

## SHORT COMMUNICATION

# Temperature alone is insufficient to understand hibernation energetics

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## ABSTRACT

Energy conservation has long been a focal point in hibernation research. A long-standing assumption is that ambient temperature ( $T_a$ ) largely defines the rate of energy expenditure because of well-known relationships between  $T_a$ , metabolic rate and frequency of arousal from torpor. Body condition and humidity also affect energy expenditure but are usually considered secondary factors. We held tricolored bats (*Perimyotis subflavus*) in captivity under multiple environmental conditions to directly compare the importance of  $T_a$ , fat mass and humidity for hibernation energy expenditure. Fat mass was the best predictor of female mass loss, followed by  $T_a$  and humidity. However, males had less fat and adopted a more energetically conservative hibernation strategy. Our results demonstrate that understanding the evolution of behavior, physiology and ecology of hibernation requires disentangling the relative contributions of multiple drivers of hibernation energetics, and that  $T_a$  is not always the most important factor driving energy expenditure.

**KEY WORDS:** Body composition, Torpor, *Perimyotis subflavus*, Potential evaporative water loss, Water vapor deficit, White-nose syndrome

## INTRODUCTION

Hibernation has long been considered primarily an energy conservation strategy used by some mammals (and at least one bird) during periods of low energy availability. During hibernation, periods of energetically conservative torpor are regularly interrupted by energetically expensive euthermic arousals. Both torpid metabolic rate (TMR) and arousal frequency are strongly affected by ambient temperature ( $T_a$ ), so seasonal hibernators often choose cool hibernacula to take advantage of  $Q_{10}$  effects that cause chemical processes to slow as body temperature drops near  $T_a$  (Nedergaard and Cannon, 1990). Most studies on energy expenditure during hibernation have therefore assumed energy expenditure over the course of a season of hibernation can be effectively estimated with a few basic parameters describing  $T_a$ , TMR and arousal frequency (e.g. Boyles and Brack, 2009; Humphries et al., 2002).

In addition to  $T_a$ , a host of other factors, both endogenous and exogenous, likely play underappreciated roles in determining energy expenditure during hibernation. For example, dehydration might play

a role in arousal frequency (Thomas and Geiser, 1997) and increased arousals associated with water loss might drive increased energy expenditure (Ehlman et al., 2013). In socially hibernating species, clustering during hibernation can decrease energy expenditure through social thermoregulation (Boyles et al., 2008) and reduced water loss (Boratyński et al., 2015), or can increase energy expenditure if disturbances from other individuals cause increased arousal frequency (Turner et al., 2015). Further, hibernators may not attempt to maximize energy conservation (Boyles et al., 2020; Humphries et al., 2003), and individuals in better condition (i.e. with larger energy stores) often make behavioral decisions that lead to increased energy expenditure (Boyles et al., 2007; Wojciechowski et al., 2007). Although experiments have tested the individual effects of water loss, social interactions and behavioral decisions on energy expenditure during hibernation, few have been designed to evaluate the interactions and relative importance of these or other factors on seasonal energy expenditure.

Bats are increasingly a preferred model taxon for studying the physiology and ecology of hibernation because they are amenable to a wide range of lab and field studies (Boyles et al., 2020). Interest in the hibernation dynamics of bats has further grown over the last decade since the discovery of white-nose syndrome (WNS), a catastrophic disease caused by the fungal pathogen *Pseudogymnoascus destructans* (Frick et al., 2010; Warnecke et al., 2012). Infection with *P. destructans* leads to a cascading series of physiological and behavioral changes in hibernating bats that ultimately leads to death (Field et al., 2018; Langwig et al., 2012; Warnecke et al., 2013). However, species are affected by WNS to different degrees, with some species suffering catastrophic population declines while others are relatively unaffected (Frick et al., 2015; Langwig et al., 2016; Moore et al., 2018). Furthermore, susceptibility varies among individuals and there is growing evidence of remnant populations and individuals that survive multiple winters of infection (Dobony et al., 2011; Kurta et al., 2020). Fat is an important predictor of WNS persistence (Cheng et al., 2019) but temperature and humidity conditions of hibernacula have also been implicated in both empirical studies (Johnson et al., 2014; Langwig et al., 2012, 2016) and theoretical models (Haase et al., 2019). Therefore, identifying the relative effects of temperature, humidity and body condition and how these contribute to the energetic demand of hibernation is essential for understanding the basic biology of hibernation, with important conservation implications for WNS (Willis, 2015).

