OPEN ACCESS Freely available online

PLoS one

# Frequent Arousal from Hibernation Linked to Severity of Infection and Mortality in Bats with White-Nose Syndrome

**DeeAnn M. Reeder[1]*, Craig L. Frank[2], Gregory G. Turner[3], Carol U. Meteyer[4], Allen Kurta[5], Eric R. Britzke[6], Megan E. Vodzak[1], Scott R. Darling[7], Craig W. Stihler[8], Alan C. Hicks[9], Roymon Jacob[1], Laura E. Grieneisen[1], Sarah A. Brownlee[1], Laura K. Muller[4], David S. Blehert[4]**

**1** Department of Biology, Bucknell University, Lewisburg, Pennsylvania, United States of America, **2** Department of Biological Sciences, Fordham University, Armonk, New York, United States of America, **3** Pennsylvania Game Commission, Harrisburg, Pennsylvania, United States of America, **4** U.S. Geological Survey–National Wildlife Health Center, Madison, Wisconsin, United States of America, **5** Department of Biology, Eastern Michigan University, Ypsilanti, Michigan, United States of America, **6** U.S. Army Engineer Research and Development Center, Vicksburg, Mississippi, United States of America, **7** Vermont Fish and Wildlife Department, Rutland, Vermont, United States of America, **8** West Virginia Division of Natural Resources, Elkins, West Virginia, United States of America, **9** New York State Department of Environmental Conservation, Albany, New York, United States of America

## Abstract

White-nose syndrome (WNS), an emerging infectious disease that has killed over 5.5 million hibernating bats, is named for the causative agent, a white fungus (*Geomyces destructans* (Gd)) that invades the skin of torpid bats. During hibernation, arousals to warm (euthermic) body temperatures are normal but deplete fat stores. Temperature-sensitive dataloggers were attached to the backs of 504 free-ranging little brown bats (*Myotis lucifugus*) in hibernacula located throughout the northeastern USA. Dataloggers were retrieved at the end of the hibernation season and complete profiles of skin temperature data were available from 83 bats, which were categorized as: (1) unaffected, (2) WNS-affected but alive at time of datalogger removal, or (3) WNS-affected but found dead at time of datalogger removal. Histological confirmation of WNS severity (as indexed by degree of fungal infection) as well as confirmation of presence/absence of DNA from Gd by PCR was determined for 26 animals. We demonstrated that WNS-affected bats aroused to euthermic body temperatures more frequently than unaffected bats, likely contributing to subsequent mortality. Within the subset of WNS-affected bats that were found dead at the time of datalogger removal, the number of arousal bouts since datalogger attachment significantly predicted date of death. Additionally, the severity of cutaneous Gd infection correlated with the number of arousal episodes from torpor during hibernation. Thus, increased frequency of arousal from torpor likely contributes to WNS-associated mortality, but the question of how Gd infection induces increased arousals remains unanswered.

**Citation:** Reeder DM, Frank CL, Turner GG, Meteyer CU, Kurta A, et al. (2012) Frequent Arousal from Hibernation Linked to Severity of Infection and Mortality in Bats with White-Nose Syndrome. PLoS ONE 7(6): e38920. doi:10.1371/journal.pone.0038920

**Editor:** Raphaël Arlettaz, University of Bern, Switzerland

**Received** September 20, 2011; **Accepted** May 16, 2012; **Published** June 20, 2012



**Funding:** The temperature tracking portion of this study was supported by State Wildlife Grant funds awarded through the Northeast Association of Fish and Wildlife Agencies (NEAFWA) Regional Conservation Needs grant program to DMR. (PI), CLF, GGT, ACH, and ERB, by funds from the Pennsylvania Department of Conservation and Natural Resources and the Woodtiger Fund to DMR, and by Graduate Fellowships from Bucknell University to LEG, SAB, and RJ. This grant and its associated conservation activities are done to support implementation of a priority action of the State Wildlife Action Plans from members of the NEAFWA. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: dreeder@bucknell.edu

