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Behavioural microclimate selection and physiological responses to environmental conditions in a hibernating bat

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Abstract

Hibernators adjust the expression of torpor behaviourally and physiologically to balance the benefits of energy conservation in hibernation against the physiological and ecological costs. Small fat-storing species, like many cave-hibernating bats, have long been thought to be highly constrained in their expression of hibernation because they must survive winter relying only on endogenous energy stores. We evaluated behavioural microclimate selection in tri-colored bats (*Perimyotis subflavus* (Cuvier, 1832)) across a three-month hibernation experiment under laboratory conditions. We also opportunistically tested for evidence of acclimatization in torpid metabolic rate (TMR). When given access to gradients in microclimate, bats tended to choose the warmest temperature available (11°C) while almost completely avoiding the driest condition available (85% relative humidity at 8°C). Further, bats held at different temperatures over the course of the hibernation showed no differences in TMR when measured under common conditions at the end of hibernation. Taken together, our results suggest selective pressures to conserve energy during hibernation are not overwhelmingly strong and further support the proposition that optimal expression of hibernation is something less than the maximal expression of hibernation unless the animal is nearing starvation.

Keywords: microclimate, optimal hibernation, *Perimyotis subflavus*, torpor, tri-colored bats

Introduction

Hibernation was long thought to be almost exclusively motivated by an overarching need to save energy (and sometimes water) during seasonal periods of low energy availability. This idea led to a proliferation of studies aimed at determining the environmental conditions necessary to maximize energy conservation (Nagel and Nagel 1991), and the idea of a cold optimal temperature for hibernation dominated the field for decades. The “colder is better” hypothesis (Johnson et al. 2021) became, in effect, the ruling hypothesis in the field, driving explanations for variance in microclimate selection. For example, when confronted with data that hibernating bats often choose temperatures other than the temperature that minimizes energy expenditure, Twente (1955) postulated that bats preferred cold temperatures during hibernation, but that finding the correct temperature is a matter of trial-and-error. Further field studies since have made it clear that an “energy-first” paradigm of hibernation, like the colder-is-better hypothesis proposes, does an exceedingly poor job of predicting the expression of hibernation (Boyles et al. 2007; Wojciechowski et al. 2007). Instead, it now appears the expression of hibernation is defined by a series of trade-offs (Humphries et al. 2003a; Humphries et al. 2003b). Energy conservation must be balanced against physiological and ecological costs of deep torpor, leading to an optimal expression of hibernation that is something less than maximal expression of hibernation in most individuals that are not near starvation (French 2000; Humphries et al. 2003b; Boyles et al. 2020).

There are several mechanisms through which a hibernating mammal can alter how they express hibernation. Hibernating mammals can make coarse adjustments to the expression of hibernation by choosing microclimates with different temperatures or air moisture. Choosing microclimates warmer than those that minimize torpid metabolic rate (TMR) is common in some

species (Boyles et al. 2007; Wojciechowski et al. 2007; Boyles et al. 2017). Hibernators might also choose locations with different microclimates throughout the winter (Daan and Wichers 1968; Ransome 1968), likely to adjust energy expenditure or water loss as the relative costs and benefits of torpor change through time. While theoretically possible, but completely unexplored, hibernators might change arousal frequency by selecting hibernating sites with differing levels of air moisture, as arousal frequency is likely related to evaporative water loss (Thomas and Cloutier 1992; Thomas and Geiser 1997). Bats remain inactive during torpor bouts so behavioural adjustments can only be made during euthermic arousals, and only when different microclimates are available to choose from. Therefore behavioural adjustments are necessarily crude (Twente 1955).