We collected detailed energetic data on captive hibernating tricolored bats, *Perimyotis subflavus* (Cuvier 1832), held in hibernation chambers covering a range of realistic temperature and humidity conditions as treatments. Factors in our analysis may influence hibernation energetics in multiple ways. For example, bats with larger fat stores may maintain higher torpid metabolic rate (directly increasing energetic cost), and may also arouse more frequently (indirectly increasing energetic cost). Warmer temperatures likely increase TMR and arousal frequency. Similarly,

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increased potential evaporative water loss (pEWL) may increase arousal frequency, but it is unclear whether pEWL has direct effects on energy balance. Our study design allowed us, for the first time, to experimentally measure the interactive effects of  $T_a$ , humidity and individual body composition on energy expenditure (measured through change in body mass) across an entire hibernation period.

## MATERIALS AND METHODS

### Ethics statement

All methods in this study were approved by the Institutional Animal Care and Use Committee at Texas Tech University (protocol 18032-12). Fieldwork was approved by the Mississippi Department of Wildlife, Fisheries and Parks (permit 1115181)

### Study species and handling procedures

We collected 70 tricolored bats from three culverts in Mississippi (approximately 32°N, 91°W; exact locations withheld) on 10 December 2018, following US Fish and Wildlife Service guidelines for decontamination and preventing the spread of WNS (United States Fish and Wildlife Service, 2018; <https://www.whitenosesyndrome.org/static-page/decontamination-information>). We placed bats in cloth bags inside temperature-controlled coolers and transported them directly to Texas Tech University. Immediately upon arrival, we took morphometric measurements (body mass  $\pm 0.1$  g, forearm length  $\pm 0.1$  mm) and used quantitative magnetic resonance (QMR; Echo-MRI-B, Echo Medical Systems, Houston, TX, USA) to determine initial fat and lean mass (McGuire and Guglielmo, 2010). We attached a uniquely marked, modified (Lovegrove, 2009; Reeder et al., 2012) datalogger (DS1925L iButton, Maxim Integrated, San Jose, CA, USA) to the back of each bat using ostomy cement. We inoculated each bat by spreading 20  $\mu$ l of *P. destructans* solution ( $5 \times 10^5$  conidia  $\mu$ l<sup>-1</sup>) evenly across both wings, following established protocols (Lorch et al., 2011; McGuire et al., 2017; Warnecke et al., 2012).

### Environmental chambers and captive hibernation

Bats were housed across seven environmental chambers (Model 7000-33-1, Caron, Marietta, OH, USA) to create a blocked design, controlling temperature and humidity in each chamber (sample sizes and treatments indicated in Fig. 1). To test for cage effects (random differences among cages), we divided bats in each chamber into two cages (23 $\times$ 38 $\times$ 50 cm) constructed from mesh fabric (Part FMLF,

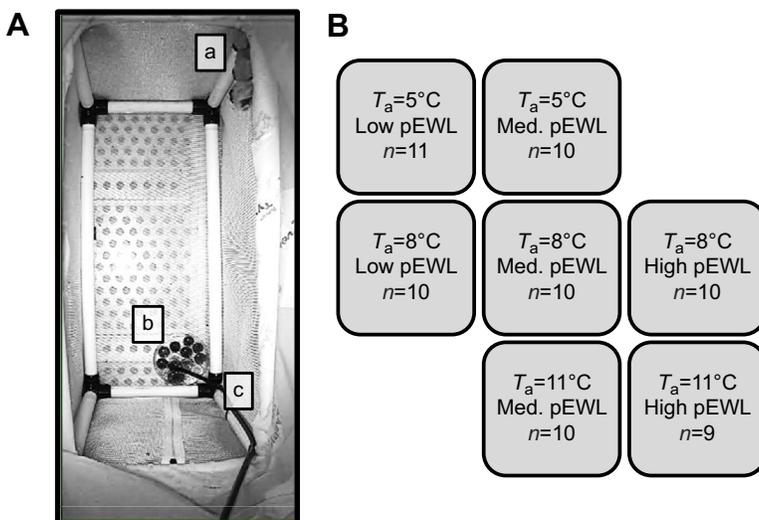
Seattle Fabrics, Inc., Seattle, WA, USA), PVC pipe and plastic sheeting. We provided *ad libitum* drinking water (Fig. 1A) but did not provide food for the duration of the 83–87 days experimental hibernation period. We assigned bats to microclimate treatments in a stratified manner to ensure equal distribution of starting body mass. Body mass was greater in females than in males, and therefore stratifying by body mass also ensured all treatments included both sexes.

