4 **ABSTRACT**

5 Torpor is an important energy saving strategy in some small birds, but it has rarely been
6 studied in natural field conditions. We compared torpor use across 43 wild-caught individuals of
7 eight hummingbird species across sites with different natural temperature regimes. Most
8 laboratory studies focus on the relationship between metabolic rate and temperature, but our aim
9 was to evaluate what environmental factors most influence hummingbird nighttime energy
10 management under natural conditions. We found that the probability of an individual entering
11 torpor was weakly correlated with mass but unrelated to nighttime temperature and that
12 hummingbirds at both warm, tropical and cooler, temperate sites used torpor. Energy savings in
13 torpor were maximized as ambient temperatures approached a species' minimum body
14 temperature, consistent with laboratory studies; energy savings ranged between 65-92% of energy
15 per hour in torpor compared to normothermy. However, regardless of the degree of energy
16 savings in torpor, variation in total nighttime energy expenditure was most significantly
17 influenced by torpor bout duration. Lab studies largely assess the effect of temperature on torpor
18 use, but our findings indicate that other environmental conditions are more important in
19 determining hummingbirds' total nighttime energy expenditure under natural temperature cycles.
20 Our results show that a small endotherm's nighttime energy management in its natural habitat is
21 more affected by torpor bout duration, which is linked to photoperiod, than by temperature. This
22 result suggests that in their natural environments hummingbirds are able to save energy in torpor
23 across a range of nighttime temperatures, indicating that they may have sufficient physiological
24 flexibility to tolerate climatic variation.

25 **KEYWORDS:** adaptive thermoregulation, avian torpor, Arizona, comparative physiology, Ecuador,
26 tropics

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INTRODUCTION

Daily torpor is an important strategy for energy management in more than 40 bird species (Ruf & Geiser, 2015). In deep, daily torpor, an animal lowers its body temperature (T_b) down to a minimum T_b (sub-zero to 30°C) as ambient temperature (T_a) decreases to a temperature equal to or below minimum T_b (Geiser, Currie, Shea, & Hiebert, 2014; Kruger, Prinzinger, & Schuchmann, 1982; Reinertsen, 1996; Richter et al., 2015; Shankar et al. In revision). Torpor allows an animal to save energy by lowering the costs of regulated heat production (Hainsworth & Wolf, 1970; Ruf & Geiser, 2015; Fig. 1). Controlled laboratory studies tend to focus on the relationships between T_a , T_b , and metabolic rate in torpor. These studies provide significant insight into the energy savings gained from torpor but tend to focus on a single environmental factor, while in the wild multiple environmental factors (temperature, photoperiod, resource availability, etc.) can vary simultaneously (Boyles, Seebacher, Smit, & McKechnie, 2011). Further, the influence of natural energy use and storage is not normally considered (Powers, Brown, & Van Hook, 2003). When animals experience the discrete temperature steps used in laboratory studies, they might not use torpor as they would under relatively predictable natural temperature cycles. As a result, laboratory studies may not always provide a comprehensive picture of torpor under natural conditions. Here we evaluate daily torpor use in eight species of hummingbirds, assessing the factors influencing nighttime energy savings under natural temperatures and photoperiods at both temperate and tropical sites.

Figure 1: Theoretical relationship between energy expenditure and ambient temperature in

an endotherm. The solid lines depict normothermic parts of the Scholander-Irving curve (Scholander, Hock, Walters, Johnson, & Irving, 1950), and dashed lines represent torpid regions (adapted from data in Hainsworth and Wolf (1970) and this study). Energy expenditure in torpor can drop down until minimum body temperature ($\text{min } T_b$) is reached, after which energy must be expended (dotted line) to maintain $\text{min } T_b$.

Hummingbirds, which are among the smallest vertebrate endotherms, are recognized for their ability to use daily torpor to reduce their nighttime energy expenditure (Kruger et al., 1982; NEE; Pearson, 1950; Schuchmann, Kruger, & Prinzinger, 1983). Laboratory studies have provided a clear understanding of the individual relationships between torpor use and the depression of T_b , the metabolic defence of minimum T_b (Carpenter, 1974; Hainsworth & Wolf, 1970; Wolf & Hainsworth, 1972), and endogenous energy stores (Hiebert, 1992; Powers et al., 2003). Laboratory measurements of metabolic rates in torpor indicate that torpid hummingbirds maximize energy savings when $T_a - T_b$ is minimized and they are at their minimum T_b (Hainsworth & Wolf, 1970; Jastroch et al., 2016). Minimum T_b varies by species (6.5-18°C,

63 Hainsworth and Wolf 1970, Carpenter 1974), suggesting that it has an adaptive role, with higher
64 min T_b in animals that experience higher T_a . However, because laboratory studies generally
65 manipulate temperature artificially to evaluate the impact of temperature on torpor metabolism
66 (Kruger et al., 1982; Lasiewski, 1963; Powers et al., 2003), patterns of T_b management might not
67 reflect torpor use under natural T_a cycles, which are generally more predictable because T_a tends
68 to decline gradually through the night, as well as across seasons. In addition, laboratory
69 measurements do not always incorporate the natural patterns of energy storage in free-living birds
70 (Auer, Bassar, Salin, & Metcalfe, 2016), which can affect whether or not torpor is used
71 (Hainsworth, Collins, & Wolf, 1977; Powers et al., 2003). Laboratory measurements of torpor can
72 also underestimate torpor bout duration and reduction of T_b (Geiser et al., 2000). Many captive
73 animals will use torpor under field conditions but not under laboratory conditions (Geiser et al.,
74 2000). A few studies have measured torpor while incorporating some of the natural conditions
75 hummingbirds experience. For instance, body temperatures of hummingbirds in torpor under
76 natural temperature cycles have been measured, but the influence of natural energy stores and
77 photoperiod on torpor were not considered (Bech, Abe, Steffensen, Berger, & Bicudo, 1997).
78 Torpor use has also been measured on hummingbirds that naturally acquired energy stores, but
79 the influence of photoperiod or temperature cycles were not simultaneously evaluated (Powers et
80 al., 2003). Thus, it is unknown whether hummingbird nighttime energy savings are most
81 influenced by torpor bout duration after reaching an energy storage threshold (Hainsworth et al.,
82 1977; Hiebert, 1992), by T_a approaching minimum T_b , or both (Geiser, 1988). Our approach
83 minimizes the potential shortcomings of laboratory studies because individuals are exposed to
84 natural conditions. We simultaneously evaluate the effects of temperature, night length, and
85 energy stores on torpor use.