Physiological adjustments potentially allow hibernators the opportunity to fine-tune the expression of hibernation by defending body temperatures (T_b) above ambient temperature (T_a) or by adjusting metabolic expenditure when maintaining a given T_b at a given T_a (Heldmaier and Ruf 1992). Torpid metabolic rates (TMR) or evaporative water loss (EWL) might also shift over time to maximize energetic efficiency or minimize water loss under the prevailing conditions of the hibernaculum (in effect, acclimatization in TMR or EWL) if either energy or water conservation impart a strong selective pressure on hibernating mammals. In other words, hibernators might have adapted to acclimatize to varied conditions. Such acclimatization could occur either through shifts in metabolic functioning (at cellular or tissue levels) or shifts in minimum setpoint temperatures during hibernation. Under this scenario, hibernating mammals would essentially adjust energetic expenditure or water loss to match environmental conditions. Conversely, if the benefit of maximizing torpor to conserve energy or water is opposed by physiological (e.g., sleep deprivation) and ecological costs (e.g., predation) or if the

physiological rates in question are largely determined by biophysical constraints, we would expect hibernators to display little ability to acclimatize to their chosen microclimate. If the non-energetic costs of hibernation are strong enough, minimizing metabolic expenditure (thereby maximizing the expression of hibernation) through acclimatization of TMR might even be counterproductive.

We collected behavioural and physiological (TMR and EWL) data from tri-colored bats (*Perimyotis subflavus* (Cuvier, 1832)) maintained in captivity under 9 different environmental regimes, some with the ability to choose different microclimates throughout hibernation, some held at constant conditions. Field studies have provided evidence that bats shift among microclimates throughout winter, but no studies have thoroughly detailed the movements of individual bats with high temporal resolution. Thus, in the subset of bats that could select microclimates, we tracked movements throughout the course of the experiment to detail behavioural adjustments to the expression of hibernation. We predicted bats with less fat at the beginning of the experiment would choose colder and higher humidity microclimates (resulting in lower torpid metabolic rate and less frequent arousals), while bats with more fat would choose warmer and perhaps drier microclimates. We also opportunistically collected TMR and EWL data under common conditions at the end of a 3-month hibernation period. The main goal of the experiment was to study the effects of microclimate on disease progression and severity of white-nose syndrome (McGuire et al. 2021), but the experimental design allowed an opportunity to test for the possibility that hibernating conditions affect physiological rates as well. We predicted that if shifts in TMR or EWL occur intraseasonally, individuals that hibernated for three months at the measurement temperature (8°C) would have the lowest TMR, followed by individuals that hibernated below the measurement temperature and individuals that hibernated

above the measurement temperature. If EWL changes over the course of a season of hibernation, we would predict the bats that hibernated in the driest conditions would exhibit the lowest EWL during measurements taken in dry air.

Materials and Methods

Animal Collection and Captive Hibernation

In early December 2018 we collected 98 hibernating tri-colored bats from highway culverts in Mississippi (approximately 32°N 91°W; exact locations withheld to protect sensitive sites). We immediately transported bats in individual cloth bags to Texas Tech University. All fieldwork followed decontamination guidelines to prevent the spread of white-nose syndrome (United States Fish and Wildlife Service 2018). Upon arrival at Texas Tech University, we recorded body mass (± 0.1 g), measured body composition by quantitative magnetic resonance, and inoculated bats with *Pseudogymnoascus destructans* (Blehert & Gargas) Minnis & D. L. Lindner following standard methods (details in McGuire et al. 2021). Importantly, few bats developed or even displayed characteristic symptoms of white-nose syndrome (Frick et al. *In Review*), and it appears experimental inoculation of *P. destructans* likely had little effect on the physiology of bats in this experiment (McGuire et al. 2021).

Bats were assigned to one of nine environmental chambers, each set to maintain different temperature and humidity conditions (**Figure 1**; Caron, Model 7000-33-1, Marietta, OH, USA). We stratified by body mass to ensure similar body mass and sex ratio in each treatment (body mass and sex were confounded because females were larger than males). We housed bats in cages constructed from mesh fabric (Part FMLF, Seattle Fabrics, Inc., Seattle, WA, USA), PVC pipe, and plastic sheeting (McGuire et al. 2017). Seven treatments confined bats to fixed

temperature and humidity conditions ($n = 9 - 11$ bats per treatment) while two treatments ($n = 14$ per treatment) allowed bats to freely move among temperature or humidity conditions (**Figure 1; Supplementary Video 1**). We monitored bats throughout hibernation with motion-sensitive infrared video cameras in each cage. Fresh water was provided *ad libitum* in a dish on the floor of each cage, with a tube running outside the environmental chamber to allow us to provide fresh water weekly without disturbing the bats during hibernation.

