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Research Article

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Tricolored Bat (*Perimyotis subflavus*) microsite use throughout hibernation

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Abstract

White-nose syndrome (WNS) has caused dramatic population declines in several bat species, including the Tricolored Bat (*Perimyotis subflavus*). Several studies have documented bats using colder roosting temperatures after infection; however, this strategy may have costs such as increased freezing risks or greater predation risks and it is unknown when during hibernation bats begin to utilize these colder temperatures. Our aim was to examine Tricolored Bat roost locations in a WNS-positive site in relation to roost microclimate and other environmental conditions throughout the hibernation season. We conducted monthly censuses of tricolored bats across 2 hibernation seasons (November to March 2020 to 2021 and October to March 2021 to 2022) in a WNS-positive hibernaculum in northwestern South Carolina and recorded skin and adjacent wall temperature, tunnel section, and distance from the entrance for each bat species. We continuously measured hibernacula temperature and relative humidity during both hibernation seasons. Most bats roosted in the back part of the tunnel where temperatures were warmer and more stable, and vapor pressure deficit (VPD) and human disturbance were low. However, >20% of bats roosted in the front, where roost temperatures were significantly colder and VPD was higher but more variable; human disturbance was also higher in this section. The proportion of bats in each tunnel section did not vary among months and we did not find evidence of significant movement to the front section of the tunnel as hibernation progressed based on marked bats; however, bats in the front section roosted higher on the wall suggesting that they may be avoiding human disturbance or predators. Our results support the notion that no optimum hibernation temperature exists for tricolored bats and that high VPD and disturbance are likely important factors driving microsite use. Protection of Tricolored Bat hibernacula that offer a range of microclimates or a network of sites in close proximity that offer different microclimates may be helpful for recovery of this species.

Key words: hibernacula, hibernation, microclimate, *Pseudogymnoascus destructans*, recreation, temperature, Tricolored Bat, vapor pressure deficit, white-nose syndrome.

Uso de micrositios por el murciélago tricolor (*Perimyotis subflavus*) durante la hibernación

Resumen

El síndrome de la nariz blanca (WNS por sus siglas en inglés) ha provocado una disminución drástica en las poblaciones de varias especies de murciélagos, incluido el murciélago tricolor (Perimyotis subflavus). Varios estudios han documentado que los murciélagos utilizan temperaturas de descanso más frías después de la infección; sin embargo, esta estrategia puede tener costos tales como mayores riesgos de congelación o mayores riesgos de depredación, y se desconoce en qué momento de la hibernación los murciélagos comienzan a utilizar estas temperaturas más frías. Nuestro objetivo fue examinar ubicaciones de descanso del murciélago tricolor en un sitio WNS-positivo en relación con el microclima de descanso y otras condiciones ambientales durante la temporada de hibernación. Realizamos censos mensuales de murciélagos tricolores durante 2 temporadas de hibernación (noviembre-marzo de 2020 a 2021 y octubre-marzo de 2021 a 2022) en un hibernáculo WNS-positivo en el noroeste de Carolina del Sur y registramos la temperatura de la piel y de la pared adyacente, la sección del túnel y la distancia desde la entrada para cada especie de murciélago. Medimos continuamente la temperatura y la humedad relativa del hibernáculo durante las dos temporadas de hibernación. La mayoría de los murciélagos se posaron en la parte trasera del túnel, donde las temperaturas eran más cálidas y estables, y el déficit de presión de vapor (VPD por sus siglas en inglés) y la perturbación humana eran bajos. Sin embargo, el >20% de los murciélagos se posaron en la parte delantera, donde las temperaturas de los sitios de descanso eran significativamente más frías y el VPD era más alto, pero más variable; la perturbación humana también era mayor en esta sección. La proporción de murciélagos en cada sección del túnel no varió entre meses y no encontramos evidencia de un movimiento significativo hacia la sección delantera del túnel a medida que avanzaba la hibernación según los murciélagos marcados; sin embargo, los murciélagos en la sección delantera se posaron más arriba en la pared, lo que sugiere que podrían estar evitando la perturbación humana o los depredadores. Nuestros resultados respaldan la noción de que no existe una temperatura de hibernación óptima para los murciélagos tricolores y que el alto VPD y la perturbación son probablemente factores importantes que impulsan el uso de micrositios. La

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protección de hibernáculos del murciélago tricolor que ofrezcan una variedad de microclimas o una red de sitios en estrecha proximidad que ofrezcan diferentes microclimas podría ser útil para la recuperación de esta especie.

Palabras claves: déficit de presión de vapor, hibernáculo, hibernación, microclima, murciélago tricolor, *Pseudogymnoascus destructans*, recreación, síndrome de la nariz blanca, temperatura.

During hibernation, bats must balance energy expenditure with physiological needs and external risk factors, including predation and disease. Survival is dependent on energy stores, energy expenditures, and duration of winter in relation to energy stores. Energy consumption during hibernation can be minimized by microsite selection, specifically through the use of roosting temperatures that reduce torpor metabolic rate (TMR; Humphries et al. 2002). The relationship between TMR and ambient temperature is U-shaped, with a sharp increase in energy consumption below a minimum set-point (Humphries et al. 2002). The use of warmer than minimum set-point temperatures can be explained by the instability of hibernacula temperatures, avoidance of predation, selection for higher humidity, huddling tendency, and reduced energetic costs of arousal at higher temperatures (Thomas and Cloutier 1992; Webb et al. 1996; Fuller et al. 2011; Boyles 2017, 2020; Ryan et al. 2019). Energy stores also influence microsite selection; bats in poorer condition and later in hibernation are more likely to use colder microsites to conserve energy (Boyles et al. 2007; Willis 2017; Ryan et al. 2019). Pathogenic infections may also influence microsite selection in response to conditions influencing pathogen viability and growth, as well as declines in body condition due to disease (Boyles et al. 2007; Hopkins et al. 2021).