## Introduction

White-nose syndrome (WNS) is estimated to be responsible for the deaths of at least 5.7 to 6.7 million hibernating bats in the eastern United States and Canada [1,2]. Clinical signs of WNS were first observed at a single cave in New York State during the winter of 2006–2007 and as of April 2012, WNS has spread to over 200 hibernacula in 19 U.S. states and four Canadian provinces (Fig. 1 [2,3]). Bats with WNS display a number of aberrant behaviors, and in many instances they have depleted fat stores. Thus far, WNS affects at least six (and possibly nine) species of hibernating insectivorous bats [2], including some classified as endangered or threatened. The little brown bat (or, little brown myotis, *Myotis lucifugus*), which was once the most common hibernating bat in the American Northeast (NE), has incurred an average of 91% mortality in sites that have been affected for at least two years [2] and mathematical models indicate that this species may go extinct in the NE within 16 years [4]. A white fungus identified as *Geomyces destructans* (Gd) grows on the muzzle, wings, and ears of bats suffering from WNS starting in late January/early February [3,5,6]. Recent laboratory experiments have demonstrated that cutaneous infection with this fungus is the cause of WNS, but it is not fully understood how such an infection produces mortality during hibernation [7]. It is hypothesized that infection by Gd disrupts normal physiological functions, such as water balance [8] or other aspects of hibernation physiology, including use of torpor [9].

For insectivorous bats that live in northern temperate zones, such as those affected by WNS, food is primarily available from late spring to early autumn and absent during winter. Bats survive