Three major hypotheses have thus far been used to explain patterns of hummingbird torpor use. Under the emergency-only hypothesis (Hainsworth et al., 1977), hummingbirds use torpor only when energy stores are below some critical threshold. This threshold is usually low, though it may be higher during migration when birds maximize fat gain (Carpenter & Hixon, 1988). Under the routine hypothesis (Kruger et al., 1982), hummingbirds use torpor every night, regardless of energy stores or day length. Under the circadian rhythm hypothesis (Carpenter, 1974), torpor is an innate circadian response to day length and season. The relative importance of these three hypotheses might be influenced by food availability, mass, degree of territoriality, and T_a. First, sufficient food is required to maintain an energy buffer to survive the night and support morning foraging, as hummingbirds appear to have an endogenous energy storage threshold that triggers torpor when crop storage and fat are depleted (Bicudo, 1996; Calder, 1994; Hiebert, 1992; Powers et al., 2003). Second, a species' degree of territoriality and size can be linked to its fat storage patterns: the territorial blue-throated mountain-gems (*Lampornis clemenciae*, ca. 8g), appeared to only use torpor in emergencies because it actively defends resources and can access and store energy for nighttime use (Powers et al., 2003). In contrast, non-territorial species in similar environments do not have easy access to resources and might use torpor more routinely (Hainsworth, 1978; Hainsworth et al., 1977; King, 1970; Powers & Wethington, 1999). For example, non-territorial Rivoli's hummingbirds (*Eugenes fulgens*, ca. 7.5g) and black-chinned hummingbirds (*Archilocus alexandri*, ca. 3g) enter torpor regularly (Calder, 1994; Powers et al., 2003).

We evaluated patterns of torpor use in wild-caught hummingbirds that naturally acquired energy through the day at several sites under natural nighttime temperatures and photoperiods. We measured their nighttime energy expenditure by temporarily keeping them in chambers

exposed to natural temperature and light cycles. We also evaluated the role of minimum T_b and torpor duration in total nighttime energy savings, asking whether nighttime energy savings (through lowering T_b) and duration differed between sites with shorter, cooler nights vs. those with longer, warmer nights. We predicted that, as nighttime temperatures got colder or hotter than minimum T_b , hourly energy savings in torpor would decrease while total NEE would increase. Thus, we predicted that temperature would have the largest effect on NEE, a prediction we tested in eight hummingbird species across five sites in North and South America. If true, climatic shifts in nighttime T_a could impact hummingbirds' ability to fine-tune their energy budget.

METHODS

Study sites and species

We collected data at five sites: three in Arizona, USA and two on the eastern slope of the western Ecuadorian Andes. The Arizona sites were Harshaw, Sonoita, and Southwestern Research Station. The Ecuador sites were Maquipucuna and Santa Lucia. We studied adult individuals from eight species (body masses ranging 3.2 – 9.6g; summarized in Table 1) and used only males to avoid potential disturbances to nesting. These birds represent a variety of foraging strategies, including territorialists, generalists, and trap-liners (Feinsinger & Colwell, 1978). Birds in Arizona were more habituated to using feeders than the Ecuadorian birds (given the numbers of birds at feeders and feeder history). The Arizona birds were studied before migratory season, during the breeding season; it is unknown when the breeding season for the Ecuador birds is. The Arizona birds were also known long-range migrants, while the Ecuadorian species are likely elevational migrants but not distance ones.

Temperature

We measured site-level ambient temperature (T_a) using iButtons from Maxim Integrated

(DS1922-L50 in Arizona and DS1920 in Ecuador) and Hobo H8 temperature loggers (Onset Corp.) in Arizona. These loggers have a resolution of $\pm 0.5^{\circ}\text{C}$. The temperature loggers were placed in insulated containers (i.e., inverted Styrofoam cups or inverted plastic grill casings) 1 meter above the ground to insulate them from solar radiation and wind, and to minimize the effects of re-radiated heat from the ground. Loggers were spread across each study site with an average of 8 loggers per site, representing a range of thermal conditions within the site.

Internal chamber temperatures (T_c) were measured to the nearest 0.1°C continuously during torpor measurements using type-T (Cu-Cn) thermocouples with a TC-1000 thermocouple reader (Sable Systems) in Arizona and thermistor probes connected to a Field Metabolism System (FMS; Sable Systems) in Ecuador. We used T_c to check how closely temperatures experienced by the birds in the chamber approximated T_a . All temperature devices were calibrated using a Percival temperature control system (model I-36VL). The temperature of the Percival was checked against a thermometer traceable to the National Institute of Standards and Technology.

Torpor measurement

We used modified Russell traps and mist nets to capture birds about a half hour before sunset to allow for near-natural daytime activities and feeding regimens for storage of endogenous energy. Birds were either banded or uniquely marked on the forehead with non-toxic nail polish to avoid repeated measurements on the same bird. After being trapped, each bird was placed into a cage (1 m³ in Arizona and 0.75m diameter x 0.75m height in Ecuador) for 15-30 minutes (until sunset) where it could feed *ad libitum* on 20% sucrose (mass/volume) to store sugar solution in its crop prior to roosting in the metabolism chamber. This was done to simulate the bird's natural practice of excessive evening foraging or hyperphagia prior to roosting for the night (W.A. Calder, Calder, & Frazier, 1990; Powers et al., 2003). From the cage, birds were transferred to