The introduction of *Pseudogymnoascus destructans* (*Pd*) to North America in 2006 precipitated dramatic population declines in several species of hibernating bats (Cheng et al. 2021). *Pd* is a psychrophilic fungus that causes white-nose syndrome (WNS) in at least 12 North American species of bats during hibernation. Invasion of the wing membrane by the fungus is hypothesized to lead to high mortality of infected bats through intensified evaporative water loss (EWL) leading to increased arousals from torpor (Warnecke et al. 2012; McGuire et al. 2016; Haase et al. 2019), physical damage to the wings leading to impaired flight, and therefore, reduced ability to forage upon emergence from hibernation, and disruptions of physiological processes associated with metabolism, thermoregulation, and respiratory gas exchange (Cryan et al. 2010; Verant et al. 2014). Based on estimated population declines and the extent of the range of species affected by WNS, Cheng et al. (2021) determined that 3 species have been highly impacted by WNS: the Northern Long-eared Bat (*Myotis septentrionalis*), Little Brown Bat (*M. lucifugus*), and Tricolored Bat (*Perimyotis subflavus*).

Following the arrival of *Pd* in North America, wintering populations of tricolored bats within the WNS-infected region declined by 93% (Cheng et al. 2021). Consequently, the species is currently proposed for listing as endangered by the US Fish & Wildlife Service (2022). Tricolored bats are particularly vulnerable to WNS for several reasons—they are one of the first species to enter hibernation and the last to emerge (Laval and Laval 1980), leaving them exposed to *Pd* for longer than other hibernating bat species. Since *Pd* load increases throughout the hibernation period, peaking at the end of hibernation (Langwig et al. 2015), tricolored bats are particularly at risk of high fungal loads (Bernard et al. 2017). Tricolored bats also use warm hibernacula (McNab 1974; Sirajuddin 2018), preferring temperatures that often overlap with the optimal temperature range for *Pd* growth (12.5 to 15.8 °C; Verant et al. 2012). For example, in a laboratory setting tricolored bats were more likely to select the warmest available temperature (11 °C) over colder chambers (5 °C or 8 °C; Boyles et al. 2022). The small body mass of tricolored bats is associated with higher rates of EWL, which is further exacerbated by WNS (Haase et al. 2021). Likely to cope with EWL, tricolored bats use areas of hibernacula with relative humidity (RH) above 80% (Ploskey and Sealander 1979; Raesly and Gates 1987). *Pd* grows better in more humid conditions although the exact relationship between *Pd* growth and humidity is unclear (Marroquin et al. 2017; Frick et al. 2022). While bats infected with WNS face increases in EWL, modeling suggests that their likelihood of survival increases in drier roosting sites (Haase et al. 2021).

Although WNS has severely impacted tricolored bats, small persisting populations have been documented at 93% of infected hibernacula 5 to 7 years after initial infection (Cheng et al. 2021) which may be due to either resistance or tolerance (Frick et al. 2017). One of the hypothesized mechanisms for survival of WNS is the use of colder roosting sites, either through a change in behavior or the persistence of individuals already using colder sites (Hopkins et al. 2021; Turner et al. 2022). For example, Lilley et al. (2016) found that persisting populations of little brown bats had lower skin temperatures during torpor than individuals during the peak WNS period or pre-WNS period. In Pennsylvania, tricolored bats shifted roosting locations toward colder sections of hibernacula after the arrival of WNS (Johnson et al. 2016). A significantly higher proportion of tricolored bats in South Carolina used the colder, front sections of a hibernaculum in the immediate years following the arrival of *Pd*, with a significant increase in the section nearest the tunnel entrance after 5 years (Loeb and Winters 2022).

Selection of hibernation roost sites by bats is a trade-off between energetic costs such as the costs of defending body temperature and arousal costs; physiological costs such as water imbalance or buildup of wastes; and nonenergetic costs such as freezing, predation, or disease risks (Boyles et al. 2020)—roost site selection may change across the season. However, most studies of bat microsite use are conducted only once or twice a season (e.g., Martínková et al. 2018; Hopkins et al. 2021; Loeb and Winters 2022; Turner et al. 2022). Our study was conducted in an easily accessible hibernaculum that allowed us to test the responses of bats to several of these costs across the hibernation season. The site had a large temperature and humidity gradient, and the coldest section was open to public visitation as well as predators. Further, bats in the hibernaculum had been infected with WNS for several years. Thus, our overall goal was to understand Tricolored Bat microsite use in response to WNS, environmental conditions, and potential disturbance across the hibernation season. Our first objective was to test whether bats moved to colder sites at the end of the season, potentially in response to increased fungal loads, or if individual bats used colder temperatures throughout hibernation. Our second objective was to better understand the hibernation roosting behavior of tricolored bats in relation to microsite and environmental conditions across space and time. We hypothesized that the number of bats in each tunnel section would vary throughout the hibernation season and predicted that the number of bats roosting near the front of the hibernaculum would increase as hibernation progressed to seek colder temperatures as *Pd* loads increased (Langwig et al. 2015) and that individual bats would move toward the entrance as hibernation progressed. We also hypothesized that bat density would vary

by hibernaculum section, temperature, and VPD and predicted that density would be highest in the back of the hibernaculum, where humidity is high and temperatures are within the range normally used by tricolored bats. We also hypothesized that roost temperature (T_R) would vary by month and location, predicting that T_R would decrease as the hibernation period progressed, inversely tracking the anticipated increase in *Pd* load (Langwig et al. 2015), and that T_R would be lowest near the hibernaculum entrance for all months. Finally, we hypothesized that disturbance would affect roost location of bats and predicted that bats in the section open to the public and most exposed to predators would roost higher on the wall than those in the protected sections.

Materials and methods.

Study site.

The study was conducted in Stumphouse Tunnel, a ~500-m long, unfinished railroad tunnel in the Blue Ridge Mountains in Oconee County in northwestern South Carolina. The tunnel has an average height of 7 m and width of 5.2 m (Fig. 1). Average winter temperatures in the surrounding area during the 2020 to 2021 and 2021 to 2022 hibernation seasons (November to March) were 8.9 °C and 9.6 °C, respectively. The 20-year (2002 to 2022) annual mean temperature in the surrounding area was 15.8 °C. The tunnel is subdivided into 3 sections by brick walls with a bat-friendly gate between sections A and B and a door-sized opening between sections B and C. Section A is open to the public year-round, section B contains a vertical ventilation shaft near its halfway point, and section C ends in a sloped dead-end. Sections B and C are closed to the public year-round.

