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RESEARCH ARTICLE | *Obesity, Diabetes and Energy Homeostasis*

White-nose syndrome increases torpid metabolic rate and evaporative water loss in hibernating bats

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McGuire LP, Mayberry HW, Willis CKR. White-nose syndrome increases torpid metabolic rate and evaporative water loss in hibernating bats. *Am J Physiol Regul Integr Comp Physiol* 313: R680–R686, 2017. First published August 23, 2017; doi:10.1152/ajpregu.00058.2017.—Fungal diseases of wildlife typically manifest as superficial skin infections but can have devastating consequences for host physiology and survival. White-nose syndrome (WNS) is a fungal skin disease that has killed millions of hibernating bats in North America since 2007. Infection with the fungus *Pseudogymnoascus destructans* causes bats to rewarm too often during hibernation, but the cause of increased arousal rates remains unknown. On the basis of data from studies of captive and free-living bats, two mechanistic models have been proposed to explain disease processes in WNS. Key predictions of both models are that WNS-affected bats will show *1*) higher metabolic rates during torpor (TMR) and *2*) higher rates of evaporative water loss (EWL). We collected bats from a WNS-negative hibernaculum, inoculated one group with *P. destructans*, and sham-inoculated a second group as controls. After 4 mo of hibernation, TMR and EWL were measured using respirometry. Both predictions were supported, and our data suggest that infected bats were more affected by variation in ambient humidity than controls. Furthermore, disease severity, as indicated by the area of the wing with UV fluorescence, was positively correlated with EWL, but not TMR. Our results provide the first direct evidence that heightened energy expenditure during torpor and higher EWL independently contribute to WNS pathophysiology, with implications for the design of potential treatments for the disease.

Myotis lucifugus; hibernation; *Pseudogymnoascus destructans*; respirometry

FUNGAL DISEASES OF WILDLIFE are on the rise worldwide (13). In contrast to viral and bacterial pathogens, which often lead to systemic infections, fungal pathogens of animals often manifest as superficial skin infections, especially among poikilothermic species. Although typically limited to infecting skin, fungal pathogens can lead to devastating physiological impacts and fatal disease across a range of taxa (1, 4, 5, 35, 36). A mechanistic understanding of pathogenesis in fungal diseases of wildlife is critical for understanding and predicting population-level impacts and developing safe and effective mitigation and management strategies.

White-nose syndrome (WNS), caused by the fungus *Pseudogymnoascus destructans*, is a recently emerged disease of hibernating bats (5) (25, 42). Since its discovery in 2007, millions of bats have been killed in eastern and central North America, leading to dramatic population declines (16) and the possibility of regional extinctions (15). Recent reviews have summarized our understanding of disease mechanisms in WNS (17, 45). A number of putative virulence factors have now been identified (14, 29), and studies of both captive (42) and free-living (33) bats indicate that the disease causes increased frequency of arousals from torpor during hibernation, emaciation, and death. Infected bats also exhibit signs of altered fluid, electrolyte, and pH balance (10, 11, 26, 41, 43), leading to development of two complementary mechanistic models of WNS pathophysiology (41, 43).

Symptoms of WNS develop in a progressive manner (26), and the most pronounced symptoms are only apparent relatively late in hibernation (41, 43). Increased arousal frequency and arousal cascades that may reflect conspecific disturbances (40) in later stages of infection lead to dramatic increases in energy expenditure and are thought to be a primary cause of emaciation (43). This pattern is described in a pathophysiological model proposed by Warnecke et al. (43). The model proposes that lesions in wing tissue, which occur in later stages of fungal infection, lead to altered blood chemistry and hematology and increased water loss, respiratory rate, and energy consumption. More recently, however, Verant et al. (41) found evidence of increased energy turnover at an earlier stage of disease, before a detectable increase in arousal frequency was observed. They proposed a model of earlier-stage disease based on these findings. Their model suggests that increased metabolic rate following initial tissue invasion, combined with reduced excretion of CO_2, initiates the cascade of physiological responses observed by Warnecke et al. (15) in the final stages of WNS (16). Two key elements of both models are increased energy expenditure and disruption of osmotic homeostasis (41, 43). Although the cause of increased arousal frequency is unknown, observations of electrolyte and fluid depletion (10) led to the dehydration hypothesis (11, 46) that fluid loss across fungal lesions on the skin increases rates of water loss, resulting in increased arousal frequency and energy depletion. In healthy hibernators, ambient humidity and evaporative water loss (EWL) affect torpor bout duration (3, 38), which suggests that increased EWL due to wing damage could trigger increased arousal frequency and mortality in WNS (46). Thus, understanding the impacts of WNS on energy expenditure and water loss, as predicted by both pathophysiological models of WNS published to date, is a critical step in under-