plastic metabolism chambers (AZ 10.0 litre, 23.5 x 19.5 x 22.0 cm; Ecuador 6.0 litre, 26 x 18 x 15 cm). We used large torpor chambers to accommodate a perch and a feeder base containing 20% sucrose solution, allowing birds to feed *ad libitum* upon waking in the morning. These hummingbirds did not feed in the dark, but occasionally fed at the end of the night, around sunrise (*pers. obs. A. Shankar*). The chambers were placed outdoors inside open ice chests (internal dimensions 50.8 x 33.7 x 29.8 cm) to expose birds to natural temperature and light cycles while insulating them from disturbance. We used negative-pressure open-flow respirometry to measure nighttime metabolism and track the use of nocturnal torpor (Powers et al. 2003, Welch 2011). Airflow through the chamber was drawn through three 0.6 cm holes along the lower back surface, allowing the chamber to essentially serve as a large mask (Welch, 2011). The negative-pressure, “mask” design of our system and placement of the bird’s perch near the chamber’s air outlet tube resulted in initial equilibration times of ~8 minutes (when fractional O₂ readings stabilised)—much less than the equilibration times typically associated with a positive-pressure, large chamber design (See Supplement S1: Fig. S1 for equilibration times).

Mass-flow rate through the torpor chambers in Arizona was 800 ml/min and was controlled using a Mass Flow System (MFS-2; Sable Systems) pump. The flow meter was calibrated using a bubble meter (Lighton, 2019). Chamber air was discharged into a syringe barrel, where it was sampled for gas analysis at 300 ml/min using a mass-flow controlled sub-sampling pump (SS4 or FOXBOX; Sable Systems). The sub-sampling pump pushed the air through a humidity sensor (RH-100, Sable Systems or a LI-COR LI-6262 CO₂/water Analyser), which measured water vapour pressure (kPa). At Harshaw and Sonoita we scrubbed the sampled air of water (Drierite) and CO₂ (Ascarite or Decarbite) after it passed through the humidity sensor. Finally, we measured oxygen content of the sampled air (O₂, ml/min) using an Oxzilla O₂

178 Analyser (Sable Systems), allowing us to calculate rates of O₂ consumption (VO₂, ml/min). We
179 baselined using outside air every 30 minutes to correct for drift in the oxygen sensor. We
180 calibrated the humidity sensor using nitrogen gas as the zero-span, and spanned using a bubbler
181 set to produce humidified air at a constant dewpoint between 21-23°C (Lighton, 2019). In
182 Ecuador, mass-flow rate through the torpor chamber was 1000 ml/min (Cole Parmer,
183 vacuum/press pump, 1.2CFM) controlled by a mass flow controller (Alicat MC-10SLPM-D/5M,
184 0 - 10 SLPM). The air was then discharged into a syringe barrel from which a Field Metabolic
185 System (FMS; Sable Systems) sampled at 400 ml/min. The FMS then measured water vapour
186 pressure (kPa) and VO₂ (ml/min) in the air. We measured water vapour pressure, VO₂, flow rate,
187 and chamber temperature (T_c) continuously throughout the nocturnal fast and baselined using
188 outside air every hour. We recorded all data using Warthog Lab Helper Software
189 (warthog.ucr.edu).

190 To analyse the torpor data from the Southwestern Research Station and Ecuador, flow rate
191 was corrected to dry flow by subtracting the contributions of water to the measured flow rate;
192 because errors due to the presence of CO₂ are small, it was omitted from the calculation (Lighton,
193 2019; p. 85). In Ecuador, rather than scrubbing the sub-sampled air of water vapour, we
194 mathematically corrected our values of fractional gas composition (Lighton, 2019; p. 85). We
195 obtained VO₂ (ml/min) values for analysis during times when the bird was in equilibrium.
196 Because the birds rarely showed stable VO₂ values until the end of the first hour of
197 measurements, we monitored their VO₂ until it stabilised, and then used the stable VO₂ values at
198 the end of the hour (which was always before torpor entry) as the value for the entire first hour. If
199 we captured a bird after sunset (such that we could not obtain a full night's data) but our
200 measurement included both entry into and exit from torpor, average normothermic values from

the remainder of the night were used to scale total nighttime energy to a full night's measurement.

Even though we used natural temperature patterns and photoperiods, we cannot rule out the possible effects of capture, handling and behavioural differences between species, which could affect patterns of torpor use in these birds. However, we believe that our methods better approximate what hummingbirds experience in the wild than standard laboratory protocols. We assumed birds entered into torpor when metabolic rate fell below resting normothermic values (a minimum of 0.4 O₂ mL/min change, and an average of 1.1 mL O₂/min in 30-90 minutes; Hiebert, 1990; Powers et al., 2003). We terminated measurements about 30 minutes after rewarming ended but no earlier than 30 minutes prior to sunrise (Calder, 1975) and allowed birds to feed in holding cages for 30 minutes prior to release. We calculated rewarming costs by integrating the area under the curve starting when VO₂ began to steadily increase and ending when VO₂ values peaked as the birds stopped actively increasing their metabolism (Bartholomew & Lighton, 1986; Fig. S2).

Laboratory torpor measurements

For broad-billed hummingbirds (n=15), we additionally measured energy expenditure under controlled laboratory conditions from 5-30°C in decreasing 5°C increments in the laboratory to test the effect of temperature on torpid energy expenditure in a controlled setting, as well as to compare laboratory and field measurements. We used a setup similar to the Arizona setup described above but placed the bird in a chamber in the lab (5.7 x 15.6 x 5.7 cm) and controlled the temperature using a Peltier device (Pelt-3; Sable Systems). These individuals were different from the individuals used in the field study. Birds were allowed to acclimatize to each temperature as needed to obtain steady-state metabolic rates before we started recording measurements, with each bird allowed to acclimatize for at least 30 minutes. We used VO₂ measurements once they had stabilized for at least 10 minutes, and analysed the files as below for

both field and laboratory measurements.