Historically Stumphouse Tunnel was the third largest known hibernation site for tricolored bats in South Carolina, with the Middle Tunnel—located approximately 1 km away—being the second largest. Winter counts of tricolored bats at the site prior to the arrival of WNS were over 300 (Loeb and Winters 2022). *Pd* was first documented in the tunnel in 2014, and WNS was first detected in 2015; by 2018, winter counts had declined by 90% (31 bats; Loeb and Winters 2022).

Hibernaculum temperature and vapor pressure deficit.

Hibernaculum temperature and RH were measured every 60 min with Hygrochron iButtons (Maxim Integrated, San Jose, California) placed approximately every 100 m within the tunnel (Fig. 1). We placed 2 iButtons in section A, 2 in section B, and 3 in section C. Vapor pressure deficit (VPD) was calculated for all hourly temperature and RH data by calculating the saturation water vapor pressure and subtracting the actual water vapor pressure (Allen et al. 2005).

Bat locations and roost microclimate.

We surveyed bats in Stumphouse Tunnel monthly from November 2020 through March 2021 and from October 2021 through March 2022. Four to five people conducted a total count of all bats. Two or more observers scanned each side and the ceiling, and 1 person measured the distance from the entrance and recorded data. For each bat, we recorded the species, distance from the entrance, tunnel section (A, B, C), location (left, right, ceiling), relative wall height (lower, middle, or upper third), and skin and wall temperatures. Skin and wall temperatures were measured with an infrared thermometer (Fluke 62 Max).

All personnel that participated in the monthly census followed WNS decontamination protocols set forth by the WNS Decontamination Team to prevent the spread of WNS (https://www.whitenosesyndrome.org/mmedia-education/national-wns-decontaminationprotocol-u-s). All methods were approved by the Clemson University (2020-051) and US Forest Service (2021-001) Institutional Animal Care and Use Committees and followed the guidelines of the American Society of Mammalogists (Sikes et al. 2016).

Individual bat movement.

During the second hibernation year (2021 to 2022) we measured individual bat movements. Each month, bats within reach were marked with a 1-cm diameter circle of colored and numbered reflective tape (Stebbings 2004; Martinez 2015; Kirkpatrick et al. 2020). Bats were not removed from the wall during marking. The tape was affixed with tweezers to the fur between the scapulae of the bat using cyanoacrylate adhesive (superglue; Loctite, Rockyhill, Connecticut). The color of the tape corresponded to the tunnel section where each bat was initially marked. Each subsequent month, we recorded the tag color and number and the distance from the entrance, wall height, and tunnel section for each resighted tagged bat. We recorded bats as disturbed in the month that they were marked and if they were swabbed for *Pd* (in either November or February as part of a different project); while bats were not removed from the wall, bats did arouse during both marking and swabbing which may have increased the likelihood that they would change locations before the next census.

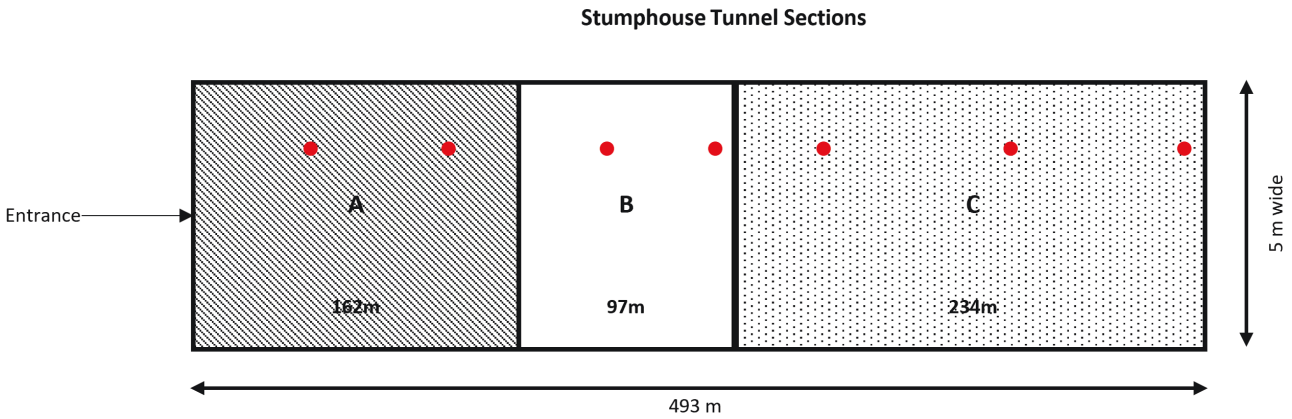

Fig. 1. Cross-section of Stumphouse Tunnel, a Tricolored Bat hibernaculum in Walhalla, South Carolina. The tunnel is subdivided into 3 sections with brick walls; the back of section C is closed. Small dots represent iButton locations.

Analyses

We calculated average daily temperatures for each tunnel section by averaging all hourly temperatures for iButtons for each day within each section. Daily minimum temperatures were calculated for each section using the coldest temperature recorded on any of the iButtons for each day within a section and the daily temperature variance was calculated for each section. We also calculated daily maximum VPD and daily and monthly means of temperature and VPD by tunnel section and hibernation year.

To determine if bats moved forward as hibernation progressed (objective 1) and whether bat microsite use was affected by disturbance (objective 2), we used a generalized linear mixed-effects model to compare bat counts between sections, among relative roost heights, across months, and the interactions. We included hibernation year as a random effect and used a Poisson distribution and a log-link. We conducted post hoc analyses using Tukey's adjustment. Due to low numbers of bats roosting in section B, we did not include this section in the model (no model convergence). We determined the number of bats that moved between sections each month. To determine if disturbance of individual bats (either from being marked or from *Pd* swabbing) affected the distance they moved from 1 month to the next (absolute value of distance), we used a linear mixed-effects model with disturbance as a predictor and individual bat as a random effect.