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standing the mechanism by which fungal infection leads to bat mortality.

We conducted an experimental inoculation to test the hypothesis that WNS causes increased energy expenditure and EWL during torpor bouts, as predicted by mechanistic models of WNS (41, 43). Specifically, we predicted that bats inoculated with *P. destructans* would have *1*) higher torpid metabolic rate (TMR) and *2*) increased EWL compared with healthy controls. We also measured TMR and EWL in both dry and humidified air to assess the impact of environmental conditions on WNS pathophysiology.

METHODS

Animal collection and housing. Twenty-eight male little brown bats (*Myotis lucifugus*) were collected from a WNS-negative hibernaculum in central Manitoba, Canada on 7 November 2013, transported to the University of Winnipeg, and randomly assigned to a control ($n = 14$) or a treatment ($n = 14$) group matched for body condition. Bats in the treatment group were inoculated with *P. destructans*, and controls were sham-inoculated, as described previously (42). The two groups were placed in separate nylon mesh cages (23 × 38 × 38 cm; modified from Exo Terra, Rolf C Hagen, Montreal, PQ, Canada) in an environmental chamber (model 6040-1, Caron, Marietta, OH) maintained at 7°C and 98% relative humidity (RH; i.e., water vapor pressure = 0.982 kPa). Water was provided ad libitum in a dish that was flushed weekly with fresh water using tubes that ran outside the chamber to avoid disturbance. Hibernating bats were monitored by infrared motion-activated video cameras (model HT6501RVFHQ, Speco Technologies, New York, NY). The two cages were placed beside each other (within a few centimeters) in a single environmental chamber to minimize potential confounding cage effects. Thus, any disturbances or environmental fluctuations that could have influenced our results were experienced equally by both groups. Within the 1st wk of hibernation, there was an unavoidable disturbance to repair one of the cages, but this repair was completed as quickly and as quietly as possible. The only other potential disturbances occurred when individual animals met humane intervention criteria for assessment [i.e., isolated from other bats, low in the cage with wings spread out (42)]. Three bats in the inoculated group met these criteria before termination of the experiment and were removed. Any disturbance was always replicated in both experimental cages, and, apart from these events, hibernating bats were not disturbed for >113 days. The forearm and muzzle of each bat were swabbed to test for *P. destructans* DNA (9) to confirm that all bats were WNS-negative before our study.

We used infrared videos to identify arousals of individual bats. In multiple previous experiments when we have recorded skin temperature alongside video recording, our motion-activated video system has never failed to detect an arousal by any hibernating bat in this experimental setup (6, 44). We used a linear mixed-effects model to test for differences in torpor bout duration (and, hence, arousal frequency) between inoculated and control groups. We recorded the duration of all torpor bouts that occurred in the final 50 days of hibernation before respirometry (fewer days for bats removed for humane intervention), and tested for effects of date and treatment, including individual as a random effect to account for repeated measures. We compared rate of mass loss between infected and control bats using a *t*-test.

Respirometry. All bats were kept under the same environmental conditions for the duration of captive hibernation (7°C, 98% RH). After ~4 mo of hibernation, the bats were removed from the environmental chamber (details below) for measurement of TMR and EWL by open-flow respirometry. We measured TMR and EWL under two conditions: humidified air and dry air. The comparison of dry and humidified air conditions allowed us to assess potential impacts of changes in environmental humidity on bats suffering from WNS, either by change in microclimate selection by the bat or, perhaps, environmental manipulation as a mitigation strategy.