We programmed environmental chambers to maintain one of three temperatures (5, 8, or 11°C) and one of three potential evaporative water loss (pEWL) conditions (low pEWL [high RH] = 0.05 kPa, medium pEWL = 0.10 kPa, high pEWL [low RH] = 0.15 kPa). For the two treatments in which bats could select microclimate, cages in adjacent environmental chambers were connected by short (~45cm) PVC pipes, allowing bats to move among environmental chambers (**Supplementary Video 1**). One group of bats ($n = 14$) was free to move among three chambers with a common temperature (8°C) but different humidity (pEWL = 0.05, 0.1, or 0.15, corresponding to 85, 90, and 95% RH). A second group of bats ($n = 14$) was free to move among three chambers with a common pEWL condition (0.1 kPa) but different temperatures (5, 8, or 11°C). At the beginning of the experiment, we placed bats into the center chamber and observed bats freely exploring among the three chambers to which they had access, confirming bats were aware of the adjacent chambers and willingly moved through the PVC pipe connecting the chambers.

Respirometry

After 83 days of hibernation, over a period of four days, we removed bats from their cages and used open-flow respirometry to measure torpid metabolic rate (TMR) and evaporative water loss

(EWL) at 8°C. We selected a subset of seven bats from each treatment for respirometry measurement, prioritizing individuals with the greatest body mass to avoid bats potentially on the cusp of starvation. We placed bats in individual chambers (350 mL glass jars), with mineral oil in the bottom to prevent bat excretions from biasing water vapor pressure (WVP) measurements. We fitted each chamber with 0.2 µm in-line air filters (Cole Parmer, #15945-42) to prevent *P. destructans* contamination.

We initially maintained flow rate (Flowbar FB-8, Sable Systems) at ~300 mL min⁻¹ and reduced flow rate to ~120 mL min⁻¹ when individuals entered torpor. We used two respirometry systems (FMS Field Metabolic System, Sable Systems) with multiplexers (RM-8, Sable Systems) to cycle among seven animal chambers and a baseline channel (14 individuals per trial) at three-minute intervals. Incurrent air was scrubbed with Drierite to remove water vapor, soda lime to remove CO₂, and magnesium perchlorate to remove additional water vapor released by the soda lime. We allowed bats to settle in respirometry chambers for approximately eight hours, followed by approximately four hours of measurement. Excurrent air was subsampled at approximately 75 mL min⁻¹ for measurement of water vapor pressure and CO₂.

We processed all respirometry recordings in Expedata (v1.9.13, Sable Systems). After correcting for lag and drift, we used equation 10.5 from Lighton (2008) to calculate $\dot{V}CO_2$ and equation 10.9 to calculate evaporative water loss. We used the oxyjoule equivalent method to convert $\dot{V}CO_2$ to metabolic rate in mW, assuming fat oxidation (respiratory quotient = 0.7). We visually inspected all metabolic rate data and excluded values with TMR > 3 mW because these bats were assumed to not be torpid at the time of measurement (**Figure S1**).

All fieldwork and experiments were approved by the Texas Tech University Institutional Animal Care and Use Committee (protocol 18032-12) and conducted under permission from the

Mississippi Department of Wildlife, Fisheries, and Parks (permit 1115181). All research followed best practices for decontamination and preventing spread of *P. destructans*, as recommended by the United States Fish and Wildlife Service.

Statistical Analysis

Among the bats that were able to move among conditions, we quantified the number of days each bat spent at each condition. We used Chi-squared tests to determine whether bats spent a disproportionate amount of time at certain conditions. We used linear regression to determine whether fat mass at the start of hibernation was related to the proportion of time spent at the warmest temperature or the lowest pEWL condition, and used a t-test to determine whether time spent at these conditions differed between males and females. The relatively small sample size ($n = 14$) combined with little variation in movement patterns among individuals precluded more complex statistical analysis. Therefore, we present qualitative descriptions of movements among treatments rather than quantitative analysis.