We set humidity to achieve specific levels of water vapor deficit (WVD) between the surface of the bat and the environment (Kurta, 2014). Higher WVD is indicative of drier air, and thus results in higher pEWL. The seven microclimate treatments were combinations of  $T_a=5, 8$  or  $11^\circ\text{C}$  and  $\text{WVD}=0.05, 0.10$  or  $0.15$  kPa (Fig. 1B). The environmental chambers control relative humidity (RH) in whole percentage points, which we set to the value closest to target WVD. We verified the  $T_a$  and RH in each chamber at 10 min intervals (Hobo Model U23-001, Onset Computer Corporation, Bourne, MA, USA). One environmental chamber ( $11^\circ\text{C}$ , high pEWL) had unexplainably higher variation in temperature (fluctuations of  $\sim 2^\circ\text{C}$ ) than the other chambers (fluctuations  $< 0.3^\circ\text{C}$ ), but analysis excluding this chamber yielded the same qualitative results reported here.

A motion-activated infrared camera (Model HT5940T, Speco Technologies, New York, NY, USA) was secured above each cage to monitor bats throughout hibernation. Within the first few days of the experiment, the camera in one chamber stopped working so we combined the bats for that treatment into a single cage. We replicated this disturbance among all environmental chambers to ensure the unanticipated entry into the environmental chamber did not bias our results. We reviewed video recordings daily throughout hibernation to monitor bats without disturbance. We noted any bats that aroused for later cross-referencing with skin temperature data.

### Quantifying periodic arousals

We quantified arousals based on skin temperature ( $T_{sk}$ ) and video recordings (Fig. 1A). To allow adjustments (i.e. bats settling into new cages) and disturbances (as bats were placed into, or removed from environmental chambers) that may have occurred on the first and last days of the experiment, we excluded arousals before midnight on the first day, and after midnight on the final day of hibernation. None of our analyses required precise determination of arousal durations; therefore, we used a simple temperature threshold



**Fig. 1. Experimental setup.** (A) Screenshot of a cluster of hibernating bats (a) in the top corner of the cage. Fresh water was provided in a dish (b) supplied by a tube (c) running outside the chamber to avoid disturbance to hibernating bats. (B) We housed bats in 7 environmental chambers with combinations of three temperatures (5, 8,  $11^\circ\text{C}$ ) and three air saturation levels. Water loss conditions were based on a water vapor deficit of 0.05 kPa (low potential evaporative water loss, pEWL), 0.10 kPa (medium pEWL) or 0.15 kPa (high pEWL).

method for initial identification of arousals. Differences in datalogger attachment (e.g. placement, thickness of fur, amount of glue) can result in differences in  $T_{sk}$  measurement; therefore, we defined the arousal threshold based on median  $T_{sk}$  for each bat individually. We initially identified arousals when  $T_{sk}$  increased  $7^{\circ}\text{C}$  above median  $T_{sk}$  then cross-referenced these arousals with those observed on video. We are confident we recorded all arousals using these two methods in conjunction.

Despite inoculation with a standard dose of *P. destructans*, there were few signs of morbidity and only five bats displayed standard histopathological signs of WNS disease (W.F.F., E.M.J., J.G.B. and L.P.M., unpublished observations). Thus, we are confident the hibernation dynamics described herein are representative of healthy individuals with little (if any) influence from *P. destructans* inoculation. Three bats died before the end of the experiment (all from separate cages) and were excluded from the analysis, but there was no indication that WNS was a contributing factor.