To examine how the patterns of bat density in each section (# bats/linear m) related to environmental conditions (objective 2), we used an information-theoretic approach to compare a set of models that included section, month, and measures of temperature and VPD as predictor variables. We averaged environmental conditions for each month, year, and tunnel section (Table 1) and calculated the density of bats in each tunnel section. We checked for collinearity between the predictor variables using correlation ($|r| > 0.60$). Collinearity between environmental variables, section, and month limited our ability to utilize a series of sequential nested models; for example, section could not be in a model with any environmental variables, and minimum average temperature and temperature variance could not be used in a model together. We therefore developed 6 simple models based on a priori hypotheses (plus an intercept-only) model to predict bat density (Table 1). The models involved predictors that were found to be correlated with bat density, and not correlated with each other. Numeric variables were scaled and centered for the models. We used linear mixed-effects models with hibernation year as a random effect for all models. We ranked the models using Akaike Information Criterion adjusted for small sample sizes (AICc) and used AICc weights to compare support for each model. We used a cutoff of 2 ΔAICc for top models.

We conducted 3 analyses to compare roosting temperatures across time and space for the population and individual bats (objective 2). First, we compared mean wall temperatures at bat roosts (T_R), using a linear mixed-effects model, with main effects of month, tunnel section, relative wall height, and the interactions between month and section and section and wall height and a random effect of hibernation year. We included data only from sections A and C for this analysis due to the low numbers of bats roosting in section B. Second, we used mixed-effects linear regression to test if T_R was correlated to distance from the entrance with month nested in year as a random effect. And finally, for individually marked bats, we used a linear mixed-effects model to determine if roost temperature was predicted by month and distance moved toward the entrance with bat as a random effect. We calculated change in roost temperature for each bat from the previous month, with negative numbers indicating a reduction in roost temperature (excluding any bats that had not been seen the previous month) and calculated the distance they moved from their previously sighted location, with positive numbers indicating movement toward the front of the tunnel.

Statistical analyses were conducted in R (v 4.1.1), except for the analyses of marked bats, which were conducted in JMP Pro (16.2.0). We present mean ± SD for descriptive statistics. We used the "lme4" package in R for mixed-effects models and used the "emmeans" package to calculate least-squared means and report least-squared means ± 1 SE. We used an alpha of 0.05 to determine statistical significance.

Table 1. A priori models to predict hibernating Tricolored Bat density in Stumphouse Tunnel (South Carolina, United States) in the 2020 to 2021 and 2021 to 2022 hibernation seasons (October to March). All models included hibernation year as a random effect. T_A, ambient temperature; T_V, temperature variance.

Model	Variable definitions
Min T_A + Mean VPD	Min T_A = monthly mean minimum daily section temperatures Mean VPD = monthly mean daily VPD
Min T_A	Min T_A = monthly mean minimum daily section temperatures
Mean T_V	Mean T_V = monthly mean daily temperature variance
Mean VPD	Mean VPD = monthly mean daily mean section VPD
Month * Section	Month = month Section = tunnel section (A, B, C)
Section	Section = tunnel section (A, B, C)
Null	Intercept-only

Results

For both hibernation years, section C had the warmest average temperatures (13.1 ± 1.0 °C and 13.4 ± 0.9 °C, respectively) compared to sections A (7.6 ± 2.5 °C and 8.4 ± 2.6 °C, respectively) and B (9.1 ± 1.9 °C and 9.5 ± 2.2 °C, respectively). Eighty-three percent of hourly temperature readings in section C fell between 12.5 and 15.8 °C, the optimal range for *Pd* growth, compared to 8% of hourly readings in section A and 14% in section B (Fig. 2). Most temperatures outside the optimal growth range for *Pd* in sections A and B fell below that range except for warmer temperatures in October of the second hibernation year. VPD was lowest in section C (0.04 ± 0.07 kPa), followed by sections A and B (0.10 ± 0.10 kPa and 0.13 ± 0.10 kPa, respectively).

Monthly bat counts and density.

In addition to tricolored bats, we also observed 3 big brown bats and 2 Rafinesque's big-eared bats during the 2020 to 2021 hibernation year and 1 big brown bat and 3 Rafinesque's big-eared bats during the 2021 to 2022 hibernation year. We counted 59 ± 6 bats per month from November 2020 to March 2021 and 89 ± 10 bats per month between October 2021 and March 2022. The greatest proportion of bats roosted in section C (0.67 ± 0.08), but more than a quarter roosted in section A (0.28 ± 0.06); section B had the lowest proportion of bats (0.05 ± 0.04; Fig. 3). The number of bats per section did not vary significantly across months ($\chi^2 = 2.73$, df = 5, $P = 0.742$). However, there was a significant interaction between section and relative roosting height on bat counts ($\chi^2 = 8.40$, df = 2, $P = 0.015$). In section A, significantly more bats roosted on the upper and middle thirds of the wall than on the lower third (8.8 ± 1.59 and 7.88 ± 1.41 compared to 1.34 ± 0.44; $z = -5.58$, $P < 0.001$ and $z = -5.92$,