We tested for differences in TMR and EWL among treatments using analysis of covariance (ANCOVA), including body mass as a covariate. We further tested for specific effects of either temperature or humidity by conducting separate ANCOVA models that treated temperature or humidity (respectively) as a factor (low, med, high), including body mass as a covariate. For the temperature model, bats in the treatment that allowed behavioural selection of temperature were excluded, and likewise bats in the treatment that allowed behavioural selection of humidity were excluded from the humidity model. All statistical analysis was completed with R statistical software (R Core Team 2020)(v3.6.3; R Core Team 2020).

Results

Bats allowed to move among chambers with different microclimatic conditions were not randomly distributed among temperature treatments ($\chi^2 = 614.82$, $df = 2$, $p < 0.0001$) with most observed bat-days (659/935 bat-days = 70%) spent at the warmest temperature (11°C). One female remained at 5°C for the entire experiment, but seven bats spent a majority ($\geq 89\%$) or all of the winter in the warmest of the three chambers (11°C; **Figure 2**). Among males, the three individuals with the most fat at the beginning of the experiment also spent the most time in the 11°C chamber, while three of the four males with the least fat died part way through the experiment with each moving to a colder temperature before death.

Bats moved more across the humidity gradient than the temperature gradient (**Figure 3**). As for the temperature gradient, bats were not randomly distributed among water loss conditions ($\chi^2 = 331.5$, $df = 2$, $p < 0.0001$). Only four of the fourteen bats spent time in the low humidity chamber at any point during the experiment, and the periods spent in that chamber were relatively short, accounting for only 7% of bat-days in the experiment. All 14 bats spent most or all of the experiment in chambers with mid or high humidity. The proportion of hibernation spent at the lowest pEWL condition (i.e., highest humidity) was not related to initial fat mass ($F_{1,12} = 0.44$, $p = 0.52$) or to sex ($t_{5,2} = -0.47$, $p = 0.66$).

Including bats from all treatments, and after excluding bats that did not settle into torpor during respirometry measurements, there were 46 bats remaining for analysis of TMR and EWL. Body mass was not related to TMR or EWL in any analysis (all $p > 0.38$; **Supplementary Figure S1**). There was no effect of treatment on either TMR ($F_{8,36} = 1.30$, $p = 0.27$) or EWL ($F_{8,36} = 0.78$, $p = 0.62$). Excluding the bats that were free to move among temperature conditions, neither TMR ($F_{2,34} = 3.17$, $p = 0.055$; **Figure 4a**) nor EWL ($F_{2,34} = 0.71$, $p = 0.50$; **Figure 4c**)

varied among hibernation temperature treatments. Similarly, excluding bats that were free to move among water loss conditions, there was no evidence for acclimatization to pEWL conditions during hibernation in either TMR ($F_{2,37} = 0.28$, $p = 0.75$; **Figure 4b**) or EWL ($F_{2,37} = 0.16$, $p = 0.85$; **Figure 4d**).

Discussion

Both the behavioural and physiological patterns described in this study serve as further evidence that maximizing energy conservation is not the exclusive determinant of the expression of hibernation (Humphries et al. 2003b). Most of the tri-colored bats given the opportunity to select ambient temperature in which to hibernate explored the chambers, and then ultimately settled in the warmest temperatures available (similar to the observations of Wojciechowski et al. 2007). Conversely, bats allowed to choose hibernation locations based on air moisture tended to avoid the chamber with the lowest humidity (85% RH at 8°C) and therefore the highest potential evaporative water loss. If bats selected conditions based solely on minimizing energetic expenditure, they should have chosen the coldest temperature and highest humidity (lowest potential evaporative water loss)(Thomas and Cloutier 1992). In this experiment, tri-colored bats did not seek to minimize energy expenditure based on temperature (preference for warmer conditions) but did avoid conditions associated with highest water loss (which might be associated with increased arousal frequency and therefore energy expenditure), at least under the conditions available in this experiment. While anecdotal, the three bats that died in these treatments all moved to colder temperatures shortly before death (**Figure 2**). This indicates energy conservation became more important as their energy state worsened, and they attempted to maximize their expression of hibernation in response. This is the same response seen in

hibernating bats afflicted with white-nose syndrome in the wild (Johnson et al. 2016) and in the lab (Moore et al. 2018).