### Statistical analysis

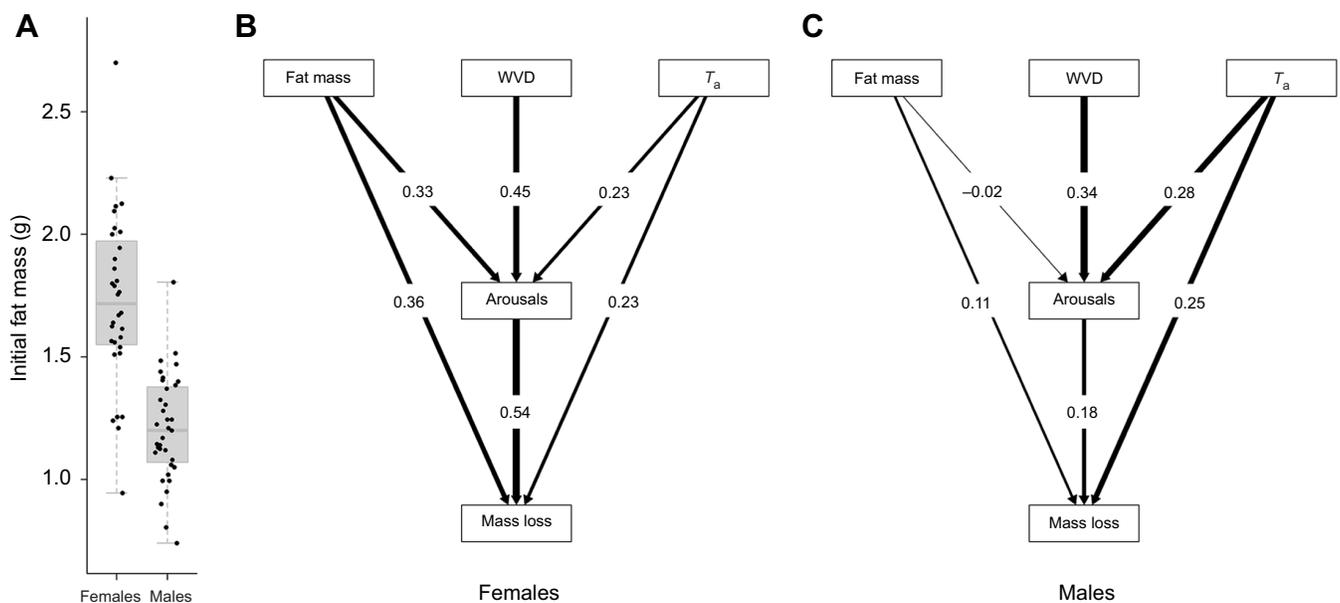
Mass loss did not differ between cage replicates in any treatment ( $t$ -tests, all  $P>0.3$ ) and therefore we lumped cages for analysis. To account for potential direct and indirect relationships among variables, we used structural equation modeling (lavaan package in R; Rosseel, 2012). Sex and body condition were confounded in our dataset with little overlap between females (fat) and males (lean) (Fig. 2A). Therefore, to address both condition and sex, we first determined optimal model structure using the full dataset of all bats, and then used that model structure to compare separate models of males and females. We fitted a saturated model (all values centered and scaled) of mass loss as related to number of arousals, and direct and indirect effects of initial fat mass,  $T_a$  and WVD. From the saturated model, we identified individual regression relationships that may not be supported in the model ( $P>0.05$ ) and fitted candidate models excluding all combinations of these relationships.

We assessed fit metrics and modification indices and excluded candidate models that did not produce a good fit (Cangur and Ercan, 2015; Grace, 2015; Grace et al., 2012). We used changes in corrected Akaike's information criterion,  $\Delta\text{AICc}$  (models with  $\Delta\text{AICc}<2$  considered equivalent) and model weight to determine the best model. With the best-fit model structure determined from the overall dataset applied to each sex separately, comparison of these models indicates similarities and differences in the hibernation of each sex, while the influence of body condition can be assessed within each sex-specific model.

### RESULTS AND DISCUSSION

At the beginning of the experiment, females ( $6.6\pm 0.1$  g, mean $\pm$ s.e.m.,  $n=32$ ) were heavier than males ( $5.7\pm 0.1$  g,  $n=35$ ;  $t_{55.2}=7.0$ ,  $P<0.0001$ ) and had more fat (Fig. 2A; females:  $1.73\pm 0.06$  g, males:  $1.21\pm 0.04$  g;  $t_{51.2}=7.2$ ,  $P<0.0001$ ). Bats aroused from hibernation between 4 and 16 times but there was not a detectable difference in the number of arousals between males and females (females:  $8.8\pm 0.5$  arousals, males:  $7.9\pm 0.3$  arousals;  $t_{53.7}=1.6$ ,  $P=0.11$ ). At the end of the experiment, males continued to weigh less (females:  $5.4\pm 0.1$  g, males:  $4.7\pm 0.1$  g;  $t_{63.0}=5.4$ ,  $P<0.0001$ ) and had less fat (females:  $0.54\pm 0.03$  g, males:  $0.34\pm 0.03$  g;  $t_{61.6}=4.4$ ,  $P<0.0001$ ) than females.

