours to acclimate to the chamber and enter torpor before the start of data collection at the beginning of their normal daily rest phase. The next morning at approximately 07:00, I began recording incurrent and excurrent O_2, CO_2, and WVP for five hours, sub-sampling from each animal chamber and reference chamber in sequence five times over that period. After five hours of recording, I switched to dry air and allowed one hour for bats to acclimate and then recorded incurrent and excurrent O_2, CO_2, and WVP for another five hours. Once the respirometry trial was completed (approximately 18:00), each bat was removed from respirometry chambers, handfed up to 40 mealworms, given water, and returned to their incubator.

I ran a total of 26 solitary trials and 13 group trials with 19 different bats over 14 days of the experiment. I excluded data from my analysis if bats did not enter steady-state torpor based on the T_{sk} data (see **Figure S.5** for T_{sk} data). Additionally, I terminated respirometry trials where I observed condensation in the respirometry chamber (through the IR videos) and excluded any physiological measurements from analyses. I excluded group trials where bats were not observed to be in direct contact with each other (based on the IR camera feed or my observations of bats when I removed them from respirometry chambers).

Data Processing and Statistical Analyses

I used *Expedata* (v 1.3.0, Sable Systems) to analyse respirometry data. I first corrected for lag (the time required for a change in gas concentrations to reach the analyser) and drift (gradual change in signal from the analysers), and washout characteristics, and then automated calculations to determine VCO_2, VO_2 and VH_2O. I used the following equations to calculate O_2 consumption ($\dot{V}O_2$ - Eq. 10.6, Lighton (2008); **Equation 3.2** below), CO_2 production ($\dot{V}CO_2$ - Eq. 10.7, Lighton (2008); **Equation 3.3** below), and total EWL ($\dot{V}H_2O$ - Eq. 10.9, Lighton (2008); **Equation 3.4** below):

$$\dot{V}O_2 = FR_i[(F_eO_2 - F_i'O_2) - F_e'O_2(F_e'CO_2 - F_iCO_2)]/(1 - F_e'O_2)$$ **(3.2).**

$$\dot{V}CO_2 = FR_i[(F_e'CO_2 - F_i'CO_2) - F_e'CO_2(F_eO_2 - F_i'O_2)]/(1 - F_e'CO_2)$$ **(3.3).**

$$\dot{V}H_2O = FR_i\ (F_eH_2O - (F_iH_2O)/(1 - F_eH_2O))$$ **(3.4).**

where FR_i is the incurrent flow rate and FR_e is the excurrent flow rate of all gases (i.e., O_2, CO_2, and water vapour) in the airstream. F_iO_2, F_iCO_2, and F_iH_2O represent the fractional incurrent flow rates of each gas while F_eO_2, F_eCO_2, and F_eH_2O represent fractional excurrent flow rates, and FR_i represents the incurrent flow rate. For VH_20, I corrected for barometric pressure, by dividing by 92.7 kPa. I identified 15 min periods in the total EWL and VCO_2 traces when values were minimal and stable, calculated the average of VCO_2 and VH_2O and for each bat or group of bats, then used these values for subsequent analysis. I used VCO_2 to calculate torpid MR (in mW) assuming fat oxidation and a respiratory exchange ratio of = 0.71 (28.008 J ml CO_2^{-1}; Gessaman, 1987). I used a standard conversion factor of 0.803 to convert volume of EWL (ml min^{-1}) to mg min^{-1} (Lighton, 2008).

I calculated body mass for each bat at the time of metabolic measurements assuming a linear decline of body mass from the start to end of trials. I was not able to measure torpid MR and total EWL for each individual bat in the group chamber, so I divided the torpid MR and total EWL of the huddle by the number of bats (Boratyński et al., 2015; Gilbert et al., 2010). For analyses including body mass as a covariate, I divided total mass of bats in the huddle by the number of individuals (Boratyński et al., 2015; Seltmann et al., 2009).

I was not able to complete enough trials for solitary bats (n = 1.37 ± 0.50 trials per bat) to allow for repeated measures in my analyses. Therefore, for bats that underwent two separate trials (i.e., on different days), I used the first metabolic trial for my analyses. Additionally, one measurement of total EWL from a solitary bat (*dry treatment* incubator, under dry air) was identified as highly influential outlier (Cook's D = 1.54; threshold of 4/n = 0.14). This value of total EWL was four-times greater than the highest measurement reported for solitary big brown bats under dry air (~0.37 mg min^{-1}; Klüg-Baerwald and Brigham, 2017). Since measurements of total EWL from this bat were unlikely to be accurate, I removed both measurements of total EWL from analyses, along with corresponding measurements for torpid MR. Thus, for bats from the *humid treatment* incubator, I was able to measure torpid MR and total EWL for solitary bats under wet air (n = 6) and dry air (n = 9) and for huddling bats under wet (n = 3) and dry air (n = 3). For bats from the *dry treatment* incubator, I was able to measure torpid MR and total EWL for solitary bats under wet and dry air (both n = 16) and for huddling bats under wet and dry air (both n = 6) (**Table 3.1**).

Using the metabolic measurements from solitary bats, I tested for an effect of acclimation (i.e., *humid treatment* or *dry treatment* incubator) using a linear mixed model (R

package ‘nlme’, Bates et al. 2015) with torpid MR as the response variable, respirometry treatment (i.e., wet or dry air) as a fixed factor, and body mass and the two-way interaction between acclimation and respirometry treatment as covariates. I included Bat ID as a random effect to account for repeated measures of bats between respirometry treatments (i.e. wet or dry air). I used a second linear mixed model with total EWL as the response variable, and respirometry treatment and torpid MR as a fixed factor, and body mass, and the two-way interaction between acclimation and respirometry treatment as covariates, with Bat ID as a random effect to account for repeated measures of bats between respirometry treatments. In both models, I removed non-significant covariates until only significant effects remained. I then used a two sample t-test to compare the body masses of solitary bats from the two acclimation treatments at the time of metabolic measurements. I calculated effect size as Cohen’s d (R package “effsize”, Torchiano 2020) to compare the magnitude of body mass effects between acclimation treatments.

I used a series of linear models to test for an effect of huddling on torpid MR and total EWL. I used separate linear models (one for bats from the *humid treatment* and one for bats from the *dry treatment*) to test for an effect of huddling on the torpid MR with respirometry treatment as a fixed factor and body mass as a covariate. I also used two linear models (again one for bats from the *humid* and one for bats from the *dry treatment*) to test for an effect of huddling on the total EWL (log transformed for normality) including respirometry treatment, and torpid MR as fixed factors and body mass as a covariate. For all linear models, I removed non-significant covariates until only significant and fixed effects remained.

All statistical analyses were conducted in R 4.0.4 (R Core Team, 2021) using RStudio (Version 1.4.1106) with graphs produced using 'ggplot2' (Wickham, H. 2016). For all statistical tests, significance was assessed at $p < 0.05$, and values are reported as mean ± SD and samples as n = number of measurements.

Results

For solitary bats, there was no effect of acclimation on torpid MR (**Table 3.2**). but, regardless of acclimation treatment, bats in wet air during respirometry had higher torpid MR (**Table 3.2**, **Figure 3.2**). There was no effect of body mass (**Figure S.6**) or the interaction between acclimation and respirometry treatment on torpid MR (**Table 3.2**; **Figure 3.2**). Bats acclimated to dry conditions had rates of total EWL 17.14% lower (**Table 3.3**; **Figure 3.3.A**) than bats acclimated to humid condition. Additionally, regardless of acclimation treatment, wet air during respirometry led to lower rates of total EWL for solitary bats (**Table 3.3**; **Fig 3.3.B**) with no effect of the interaction between acclimation and respirometry treatment (**Table 3.3; Figure 3.3.B**). Solitary bats with smaller body masses had higher rates of total EWL (**Table 3.3, Figure S.7**) and bats with higher rates of torpid MR had increased rates of total EWL. (**Table 3.3**). Solitary bats from the *humid treatment* incubator had body masses = 18.3 ± 1.1 g (range of 16.1 – 19.7 g) at the time of their metabolic measurements which was significantly higher than the 17.5 ± 0.9 g (range 16.1 – 18.7 g) for bats from the *dry treatment* incubator ($t = -2.24$, $df = 43$, $p = 0.03$, Cohen's $d = 0.78$) (**Figure 3.4.A** and **Figure 3.4.B**)

For bats from the *humid treatment* incubator, there was no effect of huddling (**Figure 3.5.A;** $p = 0.21$) on torpid MR. However, bats with smaller body masses ($p < 0.001$, **Figure S.8.A**) and bats under wet air during respirometry ($p < 0.001$) had higher torpid MR. Consistent with my hypothesis, an average bat in a huddle had significantly lower rates of total EWL (29.21% lower; **Figure 3.5.B** $p = 0.02$) compared to solitary bats. Bats from the *humid treatment* incubator under dry air during respirometry also had higher rates of total EWL ($p < 0.001$) and

showed a positive relationship between torpid MR and total EWL ($p < 0.001$). There was no effect of body mass on total EWL for these bats ($p = 0.11$; **Figure S.8.B**).

For bats from the *dry treatment* incubator, there was no effect of huddling (**Figure 3.6.A;** $p = 0.47$), respirometry treatment ($p = 0.10$), or body mass ($p = 0.10$; **Figure S.9.A**) on the torpid MR and there was no effect of huddling on total EWL (**Figure 3.6.B;** $p = 0.12$). As for bats from the *humid treatment* incubator, dry air during respirometry ($p <0.001$) and higher rates of torpid MR ($p < 0.001$) led to increased rates of total EWL with no effect of body mass on total EWL ($p = 0.11$; **Figure S.9.B**).

Table 3.2: Summary of the linear mixed model testing for an effect of acclimation on measurements of torpid metabolic rate with respirometry treatment as a fixed factor, body mass (g) and the interaction between acclimation and respirometry treatment as covariates, and Bat ID as a random factor for solitary big brown bats (*Eptesicus fuscus*; n = 27 on 19 bats). Significant p-values are bolded with displayed values from final models after non-significant predictor variables were dropped.

Variable	Coefficient	t-value	p-value
Acclimation Treatment (Humid Incubator)	-0.27	-0.99	0.34
Respirometry Treatment (Wet air)	**0.44**	**6.43**	**< 0.001**
Body mass (g)	-0.14	-1.27	0.23
Interaction term: Acclimation Treatment and Respirometry Treatment	-0.04	-0.23	0.82

Table 3.3: Summary of the linear mixed model testing for an effect of acclimation on total evaporative water loss with respirometry treatment and torpid metabolic rate as fixed factors, body mass (g) and the interaction between acclimation and respirometry treatment as covariates, and Bat ID as a random factor for solitary big brown bats (*Eptesicus fuscus*; n = 27 trials on 19 bats). Significant p-values are bolded with displayed values from final models after non-significant predictor variables were dropped.