Torpor analyses

Torpor data were processed using Warthog Lab Analyst software (warthog.ucr.edu). We obtained VO₂ values for analysis during times when the bird exhibited steady-state metabolic rate. Our VO₂ measurements were converted to energy expended in kJ/hour and summed over the night to find nighttime energy expenditure (NEE; kJ). To convert VO₂ to energy units, we assumed a respiratory exchange ratio (RER) of 1 (i.e. an energy substrate of carbohydrates) for the first two hours of night, and 0.71 (energy substrate of fats) for the remaining hours (Eberts, Dick, & Welch, 2019). We calculated the oxyjoule equivalent as: $\text{J mL O}_2^{-1} = 16 + 5.164 \cdot \text{RER}$ (Lighton, 2019).

A standardized set of measures to compare torpor use between species is lacking in the literature, though these measures have previously been used independently. We calculated the following four measures to evaluate torpor use.

1. *Frequency of torpor* is the percentage of individuals that entered torpor per species (Powers et al., 2003). Each individual was only kept for one night.
2. *Duration* is the average number of hours (to the nearest half-hour) a species used torpor per night, including torpor entry and excluding rewarming (i.e. when body temperature was relatively passively regulated). To accommodate variation in available night length, we also report the percentage of nighttime hours spent in torpor.
3. *Hourly energy savings* is the percentage of energy saved in torpor relative to normothermy for an individual and is useful for comparing across sites with variable night lengths. This measure has also been referred to as “relative torpor metabolic rate” (Ruf & Geiser, 2015) and “percentage energy expenditures” (Bucher & Chappell, 1997; Schuchmann et al.,

1983). Torpor and normothermy values were obtained by averaging hourly torpid and normothermic energy expenditure over the entire night for each individual. Note that all individuals spent at least part of the night normothermic (i.e., before torpor entry and after exiting) allowing us to obtain normothermic values for torpid individuals.

$$\text{Hourly torpid energy savings} = \left(1 - \frac{\text{Mean hourly torpid energy expenditure}}{\text{Mean hourly normothermic energy expenditure}}\right) * 100$$

4. *Total nighttime energy expenditure* is per individual nighttime energy expenditure (Lighton, 2019).

Other factors that could influence NEE and torpor use in free-living hummingbirds that we could not account for include capture stress, energetic history, species/individual coping styles to handling, and life history stage.

Bird mass

We weighed each individual three times (using a Sartorius Practum 100 x 0.001g in Arizona and a US Balance ACE 100 x 0.01g in Ecuador) to obtain a capture mass, a fed mass before placing the bird in the chamber, and an end mass when the bird was removed the next morning. We used the capture mass in subsequent analyses to best estimate the weight of each bird without including non-metabolizing, non-tissue weight.

Statistical analyses

We used the phylogeny generated by McGuire et al. (2014), pruned to match our dataset, to perform phylogenetically corrected analyses that accommodated the lack of independence caused by the species' relatedness. We used a hierarchical Bayesian approach to accommodate the hierarchical nature of individual observations clustered within species. We ran phylogenetic generalized linear mixed models using a Markov chain Monte Carlo (MCMC) sampler in the R

package MCMCglmm v. 2.24 (Hadfield, 2010). This package uses a Bayesian approach, which takes an expected prior distribution for the various parameters, runs a model incorporating the data, and returns a posterior distribution of parameter estimates. MCMCglmm explicitly models the phylogenetic relatedness between species as a random variable in the model input. We ran the MCMC chain for 5 million iterations, sampling every 1,000 generations, and checked visually for convergence. We used uninformative priors, fixing the variance for the residual at 1; this resulted in the only random effects variance being that of phylogenetic signal (“G-structure,” described below). These models required an ultrametric species tree, for which we pruned the hummingbird phylogeny generated by McGuire et al. (2014) to match our dataset. The matrices for the variance structures were:

$$R \sim \text{Inv. gamma}(V = 1, nu = 1)$$

$$G \sim \text{Inv. gamma}(V = 1, nu = 1)$$

where the R - structure is the covariance matrix of the residuals, the G - structure is the covariance matrix of random effects (phylogenetic signal in these models), V is the expected covariance structure representing the strength of belief, and nu is the degree of belief parameter for the inverse-Wishart.

For the covariance matrix B for the fixed effects, the defaults for expected value (μ) and strength of belief (V) were used (i.e. $\mu=0$ and $V = I * 1e + 10$), where I is an identity matrix of a dimension appropriate to the number of observations per species in the model.

The matrix for covariance structure of the random effect (phylogenetic signal) was:

$$g.inverse \sim \text{inv. Wishart}(invA)$$

where $invA$ is an inverse relatedness matrix and phylogenetic covariance matrix determined by MCMCglmm using the hummingbird phylogeny. This matrix is based on A , which is a

$n \times n$ matrix representing the proportion of evolutionary time shared between each species.

The first model assessed whether the probability of entry into torpor (modelled as an individual-level binary variable) was affected by individual capture mass, including species as a random effect.

$$Y_{i,j} \sim \text{Bernoulli}(p)$$

$$\text{logit}(p_{i,j}) = \alpha + \beta * M_{i,j} + s + u$$

where Y_i is response variable for the probability p of an individual i of species j using torpor, M_i is the capture mass of the individual, and α and β are the regression coefficients. The terms s and u account for two sources of variation- s accounts for phylogenetic variance using a covariance matrix from the phylogeny, while u accounts for residual variation (i.e. not explained by the other terms in the model).

The second set of models assessed which factors best predicted NEE. We tested the effects of mass, duration of torpor use, minimum chamber temperature, rewarming costs, and hourly energy savings on total NEE using the same Bayesian hierarchical MCMCglmm framework, both separately and stepwise (i.e. adding each variable incrementally). We compared these models using their DIC outputs (Table S1); lower DIC means a better model. The full model is described as:

$$Y_{i,j} \sim \text{Normal}(\mu, \sigma)$$

$$Y_i = \alpha_i + \beta_{1,i} * x_{1,i} + \beta_{2,i} * x_{2,i} + \beta_{3,i} * x_{3,i} + \beta_{4,i} * x_{4,i} + \beta_{5,i} * x_{5,i} + s + u$$

where $Y_{i,j}$ is the response variable – NEE – as a function of five dependent variables $x_{1 \text{ to } 5}$. α and β are the regression coefficients, the s term accounts for phylogenetic variance using a covariance matrix from the phylogeny, and u accounts for residual variation.