Of three candidate models, two failed multiple fit tests and were excluded. The remaining candidate model was better supported than the saturated model (AICc model weight=0.77, saturated model  $\Delta\text{AICc}>2$ ). This model included  $T_a$  and initial fat mass as both direct effects (increased mass loss in fatter bats:  $P<0.001$ , and at warmer  $T_a$ :  $P=0.03$ ) and indirect effects mediated by arousal frequency (fat:  $P=0.003$ ,  $T_a$ :  $P=0.02$ ), but WVD only influenced mass loss through the indirect effect of increased arousals at higher WVD ( $P<0.001$ ). The direct effect of WVD on mass loss was not supported, and candidate models including this term had higher AICc values and violated multiple fit metrics. The relative importance of the factors



**Fig. 2. Fat mass and model results.** (A) Females had more fat than males, with little overlap between the sexes. (B) Initial fat mass had the greatest total effect in females. Initial fat mass and ambient temperature ( $T_a$ ) had both direct and indirect effects on body mass loss (greater body mass loss at higher temperatures and in individuals with more fat), but pEWL (water vapor deficit, WVD) only indirectly affected body mass loss through increased arousal frequency. (C) Among males, the effect of temperature was similar to the effect in females, but initial fat mass had only a minor impact. Notably, the relationship between arousal frequency and mass loss was weaker in males, suggesting males with smaller fat stores compensate for increased arousal frequency with a more energetically conservative hibernation strategy. Values in each model are standardized parameter estimates.

included in the final model varied between female (fat) and male (lean) bats (Fig. 2A). Among females, initial fat mass had the largest total effect (combined standardized coefficients of direct and indirect effects=0.54) followed by  $T_a$  (0.35) and WVD (0.24) (Fig. 2B). In males,  $T_a$  was the most important factor (total effect of  $T_a$  for males=0.30). In contrast with females, initial fat mass had minimal impact on mass loss in males with comparatively smaller fat stores (total effect=0.11) (Fig. 2C). Greater WVD led to increased arousal frequency in both sexes, but the relationship of arousal frequency to mass loss was comparatively weak in male (lean) bats.

Energy expenditure of tricolored bats during hibernation was clearly determined by more than  $T_a$ . Using an experimental design that permitted disentangling effects that are usually correlated ( $T_a$  and relative humidity; Kurta, 2014), we found that  $T_a$ , humidity and body condition play interacting roles in energy expenditure during seasonal hibernation. Whether as a result of sex-specific strategies or sexual dimorphism in body condition, the relative influence of these factors varied between sexes. Multiple studies have previously confirmed that each of these factors can independently affect energy expenditure in hibernating mammals (Nedergaard and Cannon, 1990; Thomas and Geiser, 1997; Wojciechowski et al., 2007) but  $T_a$  is usually assumed to be the single largest driver of energy expenditure. Our result that body condition and water loss were equally or more important than  $T_a$  in determining mass loss among females was surprising. Previous studies have shown individuals in better body condition choose microclimates that likely lead to increased energy expenditure (Boyles et al., 2007; Wojciechowski et al., 2007). Here, we have shown that even with no opportunity to behaviorally select microclimates to alter the rate of energy expenditure, females in better body condition lost more mass than females in poorer condition. This relationship was manifested through a direct effect of initial body condition on mass loss (likely as increased whole-animal TMR) and indirectly through increased arousals. Males did not follow this pattern and adopted a more energetically conservative strategy than females. Males had smaller fat stores than females but aroused just as frequently. The weaker relationship between arousal frequency and mass loss suggests males compensated for the energetic cost of arousals (i.e. adopted alternative energy savings strategies) to maintain an energetically conservative hibernation strategy.