Our open-flow respirometry system was configured to record up to seven animal chambers plus a baseline channel. The building pressurized air system was connected to a CO_2-adsorbing system (PCDA series, PureGas, Broomfield, CO) and passed through a column of $MgClO_4$ to provide dry, CO_2-free air for the respirometry system. Air was saturated by flow through a bubbler, and RH was reduced to ~65% (0.7059 kPa) with a custom dew point generator (Polystat 12101-00, Cole-Parmer). A series of valves allowed us to supply either dry (bypassing bubbler and dew point generator) or humidified air to the animal chambers. A flow controller (model FB8, Sable Systems) regulated flow to each chamber, and all chamber temperatures were monitored with thermocouples (model TC-2000, Sable Systems). Animal chambers (370 ml volume) were housed in a temperature-controlled (8°C) cabinet. Hardware cloth (1/2 inch) was used to provide a roosting surface inside each chamber and to separate bats from a layer of mineral oil in the bottom of the chamber to prevent introduction of humidity from excreta. In-line HEPA syringe filters were placed before and after each animal chamber to prevent *P. destructans* contamination. Animal chambers were sequentially subsampled with a multiplexer (MUX, Sable Systems). The subsampled excurrent airflow was analyzed for water vapor (model RH-300, Sable Systems) before passing through a gas dryer (model ND2, Sable Systems) and $MgClO_4$ column. Finally, gas analyzers recorded CO_2 (model CA-10, Sable Systems) and O_2 (model FC-2, Sable Systems, operating in differential mode). All instruments were calibrated before measurements. Prior to measuring animals we determined the washout time for our chambers and set measurement durations accordingly (washout time <60 s, measurement time = 10 min, flow rate = 100 ml/min) (23).

We transferred groups of five to seven bats from their cage in the environmental chamber to individual respirometry chambers to measure TMR and EWL. Thus hibernation duration was 114 or 118 days for inoculated bats and 121 or 125 days for controls. We transferred bats in the evening and allowed bats to return to torpor overnight under the humidified airstream. Recording of TMR and EWL under the humidified air condition (excurrent water vapor pressure = 0.8474 ± 0.010 kPa, 81.9% RH) began on the following morning, and continued for 24 h. On the second morning we switched to a dry incurrent airstream. Dry air measurements continued overnight, except for one group of five inoculated bats. Bats in this group began to arouse, and therefore we ended the experiment after 6 h of dry air measurement. All bats were weighed (±0.1 g) before and after respirometry measurements, and rectal temperature (±0.1°C; model 80005, Sper Scientific, Scottsdale AZ) of each bat was recorded upon removal from respirometry. We used a UV lightbox (9-W, 368-nm wavelength; model BM100, Way Too Cool, Glendale, AZ) and overhead UV light to take photographs of the wings of each bat at the end of hibernation to document fluorescence characteristic of WNS (26). We measured the area of the wing affected by fluorescence in ImageJ (v1.50i) as described previously (26) and compared measured values with those reported by McGuire et al. (26) to provide a qualitative indication of disease severity for bats in our study. After UV photography, we provided all bats with dilute (1:1) unflavored Pedialyte (Abbott Nutrition, Abbott Laboratories, Columbus, OH) and Nutrical (Albrecht, Germany) nutrient supplement. All bats were then transferred to a different area of the captive colony for rehabilitation and a separate study of recovery and healing.

Analysis. We analyzed all respirometry files in Expedata (v1.3.0, Sable Systems). We corrected for lag and drift and then calculated CO_2 production using *Eq. 10.4* from Ref. 23. We converted to TMR in mW assuming fat oxidation (respiratory quotient = 0.71; see Supplemental Material for this article, available at the *American Journal of Physiology-Regulatory, Integrative, and Comparative Physiology* website). We calculated EWL using *Eq. 10.9* from Ref. 23. We used

Table 1. *Summary of experimental results*

Treatment	Body Mass, g		Late Hibernation Torpor Bout Duration, days	Respirometry Condition	TMR, mW	EWL,mg H_2O/h
	Capture	Final hibernation				
Inoculated	10.2 ± 0.2	7.7 ± 0.2	6.4 ± 0.6	Wet (n=11)	3.90 ± 0.28	6.14 ± 0.61
				Dry (n= 9)	3.74 ± 0.25	16.57 ± 1.37
Control	10.1 ± 0.2	7.6 ± 0.2	9.2 ± 0.3	Wet (n=13)	3.24 ± 0.13	4.00 ± 0.27
				Dry (n=13)	2.34 ± 0.17	10.67 ± 0.56
Significance	P = 0.22	P = 0.59	$P < 0.0001$		Treatment $P < 0.0001$	Treatment P = 0.015
					Condition $P < 0.0001$	Condition $P < 0.0001$
						Interaction P = 0.010