Variable	Coefficient	t-value	p-value
Acclimation Treatment (Humid Incubator)	**0.16**	**3.41**	**0.005**
Respirometry Treatment (Wet air)	**-0.14**	**-3.91**	**0.005**
Body mass (g)	**-0.08**	**-3.62**	**0.004**
Torpid metabolic rate	**0.15**	**3.22**	**0.01**
Interaction term: Acclimation Treatment and Respirometry Treatment	-0.01	-0.16	0.88

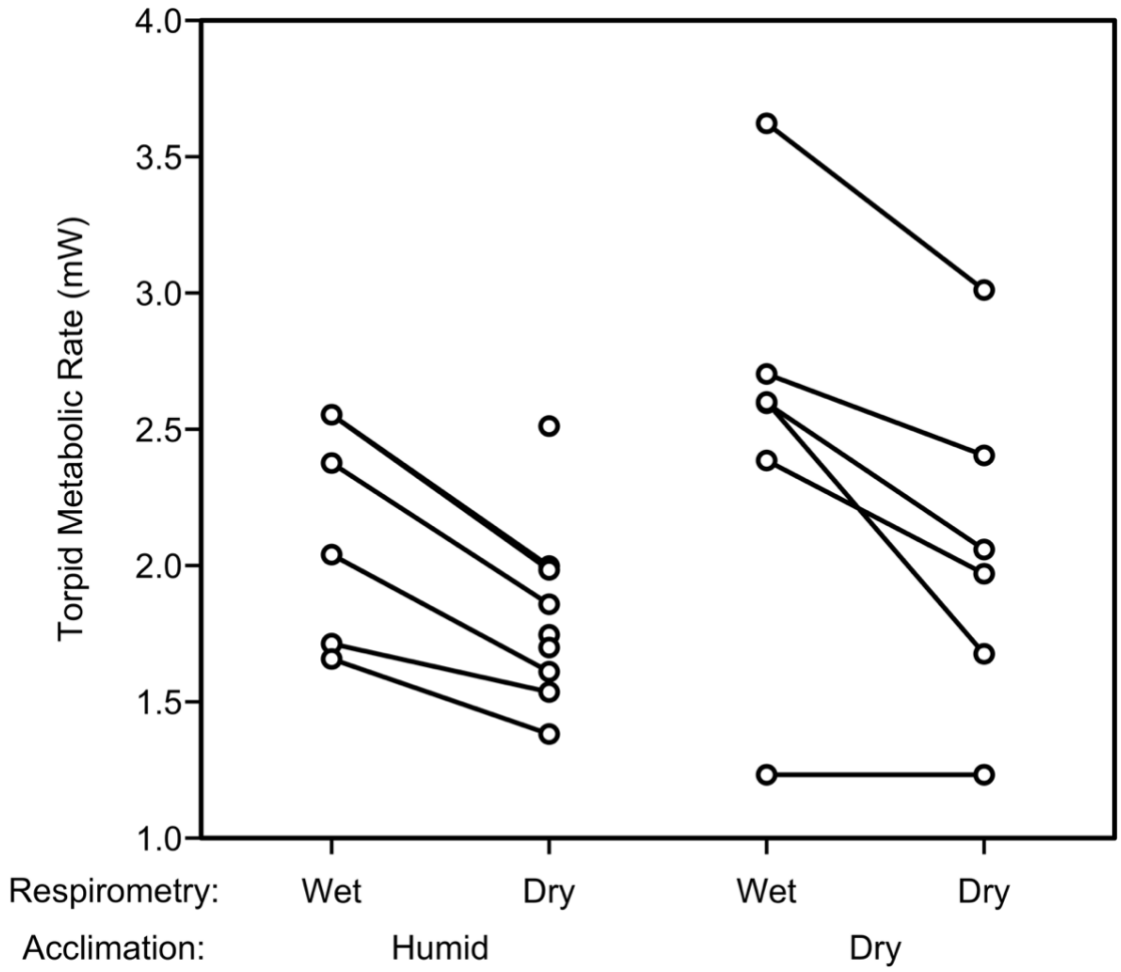

Figure 3.2: Paired scatterplot of torpid metabolic rate (mW) for solitary big brown bats (*Eptesicus fuscus*). Data were obtained from bats acclimated during the *humid treatment* and measured under wet air (n = 6) or dry air (n = 9) during respirometry and from bats acclimated during the *dry treatment* and measured under wet air (n = 6) and dry air (n = 6). Black lines indicate paired data from the same individual bat.

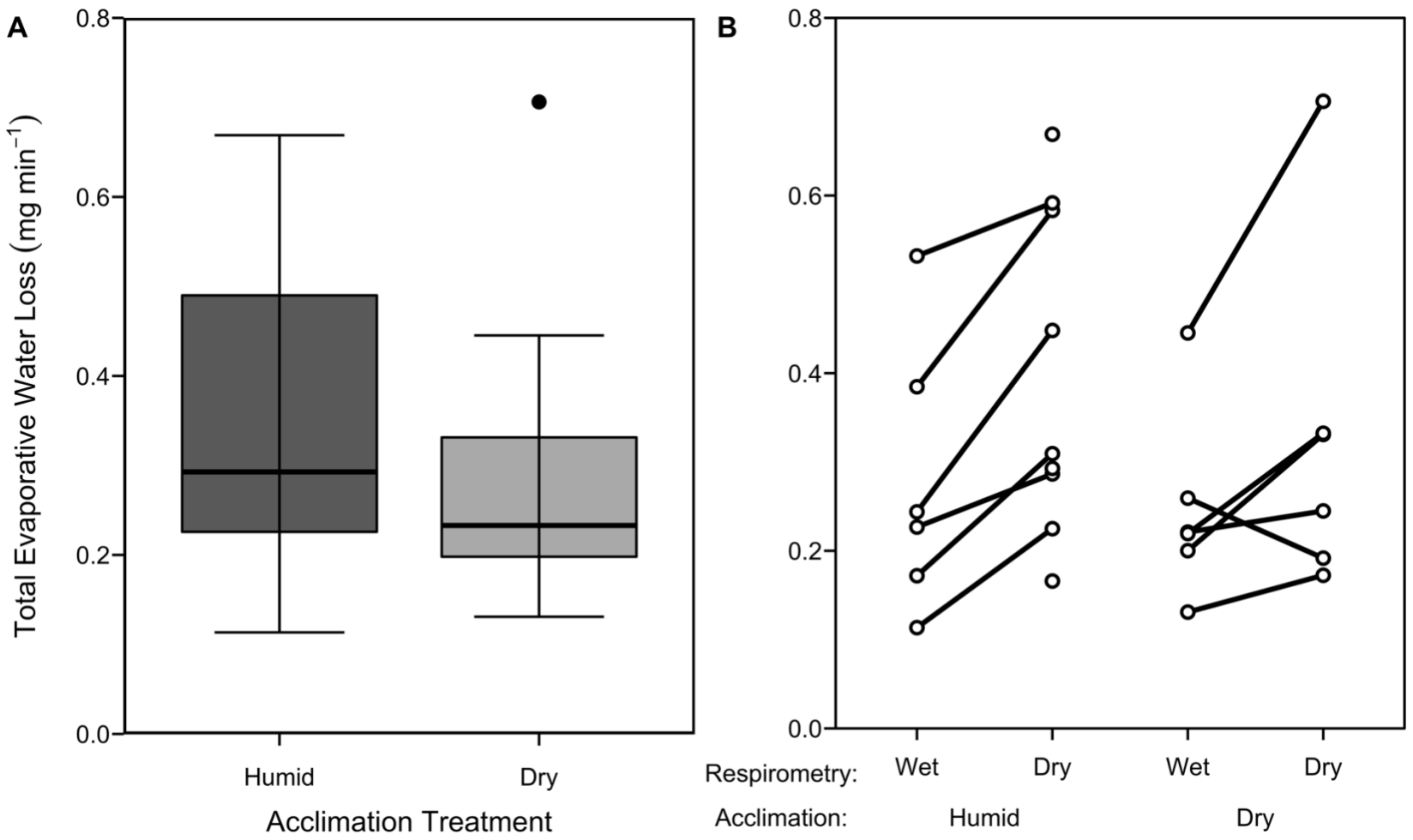

Figure 3.3: A Boxplots of the total evaporative water loss (mg min^{-1}) for solitary big brown bats from the *humid treatment* (n = 12) and *dry treatment* (n = 15). The median is represented by a solid horizontal line, the top and bottom of each box represents the 25^{th} and 75^{th} percentile, respectively. Whiskers represent maximum and minimum values. **B** Paired scatterplot of total evaporative water loss (mg min^{-1}) for solitary big brown bats (*Eptesicus fuscus*). Data were obtained from bats acclimated in the *humid treatment* and measured under wet air (n = 6) or dry air (n = 9) during respirometry and from bats acclimated in the *dry treatment* and measured under wet air (n = 6) or dry air (n = 6). Black lines indicated paired data from the same individual bat**.**