The energetic strategies adopted by males and females in our study are counter to those observed in other populations of hibernating bats, where females are the energetically thrifty sex (Czenze et al., 2017; Jonasson and Willis, 2011). This may be a difference between species (the previous studies on the topic were on *Myotis lucifugus*), but we suspect our observation relates to differing reproductive constraints on males and females in different areas. The thrifty female studies (Czenze et al., 2017; Jonasson and Willis, 2011) were performed in Manitoba, Canada, where hibernation duration is estimated to be 199 days (Hranac et al., 2021). At high latitudes, environmental demands may approach the limits of physiological capacity, forcing females to be energetically conservative if they are to reproduce successfully. As the minimum amount of fat required for hibernation approaches the maximum amount of fat that can be deposited, females must adopt thrifty hibernation strategies to ensure they emerge from hibernation in sufficient condition for reproduction in the short northern active season. Conversely, our study was conducted near the southern geographic edge of hibernating bats in North America. The predicted hibernation duration in Mississippi is only 69 days (Hranac et al., 2021) and therefore it may not be a physiological challenge to

deposit excess fat relative to the minimum required to survive the shorter duration hibernation. The discrepancy between physiological capacity and environmental demand at southern latitudes likely allows for flexibility in hibernation strategies, as reflected by the relationship between initial fat mass and hibernation energy expenditure of females. Females with relatively small fat stores at the beginning of hibernation spent comparatively little energy, while females with very large fat stores lost more mass through hibernation. However, this relationship was only observed among females. Male tricolored bats in our study had less fat than females, and initial fat mass was not a strong predictor of hibernation energy expenditure, indicating that males adopt a different hibernation strategy from females. Tricolored bats in this southern population hibernate in small groups across the landscape, and males may move repeatedly throughout the winter to mate. Males can forage regularly while doing so (Bernard and McCracken, 2017), and it may be unnecessary to build large fat stores before hibernation if these bats rely in part on foraging throughout winter.

Our results verify that cold, high humidity hibernacula allow for energetically conservative hibernation. This is expected, but under natural conditions, sites that both maximize energy conservation and minimize water loss may be rare. For example, across hibernacula in Michigan, the coolest sites tended to be those where potential water loss was highest (Kurta and Smith, 2014). Our personal experience suggests a similar pattern is common within hibernacula as well. Thus, relatively cool and humid sites may exist, but it is unlikely that any single microsite to which a bat has access would have both the single and stable temperature that minimizes energy expenditure and humidity conditions that result in minimal pEWL. There will likely be sites that are either cooler and more stable or have lower WVD. In both scenarios, hibernating bats are faced with a trade-off between energy conservation and water loss (i.e. both cannot be maximized simultaneously). Species prone to dehydration, such as tricolored bats (Davis, 1970), are likely to choose hibernacula or microsites that balance energy loss driven by water loss and energy loss driven by temperature effects. Brack (2007) noted that tricolored bats concentrated in the warmest areas of a large limestone mine and were often found covered with condensation. Species less prone to dehydration may be more flexible in microsite requirements, and thus choose cooler, drier areas in subterranean roosts.

Under some circumstances, energy conservation may be vital for survival of individual bats that are in poor body condition, such as bats with WNS. Our results suggest cool hibernacula with low potential water loss (high humidity) may be energetically favorable for bats in these circumstances. Mitigation strategies that consider manipulating hibernaculum microclimate to improve conditions for hibernation should consider both temperature and humidity. Artificially minimizing hibernacula temperature at the cost of drier air might actually increase energy expenditure in some dehydration-prone species such as tricolored bats. Indeed, WNS is a complicated conservation problem because it affects both energetics and water balance (McGuire et al., 2017), and differences in susceptibility are likely to be related to variation among species in these factors. Our findings bely the traditional understanding of hibernation energetics as being largely driven by  $T_a$  and demonstrate the need to consider a range of factors in both hibernation studies and conservation of hibernating mammals.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: L.P.M., W.F.F., J.G.B.; Methodology: L.P.M., E.M.J., J.G.B.; Formal analysis: L.P.M., E.M.J., J.G.B.; Investigation: L.P.M., E.M.J., W.F.F., J.G.B.; Resources: L.P.M., W.F.F.; Data curation: L.P.M., E.M.J.; Writing - original draft: L.P.M., J.G.B.; Writing - review & editing: L.P.M., E.M.J., W.F.F., J.G.B.; Supervision: L.P.M.; Project administration: L.P.M., E.M.J., W.F.F., J.G.B.; Funding acquisition: L.P.M., W.F.F., J.G.B.

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#### Data availability

Data are available from the Dryad digital repository (McGuire et al., 2021): dryad.fj6q573s8

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