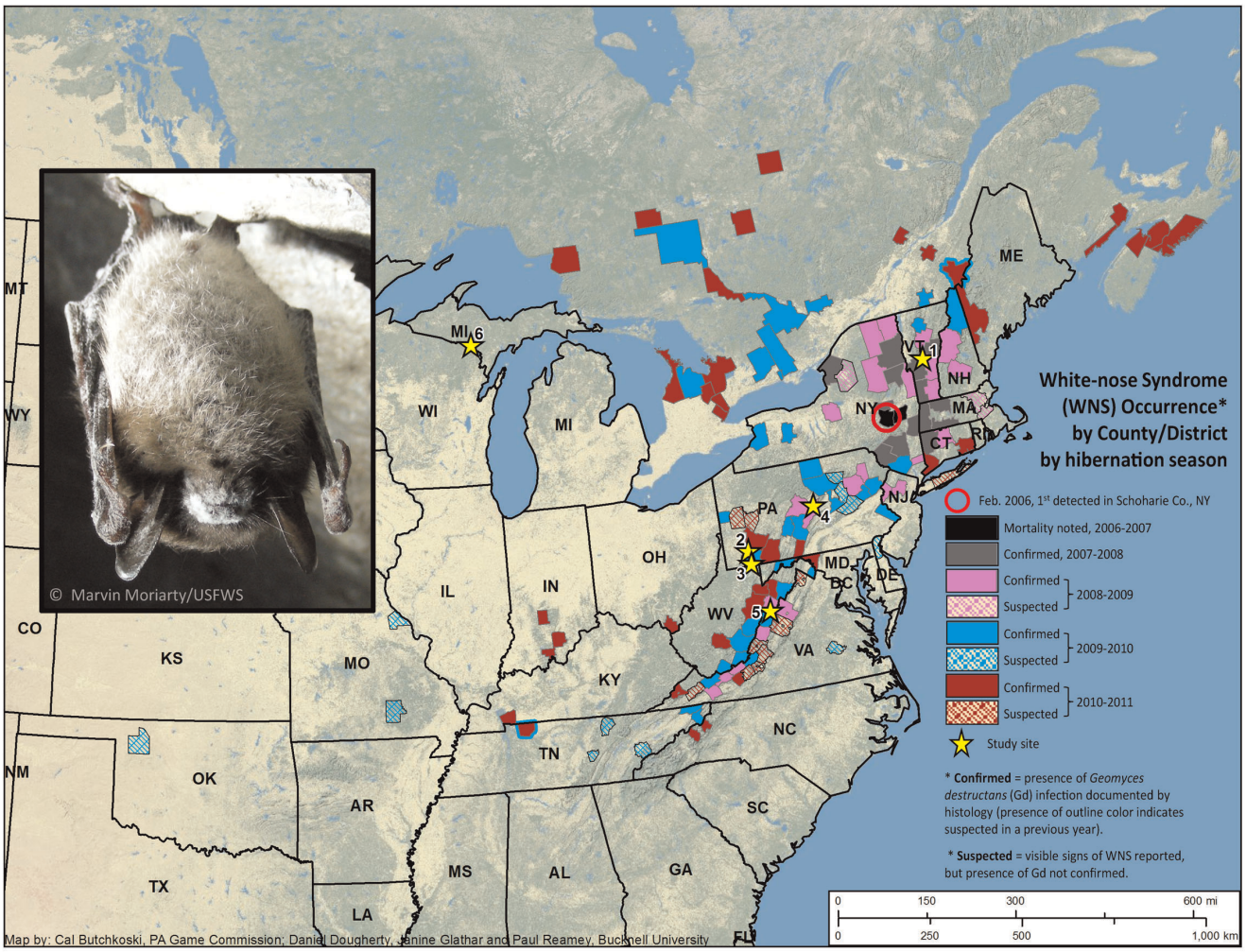



**Figure 1. Distribution and spread of WNS throughout North America.** Spread of WNS by hibernation season through the winter of 2010–2011 is shown along with locations of study sites, indicated by stars (see also Table 1). Confirmed sites have been officially reported by each state or province based upon histological confirmation of infection with the fungal pathogen *Geomyces destructans* (Gd); bats from suspect sites have clinical signs of WNS but lack laboratory confirmation. The inset shows a little brown bat infected with Gd from site #1 in Vermont. This site was WNS confirmed in 2008–2009, when bats were studied. Bats from site # 2 in Pennsylvania were studied in 2008–2009 (for 8 weeks only in the spring), when no signs of WNS were present, in 2009–2010, when a single bat from this site showed infection with Gd without mass mortality and in 2010–2011, when bats in this site were heavily infected. Bats from site #3 in Pennsylvania were studied in 2008–2009 (no WNS), 2009–2010 (when Gd was noted but without mass mortality) and in 2010–2011, when bats in this site were heavily infected. Bats from site #4 in Pennsylvania were studied in 2009–2010 (for 8 weeks only in the spring), when bats were heavily infected. Bats from site #5 in West Virginia were studied in 2008–2009, when there was no evidence of Gd presence – which was also the case for bats from site #6 in Michigan, which were studied all three years.
doi:10.1371/journal.pone.0038920.g001

this winter energetic bottleneck by building stores of body fat (depot fat) in late summer and early autumn and by conserving metabolic energy through hibernation. In little brown bats, body fat increases from approximately 7% of total mass (~6 g) during summer to 27% of total mass (~9 g) prior to hibernation, an increase of 3 g or more in body mass [10,11]. This depot fat is the sole energy source during the hibernating period, when body temperature ($T_b$) and metabolic rate are both greatly reduced. Because their energetic costs in the subsequent spring are greater than those of males, female little brown bats enter hibernation with higher body mass indexes (BMI) and manage their energy stores during hibernation more efficiently than males [12]. Minimum metabolic rates during mammalian torpor can be <5% of basal metabolic rate with $T_b$ close to ambient temperature (2° to 8° for bats) [13,14]. However, hibernators do not remain torpid throughout hibernation; instead bouts of torpor last from days to weeks, interrupted by brief arousal episodes involving periods of high metabolic rate and euthermic $T_b$ [15]. Earlier studies demonstrated that healthy, free-ranging little brown bats hibernating at ambient temperatures of 5–6°C have torpor bouts lasting between 12.4 and 19.7 days [16,17], with arousal episodes lasting 1–2 hours.

Although euthermic periods account for approximately 1% of the total time budget during winter, about 80–90% of the energy (depot fat) used during hibernation is consumed during these periodic arousals from torpor, because metabolic rate greatly increases with increased $T_b$ [13,18]. The amount of depot fat expended during each arousal episode (not including flight) for hibernating little brown bats is about 107.9 mg [18]. While the function of arousal episodes in hibernators is poorly understood and likely multifactorial [19], the fact that every mammalian

**Table 1.** Temperature-sensitive datalogger deployment and retrieval (bat recapture) data, by study year, hibernacula site number (see Fig. 1), and sex for the 504 loggers deployed.