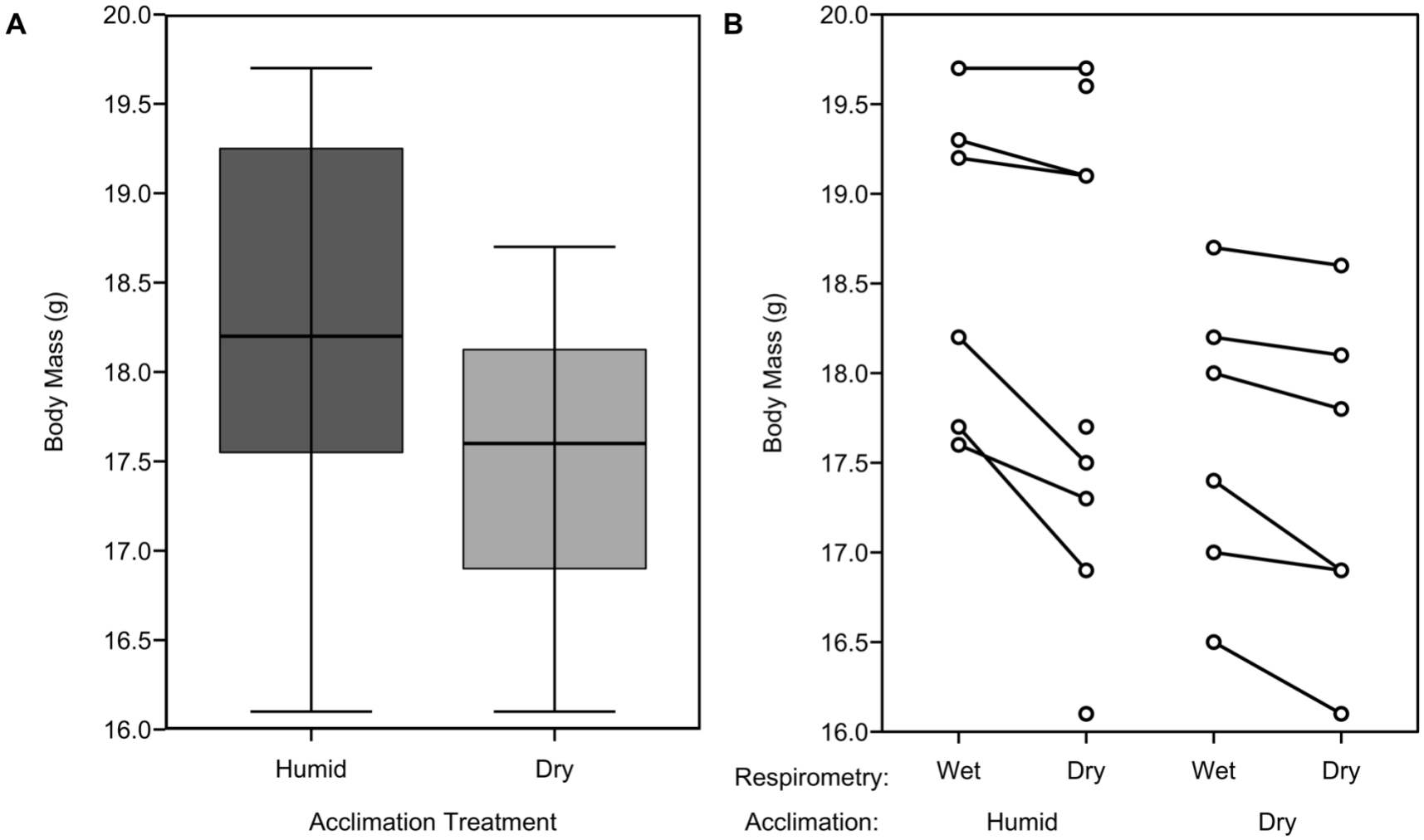

Figure 3.4: A Boxplot of body mass (g) for solitary big brown bats acclimated in the *humid treatment* (n = 15) and from bats acclimated in the *dry treatment* (n = 12). **B** Paired scatterplot of body mass (g) for solitary big brown bats (*Eptesicus fuscus*) acclimated to the *humid treatment and* measured under wet (n = 6) or dry air (n = 9) and from bats acclimated in the *dry treatment* and measured under wet (n = 5) or dry air (n = 5).

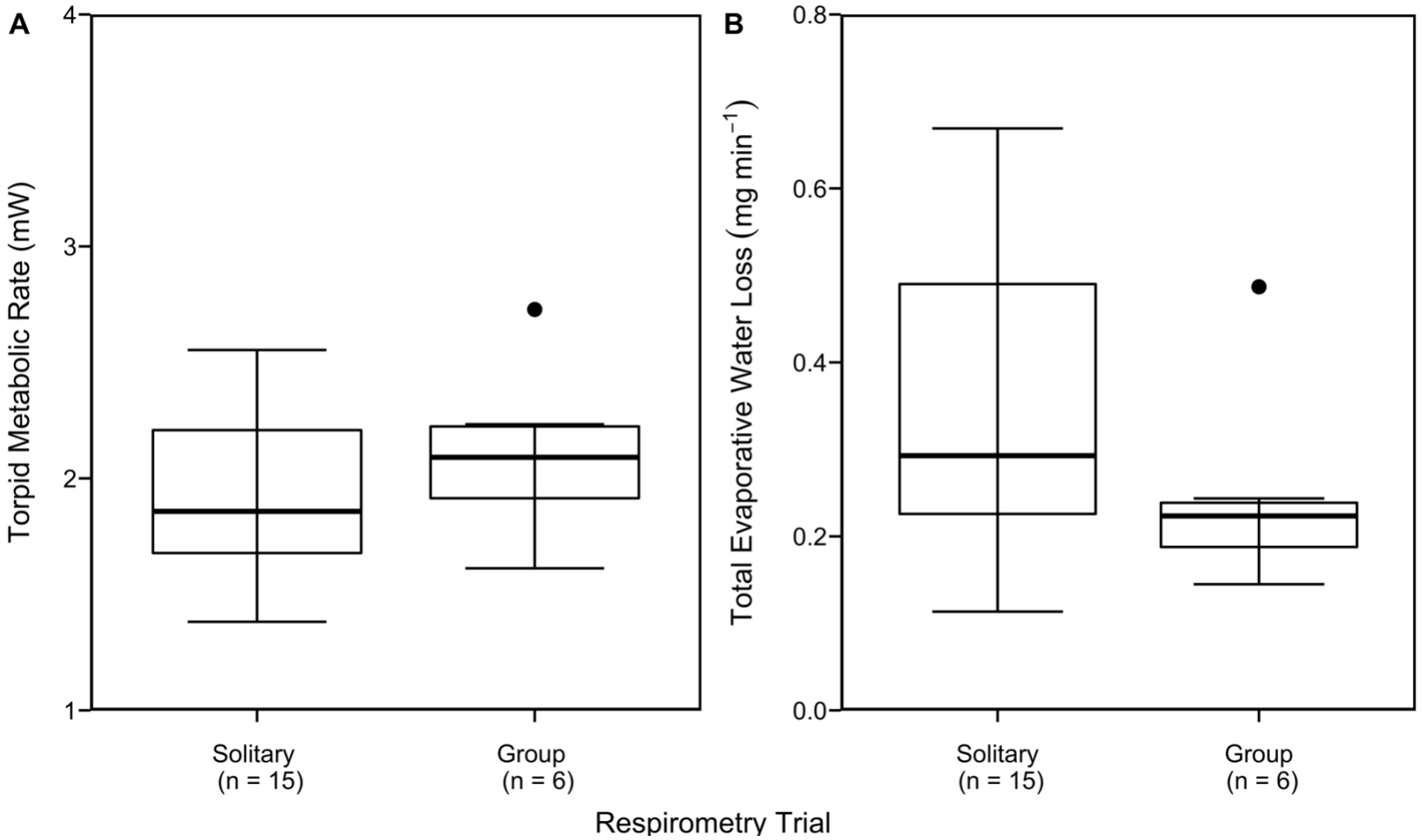

Figure 3.5: A Boxplots of torpid metabolic rate (mW) and **B** total evaporative water loss (mg min^{-1}) for big brown bats (*Eptesicus fuscus*) acclimated to humid air during hibernation. Respirometry trial (i.e., roosting solitary or huddling in a group of five) and corresponding n-values are indicated. The median is represented by a solid horizontal line, the top and bottom of each box represents the 25th and 75th percentile, respectively. Whiskers represent maximum and minimum values. Outliers are solid black dots.

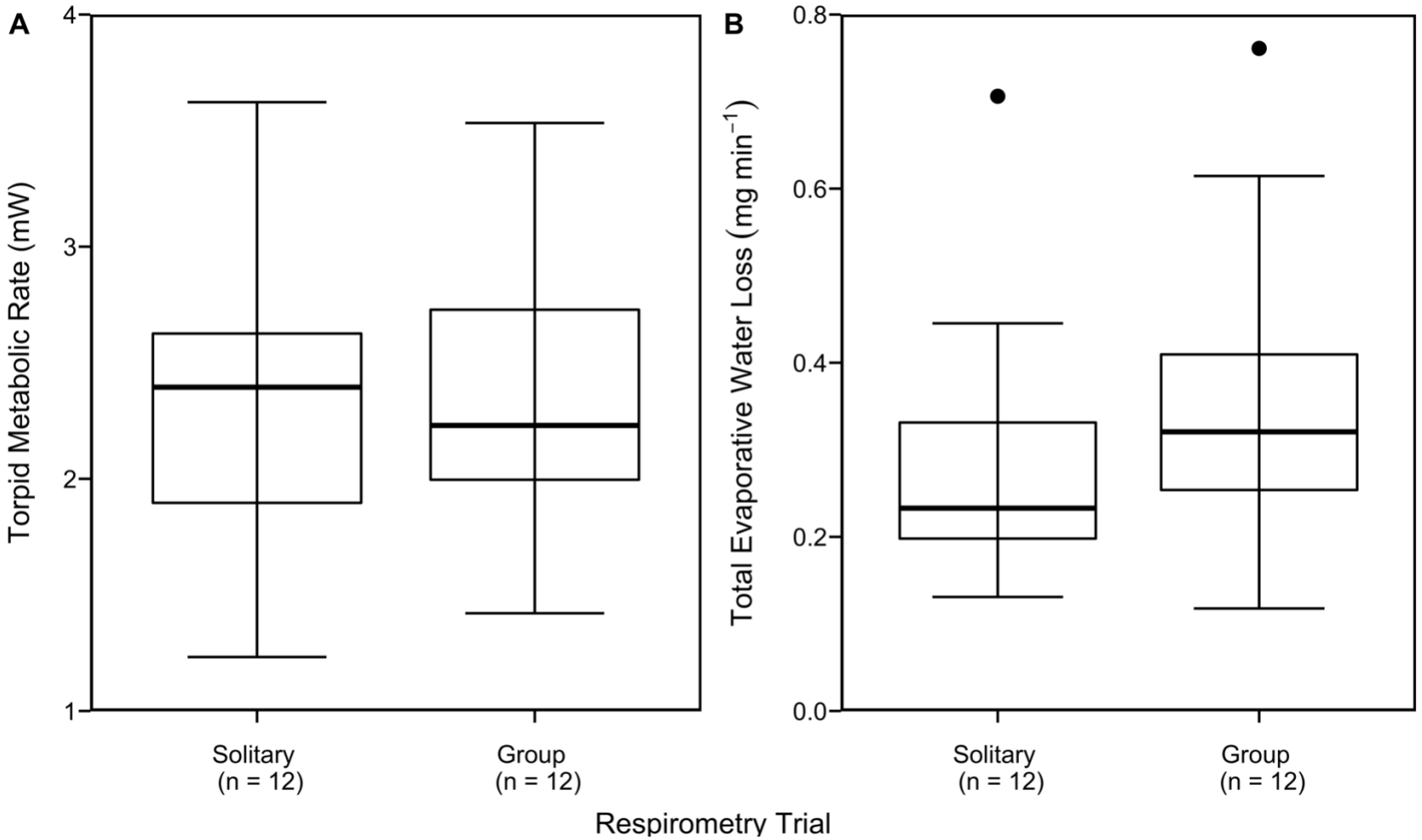

Figure 3.6: A Boxplots of torpid metabolic rate (mW) and total evaporative water loss (mg min^{-1}) for big brown bats (*Eptesicus fuscus*) acclimated to dry air during hibernation. Respirometry trial (i.e., roosting solitary or huddling in a group of five) and corresponding n-values are indicated. The median is represented by a solid horizontal line, the top and bottom of each box represents the 25th and 75th percentile, respectively. Whiskers represent maximum and minimum values. Outliers are solid black dots.