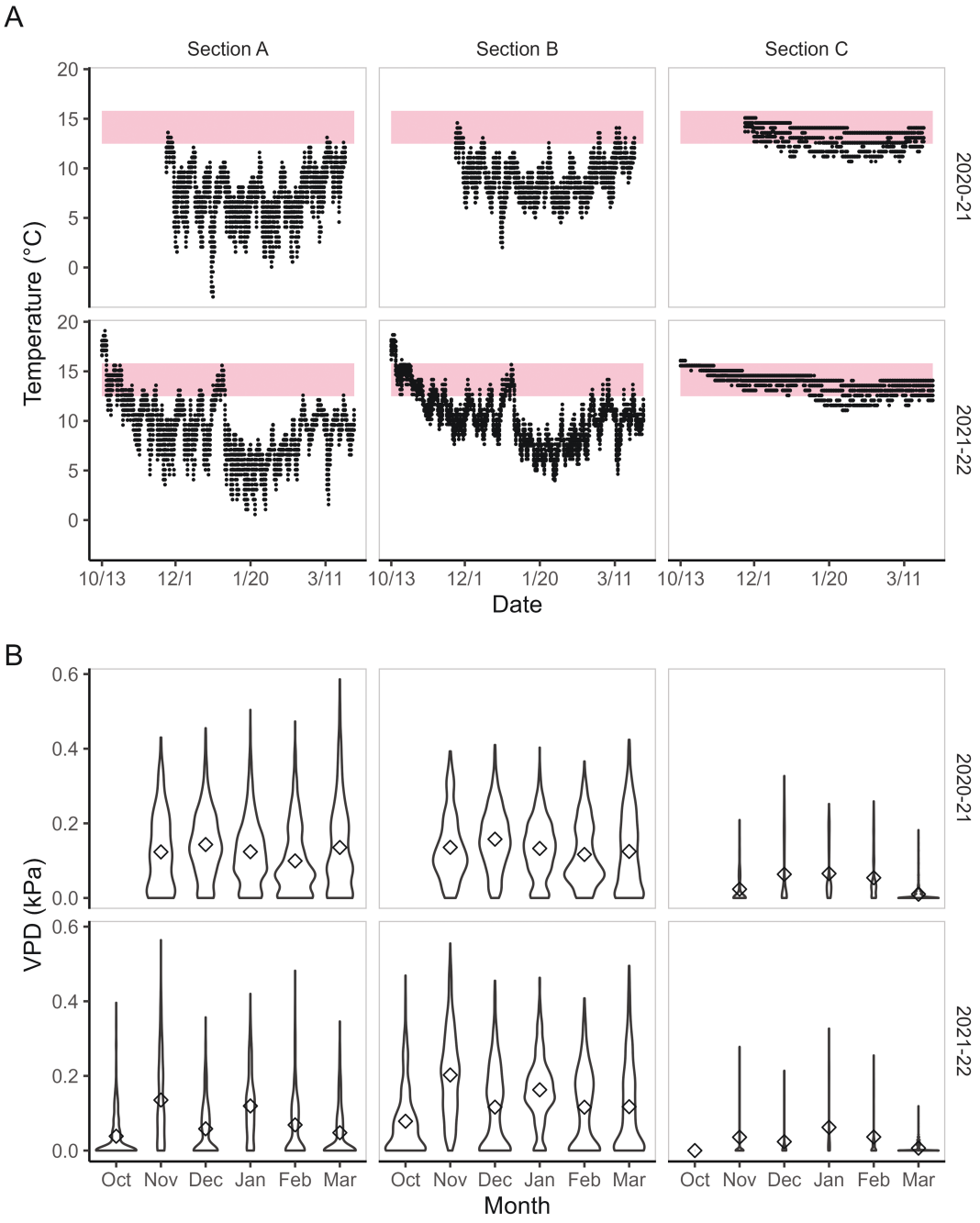

Fig. 2. (A) Hourly temperatures (°C) by day, and (B) hourly VPD by month in each section of Stumphouse Tunnel during the 2020 to 2021 and 2021 to 2022 hibernation seasons (October to March). Temperatures and relative humidity (RH) were recorded using Hygrochron iButtons (Maxim Integrated, San Jose, California), placed every ~100 m. There were 2 iButtons in sections A and B and 3 in section C. VPD was calculated from hourly temperature and RH by calculating the saturation water vapor pressure and subtracting the actual water vapor pressure (Allen et al. 2005). Temperatures for optimal *Pd* growth (12.5 to 15.8 °C) are shaded in horizontal bars (A; Verant et al. 2012).

$P < 0.001$, respectively). In contrast, significantly more bats roosted on the bottom third of the wall compared to the upper third of the wall in section C (19.52 ± 3.05 vs. 14.37 ± 2.23; z = 2.79, $P = 0.014$).

A single top model with tunnel section as the only predictor of bat density carried 100% of the cumulative model weight (Table 2). Section C had the highest density of bats (0.21 ± 0.07 bats/linear m), followed by section A (0.13 ± 0.02 bats/linear m), and section B had the lowest density (0.03 ± 0.03 bats/linear m). Despite a weight of only 0.000, the second-best model showed a significant effect of mean VPD on bat density; as mean VPD increased, bat density decreased ($F_{1,27} = 35.47$, $P < 0.0001$; Supplementary Data SD1).

Roost microclimates.

Roost temperatures varied significantly by month ($F_{5,716.4} = 293.72$, $P < 0.0001$), section ($F_{1,716.0} = 3221.12$, $P < 0.0001$), and relative wall height ($F_{2,716.1} = 60.28$, $P < 0.0001$), with significant interactions between month and section ($F_{5,716.0} = 83.5$, $P < 0.0001$; Fig. 4A) and section and wall height ($F_{2,716.1} = 26.32$, $P < 0.0001$; Fig. 4B). Roost temperatures were significantly colder in section A (9.4 ± 0.2 °C) than in section C (13.6 ± 0.2 °C), which was true for all months, with the largest differences in December (7.8 ± 0.3 °C in section A vs. 13.4 ± 0.3 °C in section C) and January (7.2 ± 0.3 °C in section A vs. 12.8 ± 0.3 °C in section C). Roost temperatures increased as relative