A third model separately tested whether energy expenditure during rewarming was

correlated with mass and chamber temperature during rewarming, using the same Bayesian hierarchical framework. We tested a mass-only model and a mass + chamber temperature model and compared their DIC outputs similarly to the NEE model. The results of the stepwise models are in Table S2.

All the MCMCglmm results are reported as post-mean with credible intervals (CI) and pMCMC; if the reported CI's overlap zero by less than 1% (Kruschke, 2010), we infer that variable does influence the structure of the data (e.g., if the post-mean and credible intervals are negative, that variable has a negative effect on the dependent variable and vice versa; Hadfield, 2010). We also used t-tests to analyse differences in torpor bout duration, hourly energy savings, and NEE between individuals at temperate and tropical sites, as well as between sites for which we had sufficient sample sizes. All t-tests were unpaired, two-tailed t-tests with equal variances, since all groups tested had equal variances.

Effect of warming temperatures on torpor

To test the potential effects of warming ambient temperatures on NEE and total nighttime energy savings, we modelled a range of torpid, normothermic, and total NEEs for broad-billed hummingbirds (*Cynanthus latirostris*) under current temperatures and with potential future nighttime warming of 3°C. We used the range of torpor durations that we measured at Harshaw and Sonoita in our study, with 2-6 hours torpor for Harshaw and 2-7 hours torpor for Sonoita. We calculated torpor and normothermic energy expenditures (EE) by taking average nighttime temperatures per hour at each site and fitting hourly energy expenditures using the metabolic rate-temperature relationships from the laboratory measurements of torpor and normothermy in the broad-billed hummingbird. We then added torpid and normothermic energy expenditures to get total NEE. To calculate changes in energy expenditures under an assumed future warming

scenario of 3°C, we added 3°C evenly to all the hourly nighttime temperature averages, recalculated torpid and normothermic energy expenditures per hour, and added these up to get total NEE.

RESULTS

Temperature

Metabolic chamber temperature (T_c) generally tracked ambient temperature (T_a), indicating that our chambers reflected natural temperature patterns (Fig. 2). The only exception was Harshaw, where T_a measurements had greater variability through the night than T_c . This was because our study site sat at the base of a canyon where cold air settles at night, causing an inverted temperature gradient. This resulted in mean chamber temperatures that were very similar to the site's minimum T_a . Nighttime T_c were generally warmer at tropical sites, more variable at temperate sites, and declined through the night at all sites.

Figure 2: Temperatures averaged per hour from all experiment nights across sites. Black lines denote the average temperature across days at that hour of night; red and blue lines respectively denote maximum and minimum temperatures at that hour. The first three facets are temperate sites; the last two facets are tropical sites. a. Ambient temperatures (T_a). b. Chamber temperatures (T_c).

Frequency and probability of entry into torpor

The overall frequency of torpor for all individuals studied was 54.8% (23 torpid/42 birds; see Table 2 for species-specific details). Two of the territorialist species—the blue-throated mountain-gem and the empress brilliant—never entered deep torpor (i.e. never dropped to minimum T_b or close to T_a), while all other species entered torpor with at least 25% frequency. Individuals exposed to the same environmental conditions varied in whether they used torpor. For example, at the Southwestern Research Station, we twice measured two Rivoli's hummingbirds on the same night, and both times one bird used torpor while the other did not. The MCMCglmm model for the probability of an individual entering torpor as a function of capture mass indicated that heavier birds had a lower chance of entering torpor (Table 3).

Duration

Torpor bout duration for all individuals that entered torpor was on average 4.9 (range 2.5 – 8) hours (Table 2). Individuals at the tropical sites had marginally higher torpor bout duration (5.9, range 3 – 8 hours) than species at the temperate (4.3, range 2.5 – 7 hours) sites ($t(14) = -2.09$, $p = 0.06$; Fig. S3). There was no difference between temperate and tropical site individuals in the proportion of the night they spent torpid ($t(17) = -0.70$, $p = 0.49$). Torpor bout duration was neither related to hourly energy savings nor minimum nighttime T_c (Supplement S1: Fig. S4). No birds entered torpor within the first hour of the experiment, potentially allowing them to begin processing their last meal. Individuals that entered torpor earlier in the night had longer torpor bout durations, and all individuals that used torpor had a single, uninterrupted torpor bout. Torpor arousal times were usually about an hour before sunrise.

Torpor energy savings

Average hourly energy savings in torpor across all species was 82% of normothermic costs (Table 2; Fig. S5). Hourly torpid energy savings were significantly higher at temperate

381 compared to tropical sites (85% average vs. 76% average; $t(13) = 2.94$, $p = 0.01$). Average hourly
382 torpid energy savings at Harshaw (Arizona) for broad-billed hummingbirds were lower than
383 hourly torpid savings at Sonoita (Arizona; $t(10) = 3.07$, $p = 0.01$). Torpor measurements with
384 broad-billed hummingbirds under both natural nighttime temperature gradients and controlled
385 laboratory conditions (5°C temperature steps) showed similar trends, though birds under natural
386 temperature conditions showed higher variability in energy expenditure in torpor, especially at
387 lower temperatures (Fig. 3). Nighttime metabolic rate decreased with decreasing T_c as broad-
388 billed hummingbirds reached their minimum T_b (~15 °C by extrapolating the linear portion of the
389 curve with a decreasing slope to its X-intercept, Fig. 3a); metabolic rate then increased below
390 minimum T_b .

Figure 3: Lab vs. field data on broad-billed hummingbird oxygen consumption in torpor. a.