Discussion

I found support for my first hypothesis that phenotypic flexibility in total EWL helps explain the tolerance of big brown bats for a wide range of humidity. Consistent with my prediction, big brown bats acclimated to dry conditions had lower rates (17.14% decrease) of total EWL compared to humid-acclimated bats. Big brown bats are unusual in their ability to hibernate in dry conditions compared to most other North American hibernating bat species (Klüg-Baerwald and Brigham, 2017). A previous study hypothesized that water loss equivalent to 5% of body mass may be a critical threshold for some species above which individuals must arouse and restore water balance (Kallen, 1964). Thus, the ability for big brown bats to maintain water loss below this threshold in dry conditions, would reduce the need for energetically expensive arousals and allow them to exploit habitats for hibernation that are unavailable to other bat species.

Bats acclimated to dry conditions may have modified the lipid composition of their skin and flight membranes. Analysis of the lipid composition in the flight membranes of desert bats show that higher proportions of ceramides (i.e., a class of lipids) in the stratum corneum (i.e., outer layer of the epidermis) are associated with reduced cutaneous EWL (*Tadarida brasiliensis* and *Myotis velifer*; Muñoz-Garcia et al., 2012). Additionally, some desert bat species have increased concentrations of cerebrosides (i.e., compounds that consist of ceramides) which, at low or moderate ambient temperatures, can sequester water molecules, decreasing water diffusion and therefore cutaneous EWL (Ben-Hamo et al., 2016; Muñoz-Garcia et al., 2008). Big brown bats in my study could have relied on similar mechanisms during dry acclimation and I

recommend that future studies compare the lipid composition of flight membranes of big brown bats following humid and dry acclimation.

I found mixed support for my second hypothesis that the primary benefit of huddling in big brown bats is to mitigate the hygric challenges associated with dry conditions. I predicted that huddling bats acclimated to both humid and dry conditions would exhibit lower total EWL compared to solitary individuals. Consistent with my prediction, humid-acclimated big brown bats did benefit from reduced total EWL with huddling (29.21% reduction). However, there was no huddling effect for dry-acclimated bats. The absence of an effect of huddling on total EWL was unexpected and contradictory to previous studies (e.g., Boratyński et al., 2015; Brown, 1999). Potentially, bats acclimated in the *humid treatment* lacked underlying mechanisms to reduce total EWL (see previous result), and thus, would need to rely more on huddling to avoid water loss, and thus dehydration. In Chapter 2 I found that the group of 10 bats in the *dry treatment* incubator huddled in a more compact huddle throughout hibernation compared to bats in the *humid treatment* incubator. One explanation for the lack of huddling effect on total EWL for dry-acclimated bats in this chapter, then, could be variation in huddle density in the groups of five bats. It is possible that humid-acclimated bats in my respirometry trials used more compact huddles while huddling in a group of 10 to compensate for any underlying physiological mechanism that the dry-acclimated bats relied on to reduce EWL (e.g., skin lipid composition). No study to date has quantified the effect of huddle density on total EWL in any animal but presumably, bats in a more compact huddle would have lower cutaneous EWL due to the decrease in exposed surface area. My criterion for huddling in this experiment was simply that bats needed to be in contact with one another. I was not able to calculate the

density of huddles as in Chapter 2 because the camera videos were not of sufficient quality. However, for a future study, I recommend testing for an effect of huddle density on total EWL during respirometry trials. .

Consistent with my prediction, there was no difference in the torpid MR of huddling bats compared to solitary bats. Torpid MR is already less than 1% of MR at normothermic T_b (Geiser, 2004) and, while previous studies have shown that huddling can reduce energy expenditure for normothermic mammals only two studies have quantified effects of huddling on torpid MR, and neither detected an effect (Boratyński et al., 2015; Brown, 1999). Since huddling does not appear to have a direct effect on torpid MR, it may function to reduce energy expenditure of individuals rewarming to normothermic T_b's. The rewarming phase is the most energetically expensive phase during hibernation and, overall, bats are known to use up to 90% of their total energy budget during arousals (i.e., rewarming, periods of normothermia, and cooling; Haase et al., 2019; Thomas et al., 1990a), however, this estimate represents bats roosting alone. By rewarming in a cluster, bats could greatly reduce energy expenditure during hibernation (Boyles et al., 2008; Geiser, 2004). Therefore, any energetic benefit of huddling is likely not a reduction in torpid MR (as observed in this study and Boratyński et al., 2015, Brown, 1999) but rather a reduction in energy expenditure during the rewarming phase of arousals combined with a possible indirect benefit of reduced EWL which could reduce arousal frequency.

Unsurprisingly, solitary and huddling bats exposed to wet air during respirometry trials had reduced total EWL regardless of acclimation conditions. Cutaneous and respiratory EWL during torpor is dependent the on WVP of the surrounding air, with high WVP reducing total

EWL (Studier, 1970; Thomas and Cloutier, 1992). Less expectedly was higher torpid MR for solitary big brown bats under wet air (2.34 ± 0.62 mW) compared to dry air (1.91 ± 0.46 mW), and for bats acclimated to humid conditions (solitary and huddling). McGuire et al. (2017) also observed higher torpid MR under wet air for solitary little brown bats suggesting the possibility that these species express maximal torpor depth (i.e. lower torpid MR) while in dry conditions. Torpid MR was calculated from respiratory gases, and respiratory EWL correlates with MR (Muñoz-Garcia et al., 2012b; Schmidt-Nielsen, 1970). Thus, bats could decrease respiratory EWL by entering deeper torpor and decreasing torpid MR. On the other hand, for Kuhl's pipistrelles (*Pipistellus kuhlii*), bats in deep torpor had rates of cutaneous EWL similar or higher than bats in shallow torpor (Muñoz-Garcia et al., 2012b) suggesting that bats have less physiological control over cutaneous EWL while in deep torpor. All bats in this study were confirmed to be in steady-state torpor during metabolic measurements based on their T_{sk} measurements. Thus, it is unlikely that increased torpor depth led to decreased rates of torpid MR under dry air.

Boratyński et al. (2015), found that torpid MR of natterer's bats did not vary for bats under wet versus dry air. Bats in my experiment, and in McGuire et al. (2017) were placed in wet air in the evening and remained in these conditions until the first physiological measurements the following morning. The air was then switched to a dry airstream and physiological measurements were taken again (after a short period of acclimation). Boratyński et al. (2015) however, first placed natterer's bats in dry air overnight, then switched to wet air for physiological measurements in the morning, followed by a subsequent measurement period in dry air (Boratyński et al., 2015). Thus, different results between studies could reflect methodology and not necessarily the physiology of bats. For solitary and huddling bats

(regardless of acclimation), as torpid MR increased, total EWL also increased. During respirometry, all bats were in steady-state torpor (confirmed with T_{sk} measurements). Since respiratory EWL correlates with MR (Muñoz-Garcia et al., 2012b; Schmidt-Nielsen, 1970), the increase in total EWL was likely due to an increase in respiratory EWL, and not cutaneous EWL.

For humid-acclimated bats, individuals with smaller body masses had increased torpid MR although there was no effect of body mass on torpid MR for dry-acclimated bats. Bats with smaller body masses would have a larger surface area to volume ratio, which could result in higher rates of heat loss and smaller bats may also have had a smaller fat store and, therefore, less insulation and higher thermal conductance. During torpor, bats still regulate T_b often slightly above the surrounding T_a (Geiser, 2004) and, if thermal conductance of small bats was higher, they would need to increase torpid MR to maintain torpid T_b. I may have detected this effect for humid- but not dry-acclimated bats because the range of body masses for the two groups happened to vary. Body mass ranged from 16.1 – 19.7 g (a 22.4% difference) for humid acclimated bats versus 16.1 - 18.7 g (a 16.15 % difference) for dry-acclimated bats.

Overall, my study provides evidence that hibernating big brown bats chronically exposed to low humidity can acclimate to dry conditions, thereby allowing for decreased rates of total EWL. My results also suggest that big brown bats which have not acclimated to dry conditions can adjust total EWL if they have the opportunity to huddle. The ability for big brown bats to reduce total EWL through acclimation may reduce the need to huddle with conspecifics to avoid water loss and thus dehydration. Big brown bats have an unusual ability to hibernate in a range of humidities (Klüg-Baerwald and Brigham, 2017) and the ability to reduce water loss physiologically, rather than behaviourally may provide a mechanism for big brown bats to use

habitats for hibernation that are unavailable to other bat species. Additionally, the ability for big brown bats to acclimatize to dry environments during hibernation could provide additional protection from *Pd* by allowing them to select relatively dry habitat unfavourable for *Pd* growth (Klüg-Baerwald and Brigham, 2017; Langwig et al., 2012). Thus the ability for big brown bats to exhibit flexibility in total EWL can allow them to roost in dry conditions and maintain water balance, which may be an underlying mechanism in their resistance to WNS.

CHAPTER 4: GENERAL CONCLUSIONS

There has been an emergence of research into the relationship between the physiology, behaviour and water balance of hibernating bats in North America. Currently, a fungal disease called white-nose syndrome (WNS), caused by the fungus *Pseudogymnoascus destructans* (*Pd*) is devastating hibernating bat populations across North America. WNS currently affects 12 hibernating species, however previous research suggests that big brown bats (*Eptesicus fuscus*) appear resistant to the disease (Frank et al., 2014; Moore et al., 2018), although underlying mechanisms are unknown. Big brown bats appear capable of hibernating in a range of humidities (Klüg-Baerwald and Brigham, 2017), in a wide range of roosting locations such as buildings and caves, (Halsall et al., 2012; Lausen and Barclay, 2006b; Reimer et al., 2014), and roost either solitary or huddling in a group (Brack Jr. and Twente, 1985; Phillips, 2014). Due to their flexibility during hibernation, big brown bats have the ability to exhibit a wide range of physiological or behavioural responses that could impact water balance and metabolism. Therefore, research analysing hibernating big brown bats exposed to varying levels of humidity is important to begin an understanding into mechanisms of WNS resistance. The overall objective of my thesis was to identify and understand the influence of huddling and humidity on the behavioural and physiological responses of hibernating big brown bats.