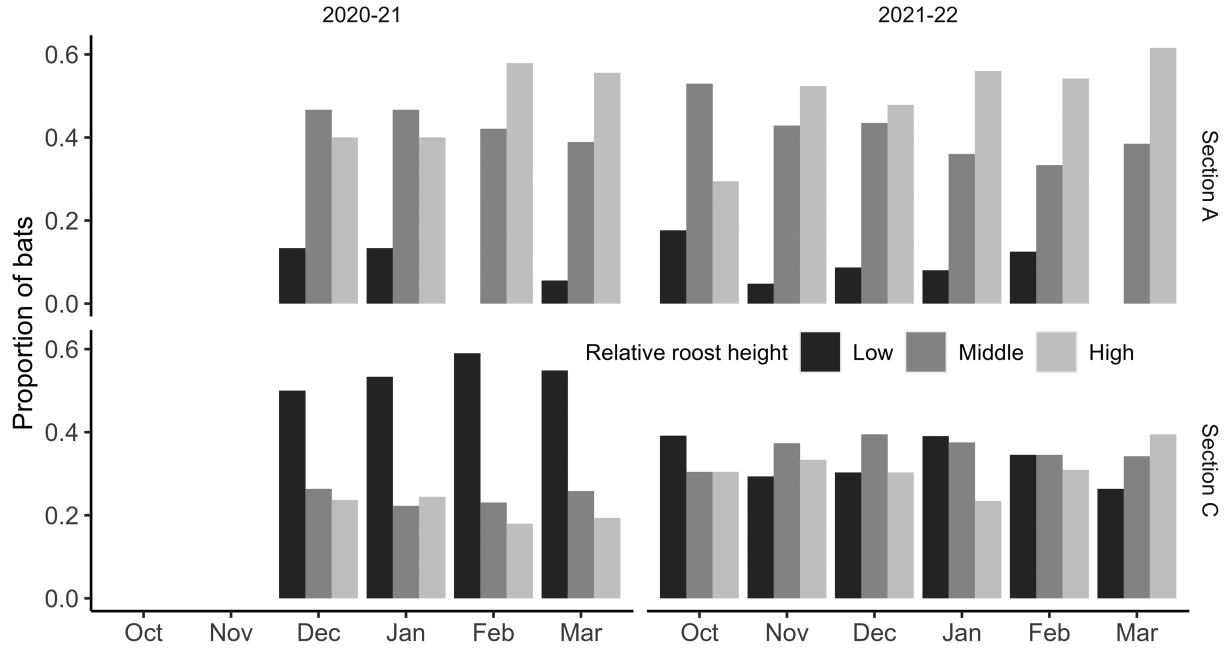

Fig. 3. Proportion of tricolored bats at each relative wall height and in the front (section A) and back (section C) of Stumphouse Tunnel (Oconee County, South Carolina) by month for the 2020 to 2021 and 2021 to 2022 hibernation seasons with SE.

Table 2. Model selection results for Tricolored Bat density (# bats/linear m) in Stumphouse Tunnel (South Carolina, United States) during the 2020 to 2021 and 2021 to 2022 hibernation seasons (October to March). All models included hibernation year as a random effect. K, number of fitted parameters in the model; AICc, Akaike Information Criterion for small sample sizes; ΔAICc, change in AICc; w_i, Akaike model weight; T_A, ambient temperature; T_V, temperature variance.

Model	K	AICc	ΔAICc	wi
Section	5	−84.8	0	1.00
Mean VPD	4	−68.6	16.19	0.000
Min T_A + Mean VPD	5	−59.6	25.22	0.000
Intercept-only	3	−55.5	29.28	0.000
Min T_A	4	−51.9	32.86	0.000
Mean T_V	4	−46.9	37.93	0.000
Month * Section	9	68.7	153.52	0.000

wall height increased, except there were no significant differences in roost temperatures between the middle and upper thirds of the wall in section C ($P = 0.244$). The largest differences occurred in section A, where roosts on the upper third of the wall averaged 0.95 °C and 1.4 °C warmer than roosts on the middle and bottom thirds of the wall, respectively. Roost temperatures increased with increased distance from the tunnel entrance ($F_{1,813} = 2564.53$, $P < 0.0001$). While this relationship held across all months, the differences between roost temperatures from the front to the back of the tunnel became greater as winter set in and only slightly decreased in March (Supplementary Data SD2).

Bat movement.

We marked 39 bats between October 2021 and January 2022: 3 bats in section A, 0 in section B, and 36 in section C. Two bats were never resighted, but 74% ($n = 35$) were resighted 3 months after marking, and 38% ($n = 21$) were resighted after 5 months (Table 3). Of the 39 marked bats, we resighted 28 until at least February; we recovered 4 tags from the tunnel floor, 2 tags were removed during netting prior to the March census, and 1 marked bat in section A was euthanized in February due to a badly broken wing. None of the bats marked in section C were observed in section A, although 10 were observed in section B; the 3 bats marked in section A were not observed in another section. Some individuals barely moved from month to month, while others moved greater distances (Supplementary Data SD3). The monthly mean changes in roost temperature of marked bats ranged from −1.0 to 0.3 °C, and the monthly mean distance moved toward the entrance varied from −0.24 to 44.57 m (Table 4). The movements that we observed were not the result of our disturbance of the bats as the absolute distance moved by bats did not significantly differ between bats that had (42.2 ± 7.8 m) or had not (39.1 ± 6.1 m) been physically disturbed the previous month ($F_{1,101.6} = 0.11$, $P = 0.74$). For every meter moved toward the tunnel entrance, roost temperature decreased by 0.01 °C ($F_{1,98.8} = 37.2$, $P < 0.0001$). The only significant difference in the change in roost temperature among months was between December and February ($F_{4,98.7} = 4.9$, $P = 0.001$); February was the only month in which roost temperature increased (0.3 °C; Table 4).

Discussion

Winter counts of tricolored bats at Stumphouse Tunnel have continued to increase since the low of 31 bats in 2018, which represented a 90% decline from pre-WNS counts (Loeb and Winters 2022). Loeb and Winters (2022) suggested that the increases they observed, starting in 2020, may have been related to increased use of the colder front section of the tunnel. Although we also found that >20% of the bats roosted in the front section, we found that the number of bats in the front of the tunnel did not vary significantly by month, contrary to our prediction. Since none of the bats marked in section C ($n = 36$) were observed in section A ($n = 3$) and vice versa in the 2021 to 2022 hibernation season, selection may have favored bats roosting at colder temperatures throughout hibernation rather than our hypothesis that bats would shift to colder temperatures later in hibernation to cope with increased fungal loads.