Tri-colored bats in our experiment had similar torpid metabolic rates (TMR) and evaporative water loss (EWL) under common conditions despite being housed in different microclimates for nearly three months. The lack of a divergence in TMR after three months of hibernation at different temperatures would suggest animals did not acclimatize and were not defending different T_b - T_a differentials during the respirometry measurements. One interpretation is that simple biophysics render the relationship between temperature and TMR relatively invariant within a species. Conversely, the lack of variation among individuals might mean that late in the experiment, when we took these measurements, most of the individuals were nearing the point of starvation. A further disturbance associated with moving the animals to respirometry measurements could have exacerbated this stress. If so, minimizing the T_b - T_a differential would maximize energy conservation by taking advantage of Q_{10} or thermoregulatory effects on metabolic rates (Heldmaier and Ruf 1992).

That only 7 of the 98 bats in this experiment died during the three-month hibernation period contradicts the longstanding belief that hibernating bats are on an energetic knife-edge between survival and death (Thomas et al. 1990; Boyles 2017). Among bats that were allowed to behaviourally select a preferred microclimate, there was clear preference for the warmest condition, and avoidance of the driest condition. Even bats held in warm and dry conditions throughout winter, with no opportunity to forage, were able to successfully hibernate for the duration of the experiment, demonstrating plasticity in hibernation behaviour and physiology beyond that historically assumed. The accumulated evidence is becoming clear that for most

individual bats, the optimal expression of hibernation is something less than the maximal expression of hibernation and varies across the season of hibernation.

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Competing Interests Statement

The authors declare there are no competing interests.

Contributors' Statement

JGB, WFF, and LPM designed the study and secured funding. JGB, LPM, EMJ, NWF, and KAS cared for animals and collected data. JGB, LPM, EMJ, and LH analysed and interpreted data. JGB and LPM drafted the manuscript, with editorial comments from all authors.

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Figure Legends

Figure 1. (left) Our experiment consisted of seven environmental chambers with tri-colored bats (*Perimyotis subflavus*) at fixed conditions of temperature and humidity, and two treatments where bats could move freely either among the humidity conditions (same temperature), or three temperature conditions (same potential evaporative water loss). **(right)** A screen shot of hibernating bats from the overhead infrared camera in an environmental chamber at 11°C and 92% relative humidity (potential evaporative water loss = 0.1 kPa). A water dish is visible on the floor and several bats are hibernating along the walls of the cage. This cage was part of the connected cages that allowed bats to move among temperature conditions, and on the left a bat can be seen entering the cage, with the corresponding movement indicated by an arrow in the left panel. See Supplementary Video 1.

Figure 2. One treatment of tri-colored bats (*Perimyotis subflavus*) was free to move among three environmental chambers all with the same potential evaporative water loss (pEWL = 0.1 kPa) but different temperatures ($T_a = 5, 8, \text{ or } 11^\circ\text{C}$). The first days of hibernation when bats moved frequently among chambers are not illustrated here. Most bats spent most or all of the hibernation period at the warmest temperature (top two rows). Only one bat spent the winter at the coldest temperature, and the three males with the least amount fat all moved from warmer to colder temperatures before death (bottom row).

Figure 3. One treatment of tri-colored bats (*Perimyotis subflavus*) was free to move among three environmental chambers all with the same temperature ($T_a = 8^\circ\text{C}$) but different potential

evaporative water loss conditions ($p_{EWL} = 0.05, 0.1, \text{ or } 0.15 \text{ kPa}$, corresponding to 95, 90, and 85% relative humidity). The first days of hibernation when bats moved frequently among chambers are not illustrated here. Most bats spent the winter at either the low or medium potential evaporative water loss condition (top two rows). Only four bats (bottom row) spent any time in the driest condition.

Figure 4: After approximately 3 months of hibernation, we measured torpid metabolic rate and evaporative water loss of tri-colored bats (*Perimyotis subflavus*). There was no evidence for acclimatization to hibernation conditions in either value.

Draft

Figure 1

292x201mm (96 x 96 DPI)

Figure 2

304x152mm (300 x 300 DPI)

Figure 3

304x152mm (300 x 300 DPI)

Figure 4

152x152mm (300 x 300 DPI)