Values are means ± SE. TMR, torpid metabolic rate; EWL, evaporative heat loss. Final hibernation body mass represents body mass upon removal from hibernation for respirometry. Late hibernation torpor bouts were quantified in the 50 days before respirometry measurements (*days 63–113* of hibernation). In late hibernation, inoculated bats had shorter torpor bouts and, hence, aroused more frequently than controls. There was no difference in initial body mass, but the rate at which inoculated bats lost mass approached significance (P = 0.10).

repeated-measures ANOVAs to test for effects of treatment (inoculated or control), condition (wet air or dry air), and body mass on TMR and EWL.

EWL will increase as a result of increasing TMR (31), but we wanted to test whether inoculation had a direct effect on EWL, independent of the effect of increased TMR. Therefore, we used structural equation modeling [lavaan package in R (34)] to compare models with only indirect (i.e., via increased TMR) and both indirect and direct effects of inoculation on EWL. Model specification and selection followed (19, 20), with the lavaan.survey package (30) used to adjust for repeated measures.

Finally, we used linear regression to test whether TMR or EWL was related to disease severity, as indicated by the percentage of wing area with UV fluorescence at the end of hibernation. All statistical analysis was performed in R (37). The experiment was approved by the University of Winnipeg Animal Care Committee and conducted under Manitoba Conservation Wildlife Scientific Permit WB15396.

RESULTS

All inoculated bats exhibited the obvious UV fluorescence characteristic of *P. destructans* infection (19.2 ± 4.5% of visible wing area, max = 47.8%). This suggests that bats in our study were in relatively late stages of infection, with many observed fluorescence values among the highest reported by McGuire et al. (26). All control bats hibernated normally, and none exhibited UV fluorescence. Inoculated bats had shorter torpor bouts and, hence, aroused more frequently than controls (Table 1; likelihood ratio = 18.10, df = 1, $P < 0.0001$). The difference in rate of mass loss between inoculated and control bats approached significance (1-tailed t-test, $t_{21.9}$, P = 0.10), with inoculated bats tending to lose mass at a greater rate than controls.

Respirometry data were compromised for one control bat due to an air leak, and two inoculated bats never entered deep, steady-state torpor, based on their elevated and variable CO_2 production during dry air measurements. Therefore, we excluded those values from our analysis. TMR was higher in bats inoculated with *P. destructans* than in controls ($F_{1,20}$ = 23.75, $P < 0.0001$) and was also higher in humidified air ($F_{1,20}$ = 31.54, $P < 0.0001$; Fig. 1*A*, Table 1). EWL was also higher in inoculated bats than controls ($F_{1,20}$ = 7.06, P = 0.015) and, not surprisingly, was higher in dry air ($F_{1,20}$ = 44.97, $P < 0.0001$; Table 1). A treatment*condition interaction ($F_{1,20}$ = 8.15, P = 0.010) indicated that the increase in EWL was greater for inoculated bats in dry air than for control bats (Fig. 1*B*).

The structural equation model that included both direct and indirect effects of treatment on EWL (Fig. 2) was better supported than a model in which EWL was affected only by TMR (ΔAICc = 9.69, χ^2_{diff} = 10.1, df = 1, P = 0.0015). There was not strong support for including a relationship between TMR and EWL in our structural equation model (z = 1.11, P = 0.26), but we elected to retain this relationship because of its likely biological importance. This analysis supports the conclusion that EWL was greater in bats infected with *P. destructans* than would be expected due to increased TMR alone.