| Site# | 2008–2009 | | | | 2009–2010 | | | | 2010–2011 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | deployed* | retrieved* | down-loaded | included in final analyses** | deployed* | retrieved* | down-loaded | included in final analyses** | deployed* | retrieved* | down-loaded | included in final analyses** |
| 1 (VT) | 16/14 [11/6/08] | 6/7 [3/17/09] | 6/7 | 5/7 | | | | | | | | |
| 2 (PA) | 20/19+[1/27/09] | 13/13 [3/24/09] | 7/7 | 3/3 | 41/41 [11/13/09] | 25/26 [3/25/10] | 13/8 | 13/8 | 22/18 [11/18/10] | 4/4 [3/10/11] | 3/1 | 3/1 |
| 3 (PA) | 15/15 [11/3/08] | 9/4 [3/23/09] | 8/2 | 8/2 | 40/30 [11/12/09] | 9/8 [3/17/10] | 7/2 | 7/2 | 22/7 [11/19/10] | 8/1 [3/2/11] | 7/1 | 7/1 |
| 4 (PA) | | | | | 35/25+[1/6/10] | 7/1 [3/11/10] | 7/0 | 4/0 | | | | |
| 5 (WV) | 21/21+[1/29/09] | 7/7 [3/23/09] | 2/4 | 2/4 | | | | | | | | |
| 6 (MI) | 15/15 [11/7/08] | 7/9 [3/21/09] | 7/5 | 7/5 | 13/13 [11/14/09] | 9/6 [3/27/10] | 4/2 | 4/2 | 14/12 [11/6/10] | 10/8 [3/26/11] | 0/1 | 0/1 |

Whether data were successfully downloaded from the logger and ultimately used in the analyses of this paper, are also described.
*# of males/# of females and date of deployment or retrieval of loggers.
**bats were occasionally excluded from analyses due to incomplete data (e.g., BMI not recorded) or problems with downloaded data.
+loggers only deployed mid-winter (January-March) as opposed to the full-hibernation season (November-March).
doi:10.1371/journal.pone.0038920.t001

hibernator periodically arouses from torpor at great energetic cost indicates the benefits must be significant.

We tested the hypothesis that WNS reduces the length of torpor bouts during hibernation in free-ranging little brown bats. We predicted that a primary cause of the increased mortality/disease state associated with WNS is abnormally shortened torpor bouts, due to more frequent arousal episodes, as was shown previously for one affected free-ranging bat in late hibernation [20] and recently for a group of experimentally infected bats held in captivity [21]. We also predicted that greater body fat stores at the beginning of hibernation, as estimated by BMI, would mediate the negative effects of frequent arousals. These predictions were tested in field studies on free-ranging little brown bats conducted at multiple sites (Fig. 1) over three hibernation seasons. Skin temperature ($T_{sk}$), which correlates well with $T_b$ in small insectivorous bats, and which has been used extensively to study mammalian hibernation [22], was measured with temperature-sensitive dataloggers attached to the backs of WNS-affected and unaffected bats. Hibernation patterns in relation to the stage of infection by Gd were also analyzed for a small sample of bats for which data were available on fungal presence (PCR) and degree of infection (histopathology).

## Materials and Methods

### Permits and Permissions

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at Bucknell University (protocol number DMR-02). In the states of VT and WV, research was conducted by state wildlife officials (SRD with Vermont Fish and Wildlife Department and CWS with WV Department of Natural Resources) on non-endangered bats; thus numbered permits were not required or issued. In Michigan, research was conducted each year under MI Scientific Collector's Permit SC620 from the Michigan Department of Natural Resources to AK. In PA, research was conducted each year under PA Game Commission permits to DMR (84-2008; 70-2009; 183-2010), in collaboration with GGT, a wildlife biologist for the state of PA. In accordance with the permits and with state wildlife policies, research was either conducted on state land or on private property, with the explicit permission of private landowners.