In Chapter 2, I analysed the influence of ambient humidity on the huddling and drinking behaviour of hibernating big brown bats. I found that big brown bats hibernating in either a dry or humid environment maintained similar patterns of periodic arousals from hibernation and thus showed a similar loss of body mass. Interestingly, the number of arousals and experimental treatment did not have an effect of body mass loss, however, bats with the

largest body mass at the beginning of hibernation, showed the greatest loss of body mass. Without needing to arouse from torpor more frequently, bats in the dry environment drank at approximately twice the rate as compared to bats in a humid environment, likely to replace any water loss to the dehydrating environment. Bats in the dry environment exhibited shorter arousal durations which may function to reduce water loss during normothermia. Notably, bats in the dry environment huddled in a denser, more compact huddle throughout hibernation, likely to reduce the amount of surface area exposed to the dehydrating conditions. Overall, findings from Chapter 2 give insight into behavioural flexibilities that have not been observed in any species of hibernating bats. The ability for big brown bats to adjust huddling and drinking behaviour can enable this species to hibernate in dry environments, without increasing the number of energetically expensive arousals to restore water balance.

In Chapter 3, I analysed the effect of acclimation to humidity and huddling on the physiological responses of big brown bats. I used open-flow respirometry to measure the torpid metabolic rate (MR) and total evaporative water loss (EWL) of big brown bats roosting solitary or huddling in a group of five. Solitary big brown bats acclimated to dry conditions during hibernation showed reduced rates of total EWL, compared to bats acclimated to humid conditions. While the underlying mechanism is unknown, by acclimating to dry conditions during hibernation, big brown bats can maintain water balance, rather than spending large amounts of energy to rewarm from torpor and drink.

Consistent with my prediction, for bats acclimated to humid conditions, huddling bats had decreased rates of total EWL, compared to solitary bats. However, for bats acclimated to dry conditions, there was no difference in rates of total EWL between huddling and solitary

bats. As predicted, for bats acclimated to humid and dry conditions, there was no difference in torpid MR between huddling and solitary bats. This provided further evidence that the main benefit of huddling during torpor is to reduce total EWL and not to provide a direct energetic benefit for individuals.

My results from Chapter 3 suggest that big brown bats have phenotypic flexibility in total EWL that help explain their tolerance to dry conditions. However, in the absence of acclimation to dry conditions, big brown bats may rely on behavioural mechanisms (i.e., huddling) to decrease rates of total EWL and prevent dehydration from occurring. The ability to decrease total EWL either behaviourally or physiologically, may be underlying mechanism that allow big brown bats to exploit habitats for hibernation that are unavailable to other bat species.

Overall, the ability for hibernating big brown bats to conserve water balance, either physiologically or behaviourally, without increasing energy expenditure in dehydrating conditions may play a role in their resistance to WNS. Previous studies have suggested that big brown bats are resistant to WNS (Frank et al., 2014; Moore et al., 2018). However, new research has identified population declines estimated at 35% (credible interval 13 – 54%), with about 32% of the geographic range affected by WNS (Cheng et al., 2021). Big brown bat population declines were highly variable and overall were lower, compared to WNS-susceptible species (Cheng et al., 2021). As WNS continues to spread across North America, conservation efforts should aim to ensure population declines of big brown bats do not continue or worsen. An understanding into the behaviour and physiology of big brown bats in variable environments can be incorporated into management decisions. Big brown bats are known to hibernate in

caves and anthropogenic structures (Halsall et al., 2012; Lausen and Barclay, 2006b; Reimer et al., 2014). Thus, understanding big brown bats tolerance to dry conditions and importantly, their roosting habitats can allow for greater insight into characterizing overwintering roosts. Conservation efforts can focus to protect critical habitats for big brown bats to prevent further population declines due to WNS.

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APPENDIX

Supplementary Figures and Tables for Chapter 2

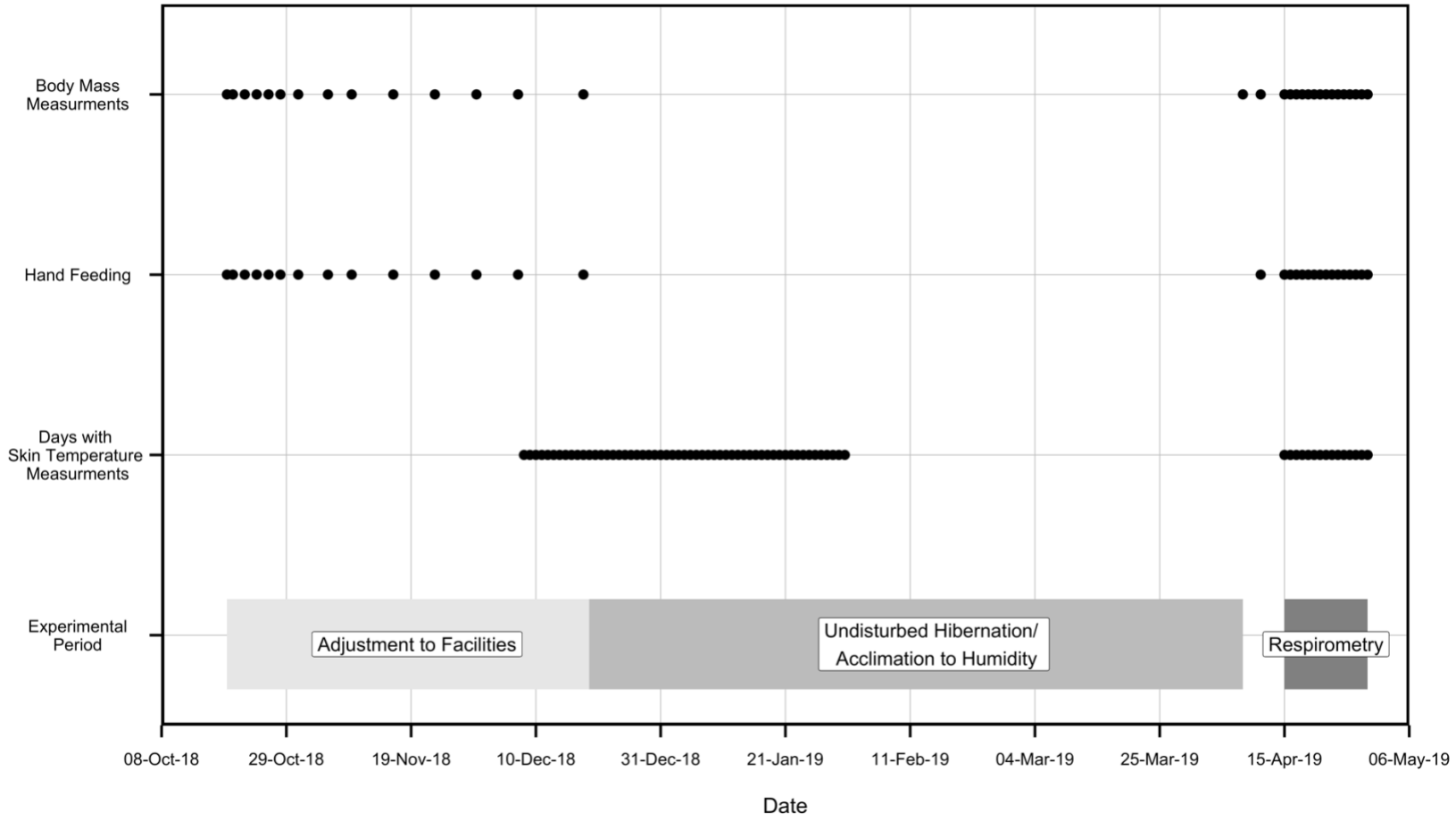

Figure S.1: Timeline of studies presented in Chapter 2 and 3 (total duration = 193 days) after the big brown bats (*Eptesicus fuscus*; n = 20) arrived at the University of Winnipeg. Solid dots represent days where bats either had body mass measurements taken, were hand fed mealworms, or had ibuttons attached that recorded skin temperature measurements. Solid bars indicate the experimental period that bats were undergoing.

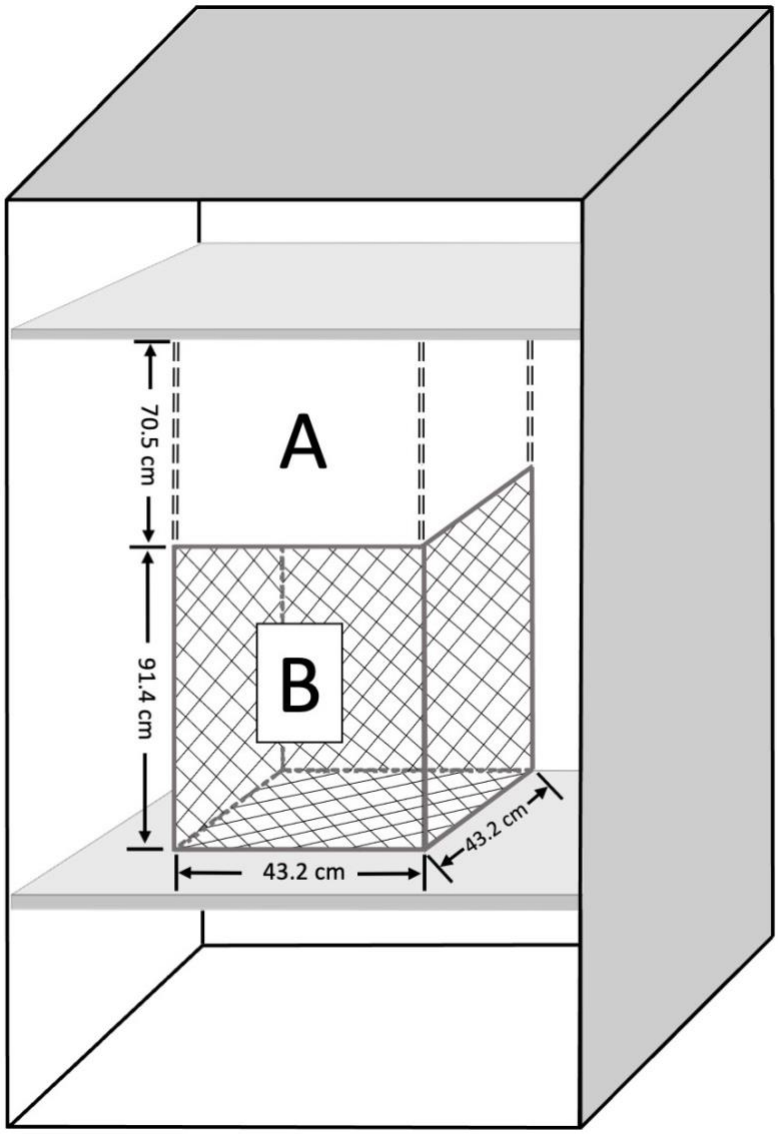

Figure S.2: Schematic diagram of one temperature and humidity-controlled incubator used to house 10 big brown bats (*Eptesicus fuscus*) during hibernation. **A** indicates the layer of plastic used to prevent bats from crawling out of view from the IR camera (not pictured) situated on the ceiling of the cage, facing downward. **B** indicates the custom-built nylon mesh cages which bats were housed in. Cage and plastic dimensions are shown but are not to scale.