We used the area of the wing showing UV fluorescence as an index of disease severity (as in Ref. 26) to test for the influence of disease severity on TMR and EWL. We report the relation-

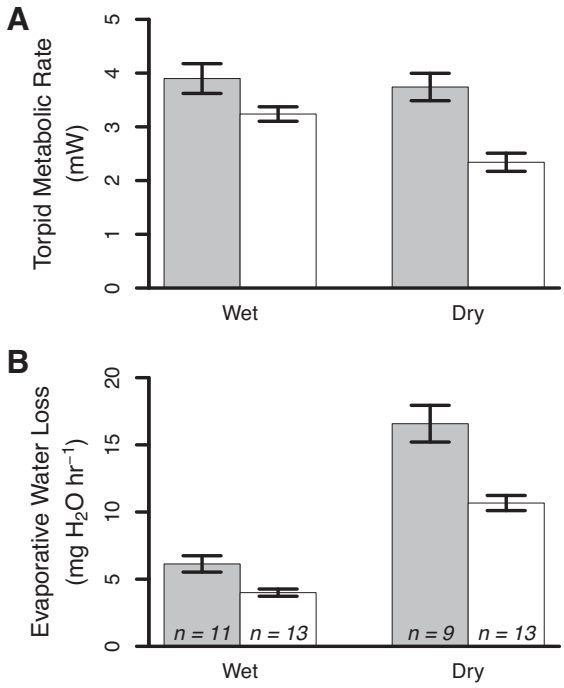

Fig. 1. Physiological responses of hibernating bats to *P. destructans* infection. *A*: torpid metabolic rate (TMR) was higher in bats inoculated with *P. destructans* (gray bars) than controls (white bars) and higher under the wet air than dry air condition. *B*: evaporative water loss (EWL) was higher in inoculated bats than controls, but an interaction indicated a greater increase in EWL in dry air for inoculated bats. Values are means ± SE; n is sample size.

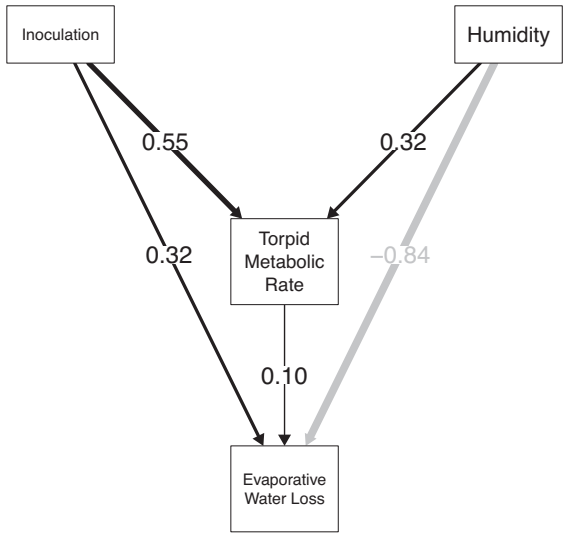

Fig. 2. Structural equation modeling indicated that increased EWL for inoculated bats was not simply an indirect effect caused by increased TMR, but that infection status directly affected EWL. Values indicate standardized parameter estimates with positive relationships indicated in black (e.g., inoculated bats have higher TMR), and negative relationships indicated in gray (e.g., increasing humidity leads to a decrease in EWL). Width of connecting arrows is proportional to the magnitude of the relationship.

ships for the dry air condition, where EWL effects were more pronounced, but the relationships were qualitatively similar for both dry and humid air conditions. We found a positive relationship between levels of disease severity, based on UV fluorescence, and EWL ($F_{1,7} = 11.56$, $P = 0.01$, slope = 0.21, intercept = 12.80, $R^2 = 0.62$; Fig. 3*A*) but not TMR ($F_{1,7} = 0.19$, $P = 0.68$, $R^2 = 0.03$; Fig. 3*B*; see Supplemental Material for the complete data set).

DISCUSSION

Our data support the hypothesis that WNS causes increased TMR and EWL for hibernating bats, consistent with predictions arising from two complementary models of WNS pathogenesis (41, 43). As predicted, TMR and EWL were greater in bats inoculated with *P. destructans*. Furthermore, infected bats were more sensitive than controls to changes in ambient humidity, with a greater increase in EWL in dry than humidified air. Importantly, our analysis confirms that increased EWL is a direct result of infection and not simply an indirect consequence of increased TMR and any associated increase in respiratory rate. This result points to wing damage as the cause of dehydration, consistent with the dehydration hypothesis (11, 46).