### Temperature Tracking

Temperature-sensitive dataloggers were programmed to read skin temperature ($T_{sk}$) every 30 min and were attached to 504 bats over the course of three winters at six different hibernacula using standard methods [22]. Temperature readings could not be collected more frequently due to constraints on datalogger memory and the need to record continuous data for up to five months. To maximize recapture rates, bats with loggers were recaptured in March of each year, several weeks prior to the 'normal' time of emergence from hibernation. Loggers weighted about 1.1 g and were either purchased commercially (iBBat or WeeTagLites, AlphaMach, Inc., British Columbia, Canada) or were constructed by the authors (DMR and GGT). Appendix S1 describes and illustrates the methods for making these dataloggers from Thermochron DS1922L iButtons (Maxim Integrated Products, Inc., California, USA), modified from the techniques of Lovegrove [23]. Table 1 provides a summary of loggers deployed, retrieved, and downloaded successfully, by site, year, and sex.

Study sites were widely distributed and located in Vermont, West Virginia, Pennsylvania, and the Upper Peninsula of Michigan (Fig. 1). Among loggers retrieved, success rates varied. WeeTagLites failed at a rate of up to 90% whereas loggers constructed by the authors failed about 20% of the time. Overall 111 of 190 loggers retrieved yielded usable data, an average of 58.4%. We expected to recover less than half the loggers placed in the field and expected datalogger failure as well, which is why so many loggers were deployed. Of the 190 bats from which loggers were retrieved, 17 were found dead (four of which were in suitable post-mortem condition to perform histology analysis). For the 173 live bats recaptured in the spring, loggers were removed, and the animal was either released (N = 126) or euthanized for measurement of immune function and other physiological parameters for a separate study (N = 25) or for histology analysis (N = 22), as described below.

## PCR and Histology

Wing skin samples (approximately 3 mm X 3 mm each) were collected from a subset of freshly euthanized animals (N = 26). Nucleic acid was extracted from each skin sample using the Gentra Puregene genomic DNA purification kit (Qiagen Inc., Valencia, CA) per the manufacturer's instructions (solid tissues protocol), with the following modifications: proteinase K was added to a final concentration of 0.5 mg/ml during the cell lysis procedure and no RNase treatment was performed. To determine presence/absence of DNA from Gd on each sample of wing skin (within the defined sensitivity limitations of the technique used), extracted nucleic acid was analyzed by PCR as previously described by Lorch et al. [24].

Wing membrane from these same animals was also analyzed by histology [5] to determine WNS infection status. The entire wing membrane was stripped from the right forearm and digits, rolled onto 2 dowels 2.5 cm in length, trimmed into three approximately 0.8 cm-wide sections, placed on trimmed edge, sectioned at 0.4 μm-thickness, and stained with Periodic Acid Schiff [5]. This preparation technique yields six whorls of wing membrane on each slide. White-nose-syndrome was diagnosed based on previously published microscopic criteria [5]. A histologic scoring system was developed to classify severity of WNS on a scale of 0 to 4 as described and illustrated in Appendix S2. Briefly, a score of 0 indicates the sample is negative for WNS, and there are no diagnostic cupping erosions in the tissues. A score of 1 indicates the tissues are positive for WNS with cupping erosions diagnostic for WNS but erosions are mild, occasional, and are limited in both depth and extent of wing membrane involved. The presence of even one characteristic WNS erosion is sufficient for a diagnosis of WNS. A severity score of 2 indicates moderate WNS with more frequent and deeper fungal cupping erosions diagnostic of WNS, but distribution over wing membrane is still limited. A WNS severity score of 3 indicates moderately severe fungal infection with deeper and coalescing cupping erosions that are deep enough to be considered ulcers, and the extent of the wing membrane with fungal invasion is greater. A severity score of 4 indicates a severe fungal infection with deep tissue invasion and coalescing of cupping erosions; as many as 100 or more erosions/ulcers can be present in one roll of wing membrane. Scores ranging from 1 to 4 were identified as WNS.

## Analyses

**Calculations and initial statistics.** Usable data for our analyses were recovered from 99 of the 504 loggers deployed (see Table 1). Although data downloaded from 111 loggers, data from 12 of these bats were removed from final analyses for a variety of reasons, including having temperature data recorded for too short of a time period to be comparable to other groups and missing body mass data. Prior to datalogger attachment, each bat was weighed using a portable battery-operated scale (accuracy to 0.1 g), and the length of their right forearm was measured (in triplicate) to the nearest mm using calipers; from these data BMI (weight in g/length of right forearm in mm) [10] was calculated. As most analyses included BMI as a covariate, only bats for which we were able to calculate BMI at the beginning of hibernation (November) were included in the final analysis (N = 83). Data from an additional 16 bats for which we had recordings from only January through March (see Table 1) are also described in the results.