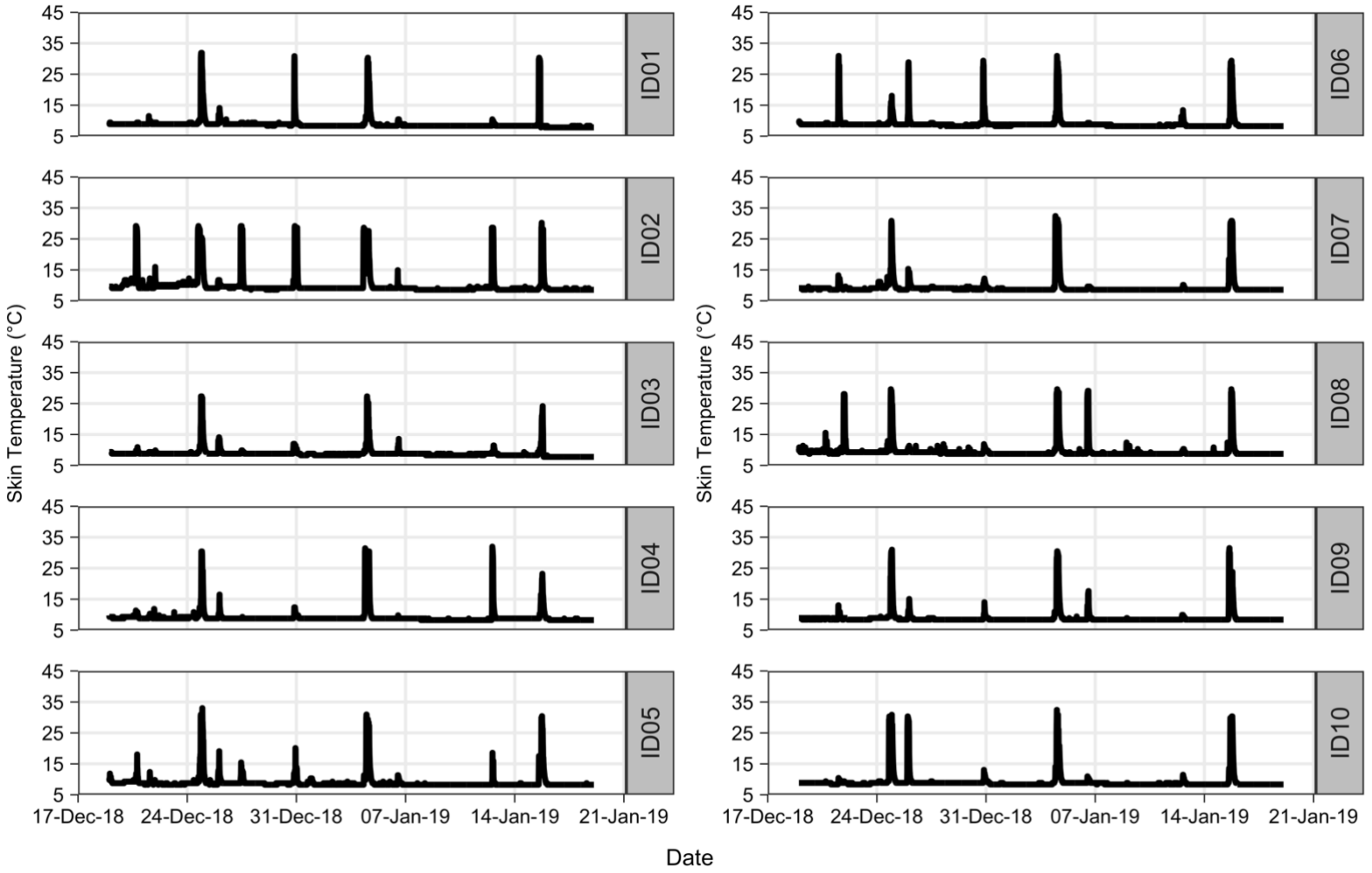

Figure S.3: Skin temperature traces (°C) of hibernating big brown bats (*Eptesicus fuscus*, n = 10) from the *humid treatment* from 18 December 2018 to 19 January 2019 (31 days of the 110-day study period). Bat ID is indicated on the right of each individual graph.

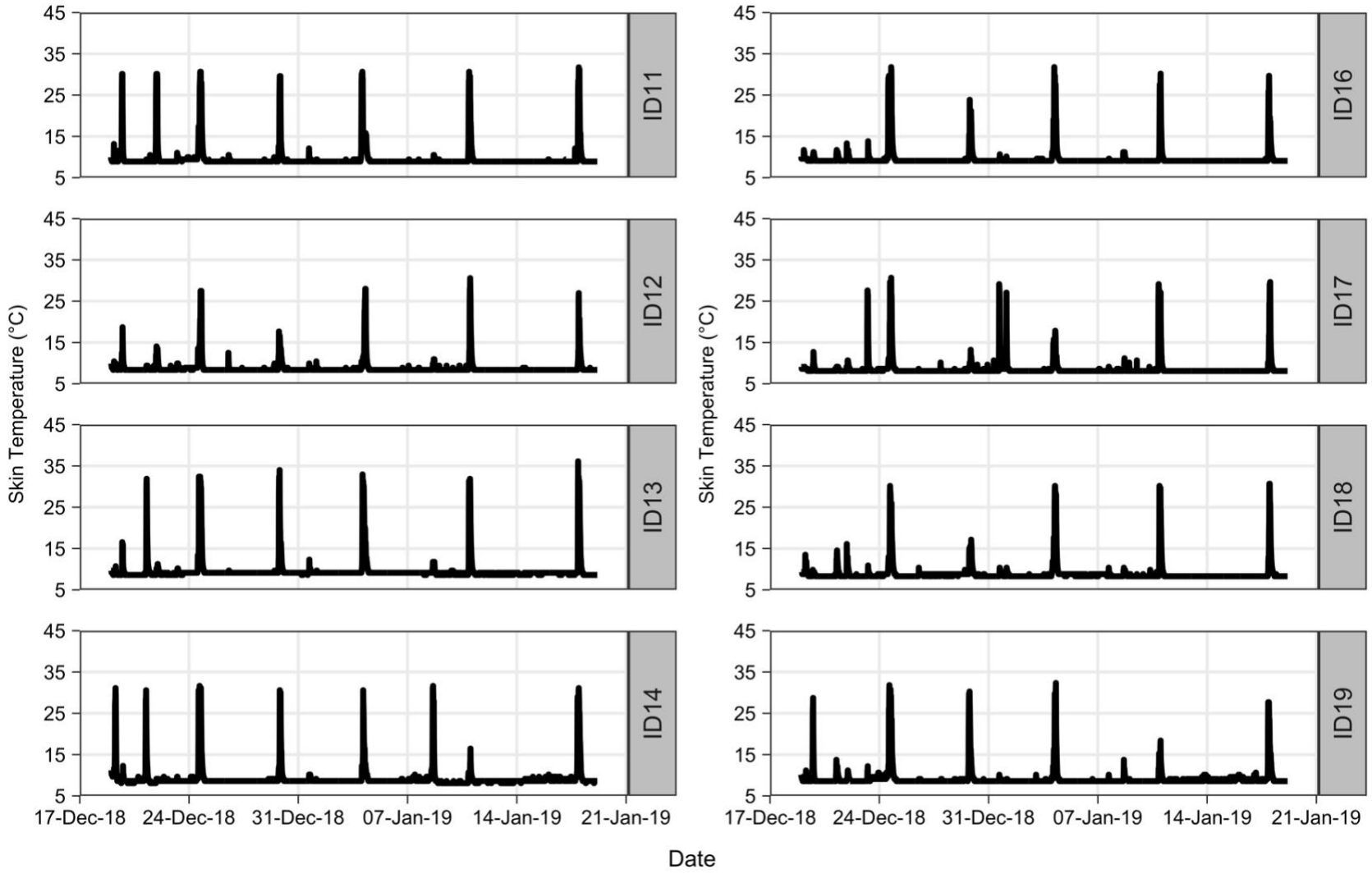

Figure S.4: Skin temperature traces (°C) of hibernating big brown bats (*Eptesicus fuscus*, n = 8) from the *dry treatment* from 18 December 2018 to 19 January 2019 (31 days of the 110-day study period). Bat ID is indicated on the right of each individual graph.

Table S.1: Summary of Bat ID, experimental treatment and date and time which skin temperature dataloggers dropped off for hibernating big brown bats (*Eptesicus fuscu*s; n = 6) during the 110-day study period.

Bat ID	Experimental Treatment	Date and time
ID01*	*Humid*	15 January 2019 15:18
ID03*	*Humid*	15 January 2019 19:40
ID05	*Humid*	21 March 2019 16:44
ID10	*Humid*	02 April 2019 14:15
ID12	*Dry*	01 March 2019 03:35
ID14	*Dry*	16 March 2019 04:27

*dataloggers dropped off bats before recording ended.