We measured total EWL, which comprises respiratory EWL and cutaneous EWL. Disruption of passive gas exchange leading to increased respiratory frequency (and respiratory EWL) has been proposed as a possible mechanism underlying increased EWL in bats with WNS (11), but our data and those of Carey and Boyles (8) do not support this hypothesis. On the basis of our structural equation modeling, the link between increased TMR (associated with increased respiratory rate) and EWL was not supported, but a direct link between infection and EWL had strong support. Increased respiratory rate may contribute to elevated EWL in relatively early stages of WNS, but in the later stage of disease exhibited by bats in our study, the relationship between TMR and EWL was not as important as the direct effect of inoculation on EWL, likely via fluid loss across wing lesions. As tissue invasion becomes more pronounced and larger areas of the wing are affected (increased fluorescence), cutaneous EWL is increased. EWL could also be affected by changes in surface lipid composition (2, 28), but the relationship between UV fluorescence and EWL, and not TMR, further supports the importance of wing lesions from WNS as a driver of EWL. If dehydration is a strong trigger underlying periodic arousals (38, 39), arousal frequency should increase as rate of EWL increases (46). As infection proceeds and wing lesions grow, the rate of EWL will increase further, resulting in more frequent arousals, as observed for bats with WNS in the laboratory (42) and field (33). Bats can drink during arousals to offset water loss, but they cannot replenish electrolytes from hibernaculum water sources. If water loss from wing lesions includes loss of electrolytes and other constituents of body fluids, this could lead to the hypotonic dehydration observed in previous studies (10, 43).

Increased TMR could also exert important influence on arousal frequency in WNS. As predicted by the models of Warnecke et al. (43) and Verant et al. (41), we observed increased TMR in our captive inoculation experiment. The same result has also recently been confirmed in studies of naturally infected bats (T. E. Tomasi, personal communication). Increased TMR is consistent with the increased energy consumption observed (41) in the absence of increased arousal frequency. Much of the emphasis of WNS pathophysiology

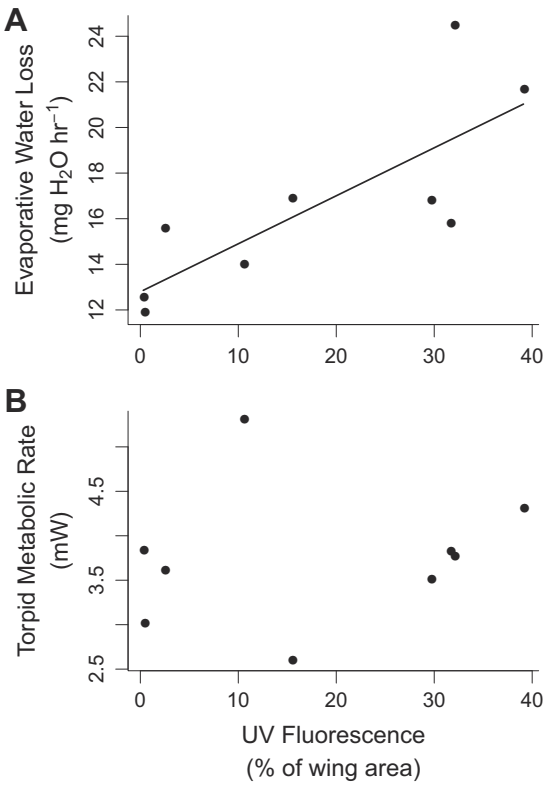

Fig. 3. Relationship between disease severity and physiological responses to *P. destructans* infection ($n = 9$). Bats with larger areas of the wing affected by WNS lesions (indicated by UV fluorescence) had higher rates of EWL (*A*), but TMR was not affected by disease severity (*B*). This suggests that fluid loss across wing lesions is the primary driver of EWL in bats with WNS.

research has focused on increased arousal frequency as a leading cause of mortality, but our results indicate that energy expenditure is increased even during the torpid phase of hibernation. Increased TMR during early stages of infection could result from a small, but persistent, immune response as bats initially attempt to fight off the fungal infection (12, 24, 27, 32). This elevation in TMR might not have a pronounced effect on overall energy expenditure, but it could strongly influence arousal dynamics. For example, at higher TMR (due to higher ambient temperature), bats are known to have shorter torpor bouts and arouse more frequently, possibly because higher TMR leads to faster accumulation of metabolic wastes (18). Thus increased TMR could indirectly increase energy consumption by increasing arousal frequency.