Oxygen consumption was measured in controlled $5^{\circ}C$ temperature steps in a chamber in the lab ($5.7 \times 15.6 \times 5.7$ cm) using a Peltier device (Pelt-3; Sable Systems). By extrapolating the lower slope to the x-axis, minimum body temperature for this species is calculated to be approximately $15^{\circ}C$. b. Data collected on broad-billed hummingbirds under natural temperature and light cycles in the field. Colours represent individuals. Birds CYLA01 – CYLA07 were measured at the colder site, Harshaw. CYLA08– CYLA15 were measured at the warmer site, Sonoita. Some of the birds at Sonoita (CYLA09, 12, 15) did not enter torpor and so are not on this graph. Minimum body

temperature here again seems to be around 15°C.

Nighttime energy expenditure

Total NEE did not significantly differ between all individuals at temperate vs. tropical sites ($t(37) = -1.93$, $p = 0.06$). Mean NEE for torpid broad-billed hummingbirds was not significantly different between Harshaw and Sonoita ($t(11) = -1.12$, $p = 0.29$; Fig. S3). At Sonoita, the total NEE of normothermic birds was almost double the total nighttime energy of torpid Sonoita birds. Rewarming was more energetically expensive at the colder site, Harshaw, than at Sonoita ($t(7) = 3.49$, $p = 0.009$), confirming that T_c affects rewarming costs within the broad-billed hummingbirds. In Ecuador, mean NEE overall was lower at Maquipucuna compared to Santa Lucia ($t(17) = -2.97$, $p = 0.008$; Fig. S3). Torpid birds at Maquipucuna had similar total NEE to torpid birds at Santa Lucia ($t(2) = 0.33$, $p = 0.78$, Table 2).

The best MCMCglmm models explaining NEE all included torpor bout duration. Hourly energy savings were significantly negatively related to NEE in a model including just energy savings; however, in both the full and best models energy savings did not significantly affect NEE—duration and mass were significant predictors of NEE. Mass had a significantly positive effect on NEE in all models. However, the best and most parsimonious model was the duration-only model, which we present here (Table 3); the remaining models are in the Supplement S1: Table S1. The duration model showed that NEE in torpor was negatively related to torpor bout duration and estimated a positive mean intercept and negative mean slope for duration (Table 3). Of the models for rewarming, the best and most parsimonious one included only capture mass and showed that rewarming costs were positively correlated to individual mass, with a negative mean intercept and a positive mean slope of 0.34 (Table 3, Fig. 4b). The average duration of elevated

metabolic rates associated with rewarming was 38 minutes (range 7-92 min). Rewarming duration was higher than has been reported for hummingbirds in the past (Hiebert, 1990).

Figure 4: We assessed whether rewarming significantly affected total nighttime energy expenditure, and assessed which factors affected rewarming costs. Colours represent chamber temperatures (°C) during rewarming. a. Rewarming costs did not significantly affect total NEE. Here, total NEE is plotted against energy expenditure during rewarming, showing no relationship. b. Mass, and not chamber temperature, affected energy expenditure during rewarming.

DISCUSSION

To evaluate existing hypotheses for hummingbird torpor and predict how wild-caught hummingbirds might adjust their torpor use in response to variable environmental conditions, we assessed the probability of an individual entering torpor and evaluated the effect of torpor bout duration, hourly energy savings, and temperature on NEE for hummingbirds across multiple tropical and temperate sites. Hourly energy savings were maximized when the difference between T_a and a bird's presumed minimum T_b was close to zero (see Figs. 1 and 5.) This finding reflects what laboratory studies have found in hummingbirds (Hainsworth & Wolf, 1970) as well as in torpid and hibernating mammals (Geiser & Kenagy, 1988; Richter et al., 2015). Total NEE was negatively related to hourly energy savings, but our best model for NEE showed that torpor bout duration was the only factor that caused significant differences in total NEE (Fig. 5). The longer a hummingbird is torpid, the lower its total NEE, regardless of hourly torpid energy savings. Across all our study sites, including those in the tropics, temperatures were cold enough to allow individuals to enter and use torpor, indicating that temperature is likely not the primary factor determining torpor use. Nighttime energy management for hummingbirds likely depends on whether the combined effects of night-length and the difference between T_a and minimum T_b result in the costs of torpor (entry, maintenance, and arousal) being less than the cost of being normothermic under the same conditions.

Figure 5: A schematic depiction of aspects of NEE within a hypothetical hummingbird species.

The most important aspects of NEE (black triangle on left) seem to be the probability of entering

torpor (influenced by mass), and the duration of torpor (influenced perhaps by night length),

rather than energy savings (which increase as ambient temperature approaches minimum body

temperature). A situation with high torpor duration and low hourly energy savings results in lower

total NEE than low torpor duration with high hourly energy savings. Arrows represent the

strength of the effect of a variable on a component of NEE. Dashed arrows represent negative

correlations; width of the arrows depict the strength of the correlation.

We predicted that temperature would be the main factor determining the probability of an

individual entering torpor; instead, mass was the main factor – heavier species had a lower

probability of entering torpor (Fig. S6). Heavier, larger hummingbird species have two energetic

advantages. First, hummingbirds have limited energy stores for nighttime use because they tend to maximize food intake and energy storage only in the late evening before they roost rather than throughout the day (W.A. Calder et al., 1990; Powers et al., 2003). However, heavier birds can store more energy than smaller birds, either in their crop (Hainsworth & Wolf, 1972) or as body fat, allowing them to avoid torpor and its associated risks. Second, since metabolic rates have a scaling exponent with body size that is <1 due to the lower surface-to-volume ratio in larger endotherms (Glazier, 2015), larger bird species should be able to use normothermy for longer from proportionally similar initial energy stores. In addition to body size, a bird's capacity for energy storage may be influenced by resource availability. The only species to always avoid torpor in our study were two large territorialist species (the empress brilliant and the blue-throated mountain-gem). In contrast, species that used torpor consisted of a mix of roles, including smaller territorialists and all non-territorialists. These results suggest that perhaps a combination of the emergency only (Hainsworth et al., 1977; Powers et al., 2003) and routine hypothesis (Kruger et al., 1982) are at play. Large, territorialist hummingbirds might be accessing enough resources in the evening to avoid torpor except in emergencies, while for smaller hummingbirds that cannot routinely store enough energy to avoid torpor at night, the routine hypothesis could perhaps better explain their torpor use patterns (though migrating birds offer exceptions to this trend; Hiebert, 1993). These results are consistent with previous findings that some combination of resource availability (Hiebert, 1992) and energy storage affects torpor use (Powers et al., 2003), but also indicate that temperature is not the main factor in determining whether an individual enters torpor or not—highlighting the importance of evaluating torpor under conditions of an organism's natural environment.