Supplementary Figures for Chapter 3

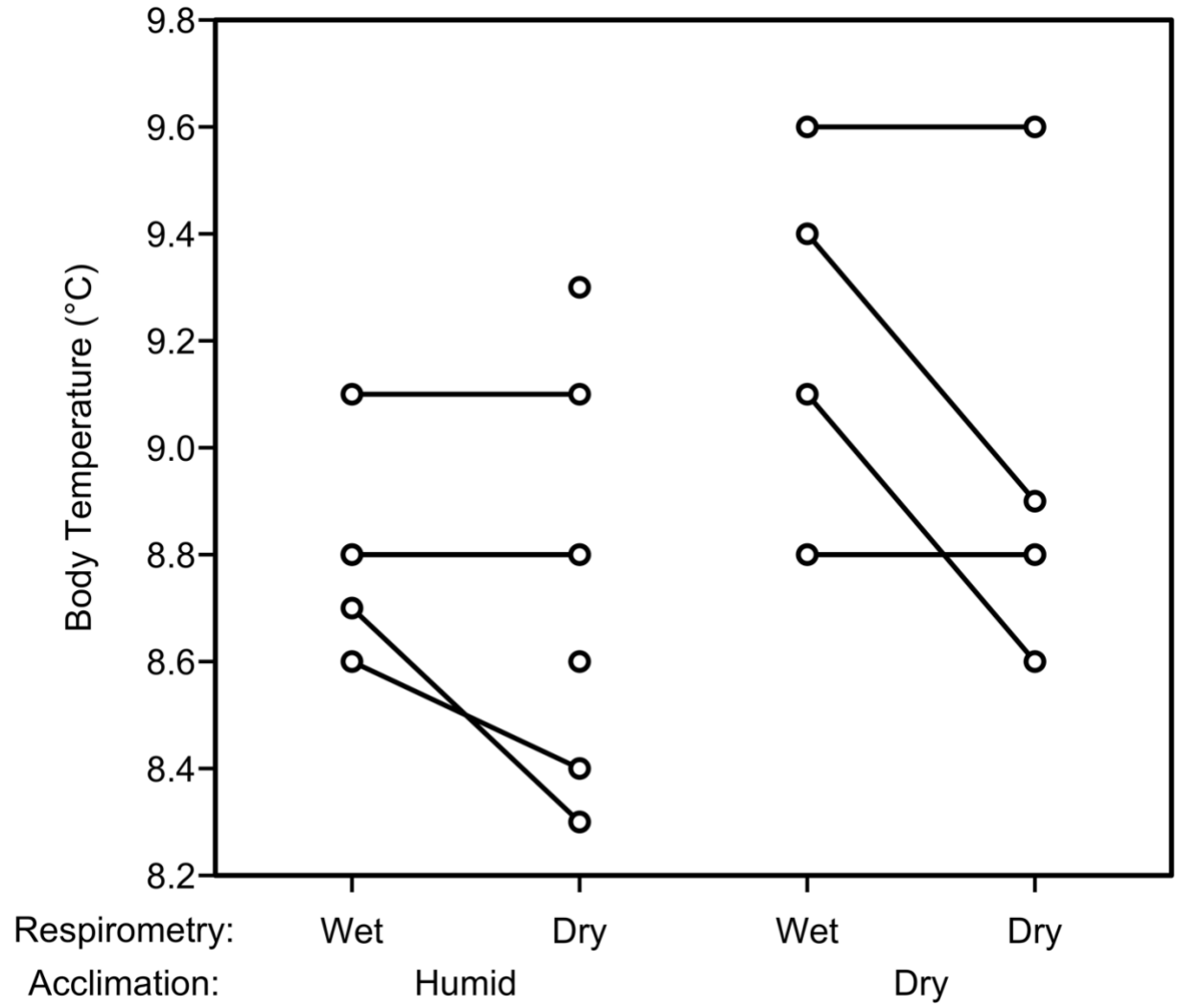

Figure S.5: Paired scatterplot of body temperature (°C) for solitary big brown bats (Eptesicus fuscus) acclimated to the *humid treatment* and measured under wet (n = 6) or dry air (n = 9) and from bats acclimated in the *dry treatment* and measured under wet (n = 5) or dry air (n = 5). Note that three bats in the humid treatment incubator and two bats in the dry treatment incubator all maintained T_{sk} of 8.8°C so those points are obscured on the graph.

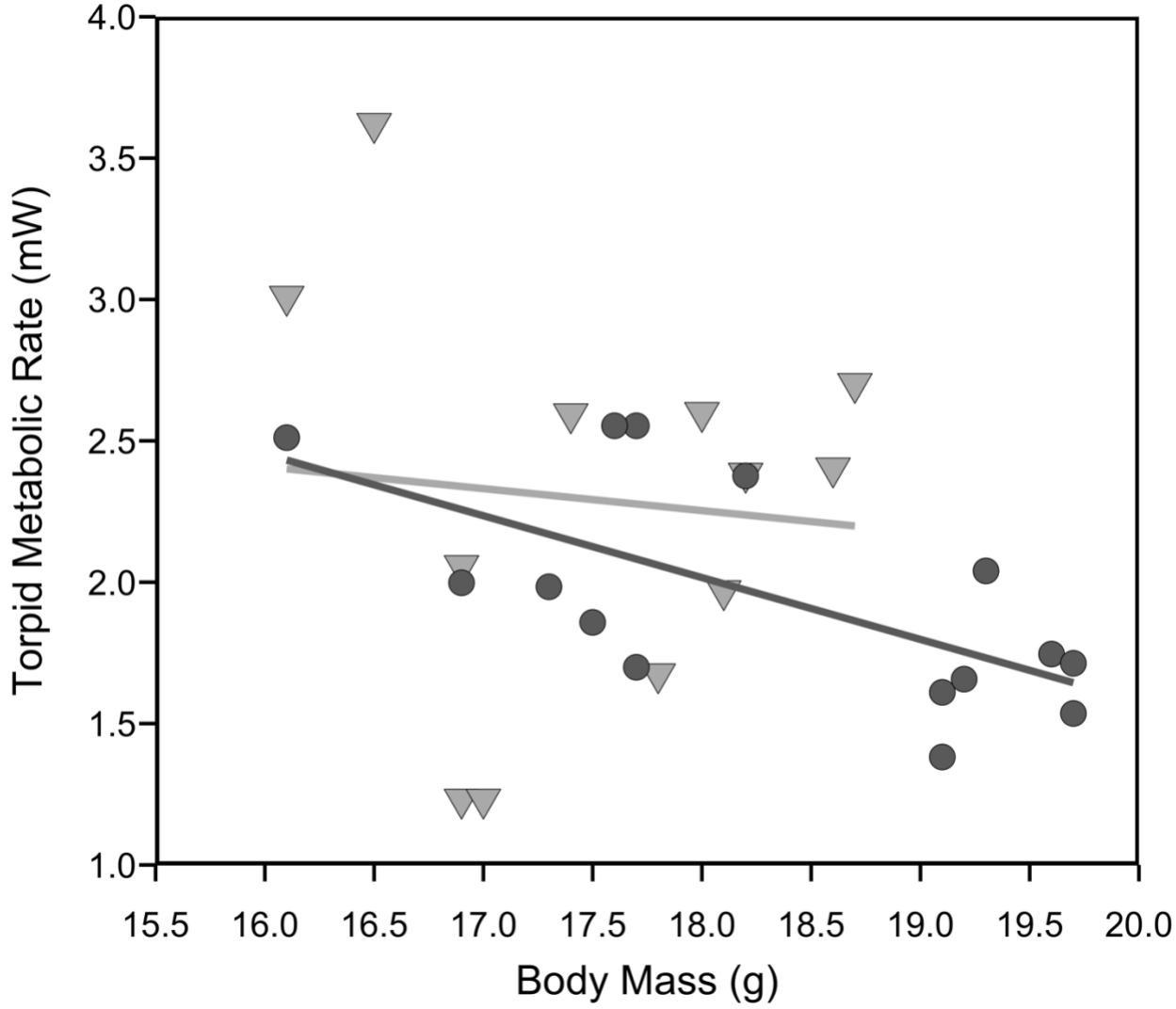

Figure S.6: Relationships between the body mass (g) and torpid metabolic rate (mW) of solitary big brown bats (*Eptesicus fuscus*). Dark grey circles represent bats acclimated during the *humid treatment* with the dark grey line representing the relationship between the two variables (R^2 = 0.66, n = 15). The light grey triangles represent bats acclimated during the *dry treatment*, with the light grey line representing the relationship between the variables (R^2 = 0.28, n = 12).