The treatment*condition interaction we observed for EWL has implications for understanding the influence of environmental conditions on bats with WNS (21, 22). Our results suggest that WNS exacerbates potentially detrimental effects of reduced humidity on bats, with EWL increasing disproportionately for bats from our infected group in dry air. Thus, not only does WNS increase EWL, but it also appears that changes in humidity inside hibernacula could disproportionately affect bats with WNS compared with healthy bats. Hibernaculum microclimate has long been recognized as a critical factor defining suitable hibernacula. Increased water loss in drier conditions may be an important driver of differences in the severity and impact of WNS among species and sites with exacerbation of EWL caused by WNS disproportionately affecting species, such as *M. lucifugus*, that have relatively high rates of EWL (46) and depend on high humidity in hibernation (38). Disproportionate increases in EWL with reduced humidity could interact with behavioral responses of bats to WNS to influence disease pathophysiology. EWL is lower in bats hibernating in clusters than solitary individuals (7), yet one characteristic response of bats with WNS is to reduce clustering and isolate themselves (22, 42). By isolating themselves (22), bats might reduce the chance of acquiring new points of infection on their wings from other infected colony mates (17) and slow the growth of *P. destructans* in drier conditions, but any benefits of this response could be overshadowed by the consequences of increased EWL. In drier conditions, the impacts of increased EWL may become more pronounced, contributing to the rapid escalation of pathophysiology observed late in hibernation (26). More work is needed to understand whether the isolation behavior shown by bats with WNS is an adaptive or maladaptive response.

Our results provide experimental support of key predictions made by mechanistic models of WNS. Support and revision of these models are key steps in understanding WNS and developing "theory to physiology to populations" (45) strategies for future research and management. Understanding the underlying physiological processes of hibernation and how they are perturbed by infection is critical to developing effective mitigation strategies. For example, microclimate modification of hibernacula is one approach that has been suggested to help mitigate impacts of WNS. Drier conditions in hibernacula could limit fungal growth rate, but, at the same time, our results indicate that dry conditions will cause a particularly large increase in EWL for bats with WNS, which could exacerbate their situation. Therefore, any mitigation that affects ambient humidity conditions inside hibernacula requires careful consideration. Similarly, a range of chemical and/or biological treatments have been proposed to help slow growth, invasion, or spread of the fungus, and a number of these treatments are being laboratory- and field-tested (https://www.whitenosesyndrome.org/us-fish-wildlife-service-funded-research-projects). Our results suggest that topical treatments, which increase EWL across the skin, could have unintended negative consequences. Ultimately, effective mitigation strategies require understanding of the underlying host physiology and disease pathophysiology.

Perspectives and Significance

WNS continues to spread across North America, invading new regions and infecting new species. While many research efforts focus on identifying potential treatment options, the underlying pathophysiology of the disease is still not fully understood. Our experimental results support key predictions of mechanistic models of the disease. Importantly, our study suggests that WNS affects both TMR and EWL independently in infected bats. Comprehension of interacting effects of microclimate and behavior will help develop an integrated understanding of the pathophysiology of WNS. As treatment and mitigation options are tested and considered, experiments addressing the pathophysiology of WNS are necessary for a more complete understanding of the underlying physiological processes involved in hibernation and disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.P.M., H.W.M., and C.K.R.W. conceived and designed research; L.P.M., H.W.M., and C.K.R.W. performed experiments; L.P.M., H.W.M., and C.K.R.W. analyzed data; L.P.M., H.W.M., and C.K.R.W. interpreted results of experiments; L.P.M. prepared figures; L.P.M. and C.K.R.W. drafted manuscript; L.P.M., H.W.M., and C.K.R.W. edited and revised manuscript; L.P.M., H.W.M., and C.K.R.W. approved final version of manuscript.

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