Among the birds that used torpor, torpor bout duration was influenced more by night length than by chamber temperature, as predicted in the circadian rhythm hypothesis (Carpenter, 1974). This finding aligns with what other, non-hummingbird studies have found—that torpor duration was best predicted by latitude and was independent of body mass in birds and mammals (Ruf & Geiser, 2015). Given that hummingbirds have limited energy stores, we expected colder nights to result in longer torpor bouts, but this was not reflected in torpor duration. Torpor bout duration was slightly shorter at the temperate sites than at the tropical sites, perhaps reflecting the shorter temperate nights. Although duration varied between sites, the proportion of the night spent in torpor was similar in temperate and tropical birds, indicating the importance of night length as a predictor of torpor duration. To confirm that torpor duration is affected by night length, additional torpor duration measurements are required for the same species either at different times of the year at temperate sites, or across latitudes with different day lengths at the same time of year.

Though the variation in hourly energy savings did not have a significant effect on total NEE, from a physiological perspective it is worth noting the relationship between energy savings and temperature. Hourly energy savings were higher at colder, temperate sites than at warmer, tropical sites and were a function of how close T_a was to the bird's minimum T_b . There was no correlation between hourly energy savings and torpor bout duration (Supplement S1: Fig. S4). The tropical green-crowned brilliant saved slightly more energy per hour of torpor at the slightly colder Santa Lucia site, where it presumably approached minimum T_b , than at the warmer Maquipucuna site. We did not measure minimum T_b for this species, but the T_a at these sites (average 25°C) was likely warmer than its minimum T_b because the highest reliable reported minimum T_b for hummingbirds is 18°C (Bech et al., 1997; Hainsworth et al., 1977), and our data

show these birds' torpid hourly energy savings continued to increase as nighttime temperatures got colder (though these increases still did not significantly impact NEE). The temperate broad-billed hummingbird saved more energy per hour of torpor at the warmer Sonoita site than at the colder Harshaw site (Figs. 3, 6; Table 4). Harshaw reached T_a and T_c below the broad-billed hummingbirds' minimum T_b , ($\sim 15^\circ\text{C}$, Figs. 2, 3, 6), while Sonoita did not, allowing for maximal torpid hourly energy savings at Sonoita and sub-maximal hourly savings at Harshaw because the birds increased their metabolic rate to maintain their minimum T_b .

With the temperature increases associated with climate change, energy savings from torpor use will likely depend on temperatures at the site. Torpor energy savings are likely to increase with warming at very cold sites, like Harshaw, and decrease with warming at warmer sites that currently have nighttime temperatures around minimum T_b (Table 4, Supplement S1: Fig. S7), including tropical sites, like Maquipucuna and Santa Lucia, and moderate temperate environments like Sonoita. However, these calculations show that warming nighttime temperatures could lower total NEE by decreasing energy expenditure during normothermic hours. This lowering of normothermic energy expenditure will have more of an effect on overall NEE than any changes in torpor energy expenditure, and have a greater effect when torpor duration is short, regardless of the levels of hourly torpid energy savings. Projections of the effects of climate change on torpor in other animals have found that torpor at temperatures warmer than T_b min could be optimal (Boyles & McKechnie, 2010); and that the ability to use torpor/hibernation could constrain future species distributions (Humphries, Thomas, & Speakman, 2002). Our findings indicate that as long as torpor can be used for long durations (contingent on night length), rising temperatures will likely not have a significant effect on torpid

energy expenditure. Instead, warming will likely have a larger effect on overall NEE by increasing the energetic cost of hours spent in normothermy (Table 4).

In hummingbirds, a few studies have reported minimum T_b ranging from 7-18°C (Carpenter, 1974; Hainsworth & Wolf, 1970), while others mention that torpor savings are maximized at T_a 's of 15-20°C (Kruger et al., 1982); however, the link between energy savings and the difference between T_a and T_b has not been explicitly articulated. Our results are consistent with some laboratory studies on mammals and birds (Hainsworth & Wolf, 1970; Jastroch et al., 2016) which have found that hourly energy savings in torpor are maximized when T_a is approximately equal to minimum T_b .

Taking all its components into account, nighttime energy expenditure was affected most by torpor duration rather than by differences in hourly energy savings. Hourly energy savings varied significantly between temperate and tropical sites, but the range of variation in energy savings did not have a significant effect on NEE. Past literature suggests that a hummingbird is likely to enter torpor on a given night if its stored energy drops below some threshold (Hainsworth et al., 1977; Hiebert, 1992; Powers et al., 2003); our findings weakly support this relationship. Once they enter torpor, hummingbirds seem to maximize torpor bout duration. The energy a hummingbird saves per hour of torpor is negatively related to $T_a - T_b$ min. Our results show that many diverse natural factors can influence torpor use, highlighting the importance of torpor field studies in understanding energetic responses to real-world conditions.

DATA ACCESSIBILITY

The datasets supporting this article are available on Dryad at <https://doi.org/10.5061/dryad.t4b8gthzh>. Code used to run analyses (on R version 3.4.4) in this article are available at <https://github.com/nushiamme/Torpor>.

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TABLES

Table 1: Characteristics of the study sites - three temperate (in Arizona, AZ; Southwestern Research Station = SWRS) and two tropical sites (in Ecuador, EC).