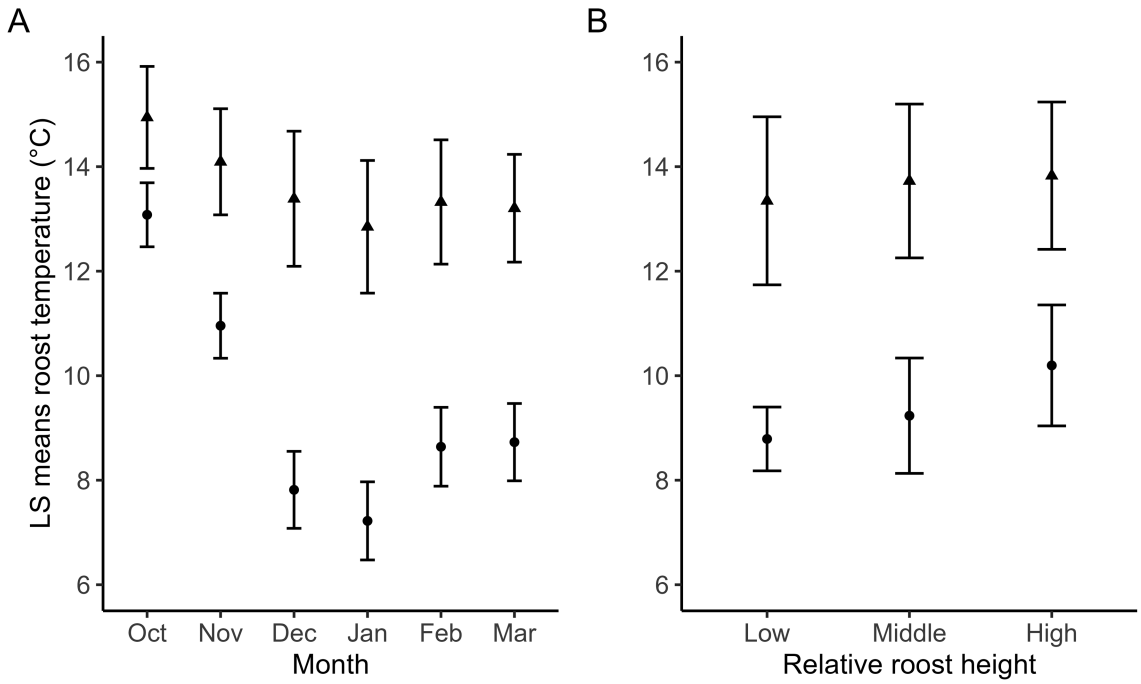

Fig. 4. Least-squared mean roosting temperatures of tricolored bats and 95% CI as a function of (A) the interaction of month and tunnel section, and (B) the interaction of relative roosting height and tunnel section in Stumphouse Tunnel (Oconee County, South Carolina) during the 2020 to 2021 and 2021 to 2022 hibernation seasons (October to March). Section A = circle; section C = triangle.

Table 3. The number of months that tricolored bats marked with numbered, reflective tape were resighted in the 2021 to 2022 hibernation season at Stumphouse Tunnel (South Carolina, United States). A total of 39 bats were marked between October 2021 and February 2022. Of the 21 bats that were monitored for all 5 months, we resighted 38.1% after 5 months (n = 8).

Number of months	Number of marked bats	Number resighted	% resighted
1	39	37	94.9%
2	39	32	82.1%
3	35	26	74.3%
4	29	16	55.2%
5	21	8	38.1%

It is also possible that the bats using section A entered hibernation in poorer condition and used the colder roosting temperatures to reduce energy consumption for the entirety of hibernation (Boyles et al. 2007).

While the proportion of bats that roosted in section A in February during our study was higher than pre-WNS, the proportion has declined since peaking in February 2020 at 46% of tricolored bats (Loeb and Winters 2022). Between February 2020 and February 2021, the number of tricolored bats in section A remained the same, while the number in section C increased by 95%. From February 2021 to February 2022, the number in section A increased by 26% compared to an increase of 41% in section C. Hopkins et al. (2021) also found that, despite a decrease in average roosting temperature post-WNS, a majority of little brown bats across 12 sites in the Midwestern United States continued to use warm roosting sites. If the use of colder roosting temperatures has played a role in the population increases in Stumphouse Tunnel, it may have been more important during peak WNS, and bats may be returning to warmer sites as resistance or tolerance to *Pd* builds in the remaining population. In a remnant population of little brown bats in New York, arousal frequencies have returned to levels observed pre-WNS, although torpor skin temperatures are lower than pre-WNS (Lilley et al. 2016). In the endemic range of *Pd* in Europe, fungal loads increase with increasing body temperature up to 5 °C and then decrease above 7 °C, which may be associated with increased resistance to invasive infection in bats that use warmer temperatures (Martínková et al. 2018).

While over 20% of tricolored bats in our study roosted in the coldest sections of the tunnel, this is not without risks including high temperature and VPD variation (Fig. 2), exposure to temperatures below the set-point, increased predation and other disturbance, more infrequent arousals leading to reduced immune function, and more energetically costly arousals (Boyles et al. 2020). Tricolored bats have historically been found in the warmest, most humid sections of hibernacula (Raesly and Gates 1987). While these conditions may have been optimal prior to WNS, they are associated with higher mortality from WNS—potentially becoming an ecological trap (Marroquin et al. 2017; Hopkins et al. 2021). Exposure to extreme cold may be particularly challenging for bats with fewer fat reserves to insulate them. Even though WNS survival has been associated with colder roosting temperatures (Lilley et al. 2016; Hopkins et al. 2021), continued persistence of the majority of bats in the back of the tunnel (section C) suggests that preference of tricolored bats for warm, humid sites is not a death sentence as witnessed by the increasing populations since 2019. In fact, a recent laboratory study found that *Pd* growth slowed and decreased at higher temperatures associated with frequent arousals (Forney et al. 2022), suggesting that warmer roost sites that are associated with shorter torpor bouts and less costly arousals may provide some benefit.

Use of different sections of the tunnel by tricolored bats may have also been influenced by disturbance and predation risk. Tricolored bats in section A were mostly found on the upper two-thirds of the wall—above approximately 2.5 m—which is out of reach for most humans and terrestrial predators. Therefore, it is possible that bats

Table 4. Distances moved (m) by and change in roosting temperature (°C) of marked tricolored bats in Stumphouse Tunnel (South Carolina, United States) during the 2021 to 2022 hibernation season (October to March); with standard deviation. Negative values of distance moved indicate movement toward the back of the tunnel; positive values indicate a move toward the front. Absolute value of distance moved disregards direction of movement.

Month	Mean distance moved (m)	Max distance moved toward back (m)	Max distance moved toward front (m)	Mean absolute value distance moved	Min absolute value distance moved	Max absolute value distance moved	Least-squared mean change in roost temperature (°C)
October to November	44.6 (72.3)	−115.5	159.1	62.7 (56.2)	2.7	159.1	−0.43 (0.26)
November to December	−0.2 (53.8)	−154.2	116.1	34.1 (41.0)	0	154.2	−1.12 (0.22)
December to January	13.5 (51.5)	−115.5	150.0	30.5 (43.3)	0	150.0	−0.58 (0.21)
January to February	16.0 (64.7)	−79.2	187.1	39.0 (53.6)	0.3	187.1	0.31 (0.21)
February to March	3.3 (60.9)	−133.2	105.5	36.9 (47.4)	0.3	133.2	− 0.38 (0.28)

selected higher roosts to avoid human disturbance and predation or bats that used higher roost sites were selected for in this section. However, bat activity was not significantly higher on weekends or holidays when human visitation to the tunnel would most likely be highest. In addition, bats that were marked the previous month and marked bats that were swabbed for *Pd* during the February 2022 census did not move significantly more or less than marked bats that were undisturbed the previous month, although they may have moved and returned to the same roost site. The bats at this site may be acclimated to higher levels of disturbance, but human activity and predation risk in section A could partially explain why most bats still roost in section C.