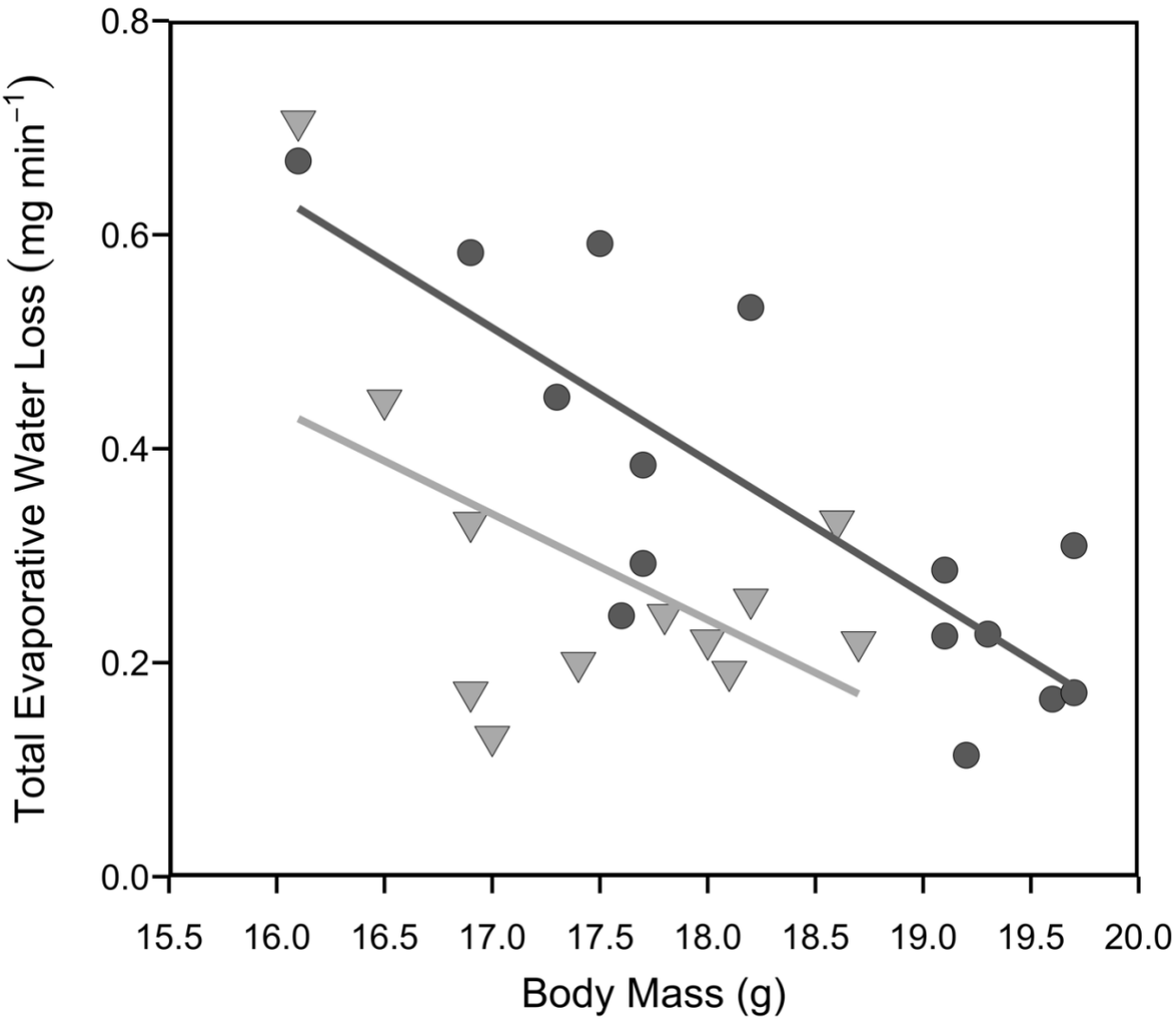

Figure S.7: Negative relationships between the body mass (g) and total evaporative water loss (mg min^{-1}) of solitary big brown bats (*Eptesicus fuscus*). Dark grey circles represent bats acclimated during the *humid treatment* with the dark grey line representing the relationship between the two variables (R^2 = 0.66, n = 15). The light grey triangles represent bats acclimated during the *dry treatment*, with the light grey line representing the relationship between the variables (R^2 = 0.28, n = 12).

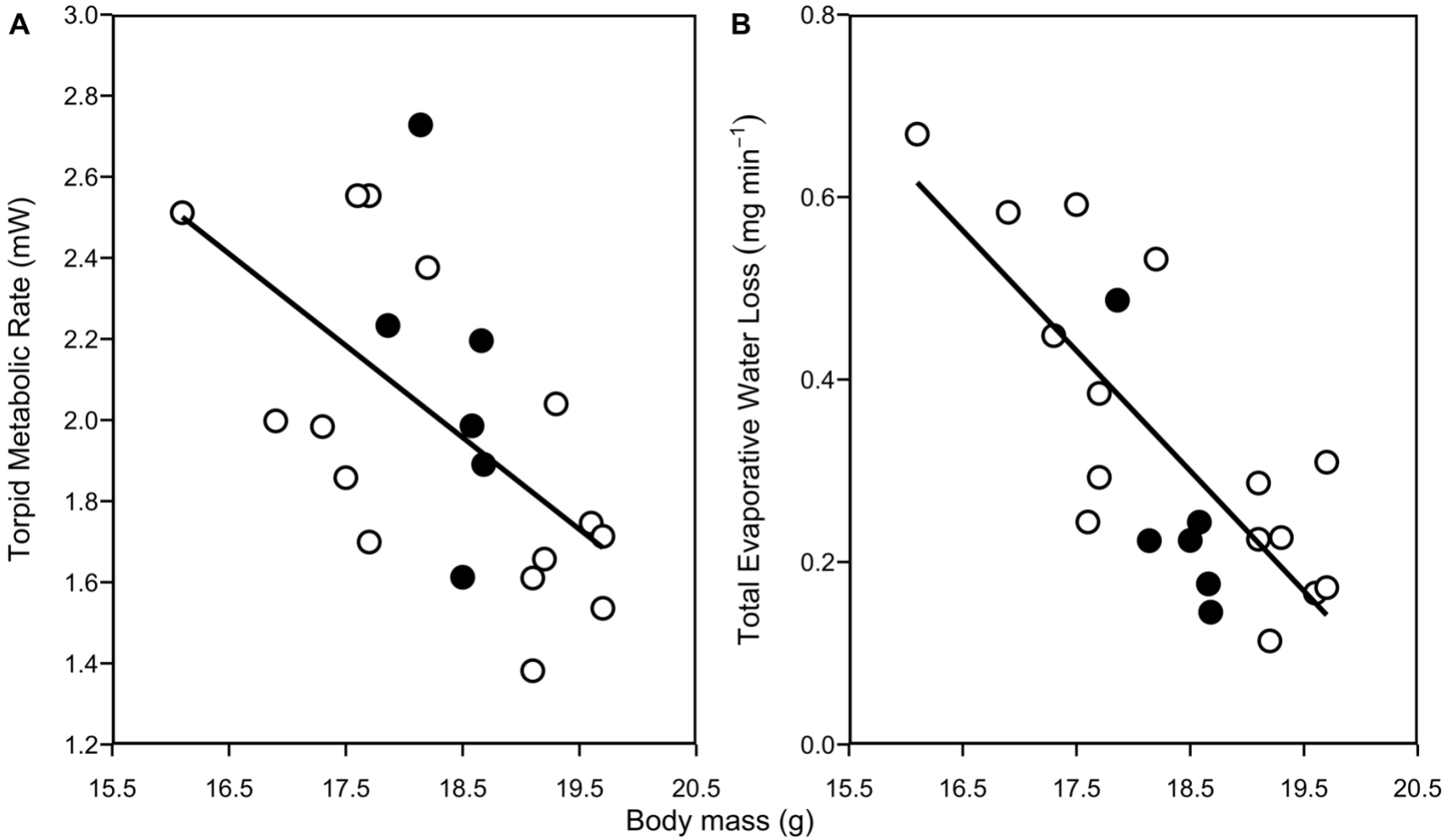

Figure S.8: A Scatterplots of the relationship between body mass (g) and torpid metabolic rate (mW) (R^2 = 0.33, solitary: n = 15; huddling: n = 6) for big brown bats (*Eptesicus fuscus*) acclimated in the *humid treatment*. **B** Scatterplot of the relationship between body mass (g) and total evaporative water loss (mg min^{-1}) (R^2 = 0.60, solitary: n = 15; huddling: n = 6) for big brown bats acclimated in the *humid treatment*. Filled circles represent the average body mass measurement for five bats huddling in a group whereas open circles represent body mass measurements from solitary bats. Black lines represent relationships between variables.

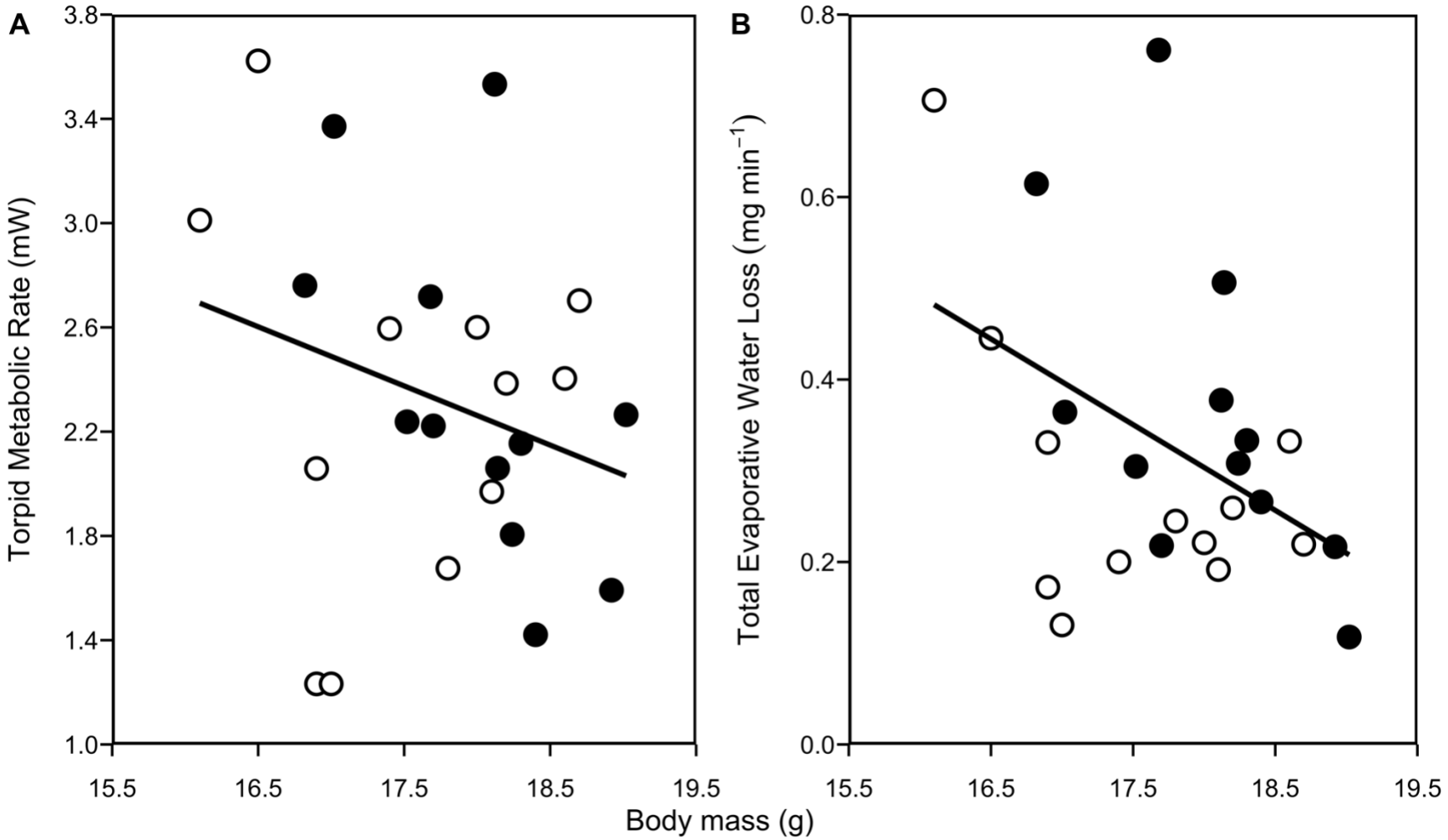

Figure S.9: A Scatterplots of the relationship between body mass (g) and torpid metabolic rate (mW) (R^2 = 0.07, solitary: n = 12; huddling: n = 12) for big brown bats (*Eptesicus fuscus*) acclimated in the *dry treatment*. **B** Scatterplot of the relationship between body mass (g) and total evaporative water loss (mg min^{-1}) (R^2 = 0.19, solitary: n = 12; huddling: n = 12) for big brown bats acclimated in the *dry treatment*. Filled circles represent the average body mass measurement for five bats huddling in a group whereas open circles represent body mass measurements from solitary bats. Black lines represent relationships between variables.