Region	Site	Species	Territorial (T)/ Non-territorial (NT)	Latitude, Longitude	Altitude (m)	Dates sampled	Night length (hours)
AZ	Harshaw	<i>Cynanthus latirostris</i> (CYLA)	NT	31.50, -110.68	1370 – 1635	Jun – Jul 2013	9
AZ	Sonoita	CYLA	NT	31.50, -110.86	1100 – 1180	Jun – Jul 2013	
AZ	SWRS	<i>Eugenes fulgens</i> (EUFU) <i>Lampornis clemenciae</i> (LACL)	NT T	31.88, -109.21	1650 – 1720	May – Jul 2014	11
EC	Maquipucuna	<i>Phaethornis syrmatophorus</i> (PHSY) <i>Heliodoxa jacula</i> (HEJA) <i>Florisuga mellivora</i> (FLME)	NT T T	0.12, -78.64	1275 – 1370	Jul – Aug 2014	
EC	Santa Lucia	HEJA FLME <i>H. imperatrix</i> (HEIM) <i>H. rubinoides</i> (HERU)	T NT T T	0.12, -78.61	1800 – 2100	Jul – Aug 2014	

Table 2: Summary of nighttime energy expenditure and torpor measures per species and site (Southwestern Research Station = SWRS). Species codes: *CYLA* = *Cyananthus latirostris* (broad-billed hummingbird), *LACL* = *Lampornis clemenciae* (blue-throated mountain-gem), *EUFU* = *Eugenes fulgens* (Rivoli's hummingbird), *HEJA* = *Heliodoxa jacula* (green-crowned brilliant), *PHSY* = *Phaethornis syrmatorphorus* (tawny-bellied hermit), *FLME* = *Florisuga mellivora* (white-necked Jacobin), *HEIM* = *Heliodoxa imperatrix* (empress brilliant), *HERU* = *Heliodoxa rubinoides* (fawn-breasted brilliant).

Site	Species	n	Torpid (T) or Normo-thermic (N)	Mean mass (g)	Mean (range) NEE (kJ)	Mean nighttime O ₂ consumption (mL/min)	Probability of entering torpor	Mean torpor duration (hours)	Mean proportion of night spent torpid	Mean hourly torpid energy savings
Harshaw	<i>CYLA</i>	6	T	3.20	3.54 (2.52 – 4.99)	167.2	100%	4.7	52%	83 %
Sonoita	<i>CYLA</i>	3	N	3.34	5.72 (4.66 – 6.54)	286.24	62.50%	4.1	46%	90 %
Sonoita	<i>CYLA</i>	5	T	3.23	3.23 (1.72 – 4.09)	155.92				
SWRS	<i>LACL</i>	4	N	8.28	21.27 (17.62 – 26.77)	1063.31	0%	NA	NA	NA
SWRS	<i>EUFU</i>	2	N	7.93	20.76 (20.2 – 21.32)	1069.15	50%	3.25	44%	86%
SWRS	<i>EUFU</i>	2	T	7.75	18.67 (18.06 – 19.27)	938.81				
Maquipucuna	<i>HEJA</i>	1	N	8.74	17.50	872.64	83%	6.8	60%	76%
Maquipucuna	<i>HEJA</i>	6	T	8.64	8.67 (6.26 – 12.21)	428.96				
Maquipucuna	<i>PHSY</i>	1	T	5.24	10.81	535.82	100%	5	45%	79%
Maquipucuna	<i>FLME</i>	1	T	6.93	12.29	613.93	100%	3	27%	66%
Santa Lucia	<i>HEIM</i>	5	N	9.60	18.35 (11.41 – 21.39)	916.52	0%	NA	NA	NA
Santa Lucia	<i>HERU</i>	3	N	8.27	18.15 (17.15 – 18.99)	909.45	25%	4	36%	82%
Santa Lucia	<i>HERU</i>	1	T	7.89	10.31	512.94				
Santa Lucia	<i>HEJA</i>	1	T	8.74	7.62	373.63	100%	7	63%	89%
Santa Lucia	<i>FLME</i>	1	N	6.90	13.31	1069.15	0%	NA	NA	NA

Table 3: The results of the three best hierarchical Bayesian MCMCglmm models, with species relatedness was accounted for: 1. tests the influence of mass on the probability of entry into torpor, 2. tests the factors influencing nighttime energy expenditure (NEE), 3. tests the extent to which mass influenced rewarming energy expenditure.

Model	DIC	Intercept Mean (CI)	pMCMC	Variable estimate (CI)	pMCMC	Species (G-structure)
1. Probability torpor ~ Individual mass (g)	7.54	220.24 (-1.24, 546.85)	0.01	Mass: -31.21 (-78.08, 0.12)	0.009	1958 (0.06, 2392)
2. NEE (kJ) ~ Duration (hr)	192.85	15.75 (8.79, 22.26)	0.0004	Duration: -1.01 (-1.39, -0.63)	<2e-04	44.46 (7.64, 109.4)
3. Rewarming (kJ) ~ Mass (g)	30.18	-0.63 (-2.48, 0.73)	0.24	Mass: 0.20 (-0.003, 0.48)	0.012	0.29 (0.0001, 1.06)

Table 4: Predicted effects of warming ambient temperatures on torpid, normothermic, and overall nighttime energy expenditure in broad-billed hummingbirds. Bold numbers are higher than their corresponding non-bold numbers in the past or future. The ranges shown assume a range from maximal to minimal torpor durations that we measured at Harshaw and Sonoita in our study, with 2-6 hours in torpor for Harshaw and 0-7 hours in torpor for Sonoita.

Site	Harshaw			Sonoita		
Measure	Whole-night EE (kJ) range, for torpid hours	Whole-night EE (kJ) range, for normothermic hours	Total nighttime EE (kJ) range (sum of previous two columns)	Whole-night EE (kJ) range, for torpid hours	Whole-night EE (kJ) range, for normothermic hours	Total nighttime EE (kJ) range (sum of previous two columns)
Current temperatures	0.22 – 0.10	2.13 – 4.91	2.34 – 5.00	0.32 – 0	1.86 – 6.10	2.18 – 6.10
Future warming	0.21 – 0.05	1.82 – 4.31	2.03 – 4.36	0.66 – 0	1.55 – 5.28	2.22 – 5.28