Higher roost sites may also protect bats from extremely low temperatures, particularly in areas with greater temperature fluctuations such as section A. Bats roosting in the section nearest the entrance tended to roost higher than those in the back of the tunnel and had significantly warmer roosting temperatures than those roosting on the lower two-thirds of the wall. Similar observations were made in a limestone cave in the Netherlands where bats roosting close to the cave entrance used higher roost sites than those in the back (Daan and Wichers 1968). Daan and Wichers (1968) also measured higher air temperatures near the ceiling compared to the ground in the entrance area and found that during the winter, the ceiling area near the entrance was a "condensation zone," with higher humidity levels than near the ground. While we did not measure environmental conditions at different heights in the tunnel, we found that VPD was a significant predictor of bat density, and this is another possible reason that bats roosted at higher sites in section A—tricolored bats may be using higher roost sites partly for microclimate selection, not just to avoid predation and human disturbance.

The second-best model to predict density included VPD rather than temperature variables. Tricolored bats are known to select humid hibernacula (Ploskey and Sealander 1979; Raesly and Gates 1987), and while a greater proportion of tricolored bats in Stumphouse Tunnel used colder microsites than pre-WNS, our results suggest that they still preferred areas with low VPD, possibly because of increased EWL related to WNS infection and lower tolerance for high VPD due to their small size. EWL leads to decreases in torpor bout length in other vespertilionid bats (Ben-Hamo et al. 2013; Haase et al. 2019), which in turn leads to reductions in body mass. While shorter torpor bouts could increase immune function (Forney et al. 2022), arousals from colder temperatures are more costly and may outweigh any benefits, which may partially explain why so few tricolored bats were observed in section B during the 2020 to 2021 and 2021 to 2022 hibernation seasons (0 tricolored bats in section B in 4 of 11 censuses). While section B offered similar temperatures to section A (mainly below the optimal temperatures for *Pd* growth), and a locked gate provided protection from predators and human disturbance, VPD was slightly higher in this section.

Recent hibernation research has focused on the complexity of microsite selection and the costs and benefits associated with deep torpor (Boyles et al. 2020; Johnson et al. 2021). Our results support the notion that no optimum hibernation temperature exists, even for a single species or colony, and that high VPD is likely a limiting factor, at least for small bats affected by WNS. Tricolored bats in our study used a wide variety of roost temperatures and VPD (Supplementary Data SD1 and SD2). For example, in January 2021 there was a 9.2 °C difference between the warmest and coldest roost temperatures, lending support for the need to manage and protect hibernacula that offer a variety of microclimates. Tricolored bats in a limestone mine in Ohio that was unaffected by WNS also used a variety of roost temperatures—while most were found in the warm, stable areas of the mine, there were always some individuals in the cold, less stable sections (Brack 2007). Under laboratory conditions, when tricolored bats were allowed to select between 5, 8, or 11 °C, they spent the most time at the warmest temperature, but some bats did use the colder temperatures, notably 3 males with low-fat reserves that moved to colder temperatures before dying (Boyles et al. 2022). Middle Tunnel, approximately 1 km away from Stumphouse Tunnel, was the largest Tricolored Bat hibernaculum in South Carolina pre-WNS but has not experienced the same level of recovery as Stumphouse Tunnel. It is a much shorter tunnel, and the lack of recovery may be related to the more uniform temperatures at the site.

Management of Tricolored Bat hibernation sites that focuses on protecting sites that offer a range of microclimates or a network of sites in close proximity that offer different microclimates may be helpful for recovery of this species. If warm, humid sites are an ecological trap for bats exposed to *Pd* (Hopkins et al. 2021), sites with diverse microclimates allow bats to shift to colder, drier microsites during arousals, which may decrease mortality from WNS and increase fitness of bats as they emerge from hibernation. Modification of existing hibernacula to provide cooler temperatures,

while controversial (Meierhofer et al. 2022; Sewall et al. 2022; Boyles et al. 2023), has resulted in increased counts of tricolored bats (as well as little brown and big brown bats; Turner et al. 2022). Climate change may increase the need for manipulations to buffer sites from rising and less predictable temperatures, particularly in the southern ranges of species (Loeb and Winters 2022). Manipulations near hibernacula entrances could also focus on reducing risks associated with roosting near the entrance such as predation and disturbance, where colder microsites are often located.

Supplementary data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1. Density of tricolored bats as a function of vapor pressure deficit (VPD) in Stumphouse Tunnel (Oconee County, South Carolina) during the 2020 to 2021 and 2021 to 2022 hibernation seasons (October to March).

Supplementary Data SD2. Roosting temperatures (°C) of tricolored bats by month and distance from the entrance (m) in Stumphouse Tunnel (South Carolina, United States) during the 2020 to 2021 (hib_yr = 1) and 2021 to 2022 (hib_yr = 2) hibernation seasons (October to March).

Supplementary Data SD3. Subset of individually marked Tricolored Bat locations in Stumphouse Tunnel (Oconee County, South Carolina) during the 2021 to 2022 hibernation season (October to March). The x axis represents distance from the tunnel entrance (far left = entrance, far right = back, 493 m), divided into tunnel sections. Bats labeled "green" were marked in section A and bats labeled "pink" were marked in section. Green shading indicates section A; gray shading indicates section B; pink shading indicates section C.

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Author contributions

Rebecca L. Brown: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Visualization. Susan C. Loeb: Conceptualization, Methodology, Investigation, Writing – Review & Editing, Funding acquisition. William C. Bridges: Formal analysis, Writing – Review & Editing. Shari L. Rodriguez: Writing – Review & Editing.

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Conflict of interest

None declared.

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