Torpor was defined as when a bat's $T_{sk}$ was 10°C or more below its highest temperature ($T_{max}$). Duration of an arousal episode (when $T_{sk}$ was within 10°C of $T_{max}$) was calculated to the nearest 30 min. Although recording $T_{sk}$ every 30 min was sufficient to detect arousal episodes, it did not provide sufficient resolution to describe precisely the true length of an arousal bout, as arousal episodes averaged less than 90 min in length (see results). Thus, we did not attempt to determine if there were significant differences in arousal episode length by WNS status. Torpor bout length (TBL, in days) was defined as the period between two arousal episodes. For both arousal bout length and TBL, values were first averaged for each bat and then averaged across all bats. Data on TBL were $\log_{(10)}$ transformed to achieve normality and homogeneity of variance, as determined by Shapiro-Wilk's test for normality and examination of skew and kurtosis and by Levene's test for equality of variances. BMI data were normally distributed. TBL data from multiple years are combined in our analysis, which is supported by the lack of a year-to-year difference in TBL in bats from a given hibernaculum when the WNS status did not change between years (e.g., from site 6 (Table 1; Figure 1): 10.52±1.62 days (2008–2009) vs. 12.47±3.09 days (2009–2010); $F_{(1,16)} = 3.091$, $p = 0.098$; partial eta squared = 0.162, power = 0.380). For all analyses, power and effect size are reported for non-significant results. All data are presented as the mean ± standard deviation (SD).

**WNS status and TBL.** For the initial analysis, bats for which we had data on TBL, BMI, and sex were grouped into three 'WNS status' categories: (1) unaffected [N = 57], (2) WNS-affected (as determined by histology and/or visible fungus) and alive at time of datalogger removal [N = 14], and (3) WNS-affected and found dead at time of datalogger removal [N = 12]. Bats were assigned to the 'unaffected' category either when the presence of fungal infection with Gd was not detected with PCR or histology [N = 10] or when they were from a hibernaculum presumed to be unaffected and not located in the WNS zone at the time of study [N = 47] (Fig. 1). Combining the two groups of 'unaffected' bats for further analyses is supported by the lack of a difference in TBL between them (17.55±4.56 days (PCR/histology) vs. 16.06±7.03 days (presumed unaffected); $F_{(1,55)} = 1.111$, $p = 0.297$; partial eta squared = 0.020, power = 0.179). Effects of WNS status on TBL were tested with ANCOVA, with BMI (random), site identity (fixed), and sex (fixed) as covariates. Post-hoc examination of sex differences in BMI was conducted using a Student's *t*-test (with df and p values adjusted for unequal variance).

**TBL and date of death.** Within the WNS-affected bats that were found dead at the time of datalogger removal, the relationships between TBL and BMI and date of death were analyzed using Pearson Product Moment Correlations (PPMC) (after confirming normality and homoscedasticity for each variable). Date of death was measured as the date on which $T_{sk} < 0$°C for the first time, since the $T_{sk}$ of little brown bats always remains

above 0°C during torpor [17,18]. P values were adjusted for multiple comparisons using sequential Bonferroni correction [25], and the coefficient of determination ($r^2$) was calculated by squaring significant correlations.

**TBL and WNS severity score.** Using a subset of animals for which a 'WNS severity score' could be calculated and for which BMI at the start of hibernation was available (N = 26), the effects of severity score, BMI, and site on TBL were examined with ANCOVA. A significant relationship between severity score and TBL was examined using the Gamma Correlation Statistic, which allows for multiple 'tied rankings' [26]. Of these 26 bats, 10 were classified in the first analysis as "unaffected" 13 were classified in the first analysis as "WNS-affected and alive at time of datalogger removal" (of these three bats received a severity score of 1, four bats a severity score of 2, two bats a severity score of 3, and four bats a severity score of 4), and three were classified in the first analysis as "WNS-affected and found dead at time of datalogger removal" (of these two bats received a severity score of 2 and one bat a severity score of 3).

## Results

### Arousing to Euthermic Temperatures

During the course of this study, when bats aroused from torpor, they remained at euthermic temperatures for a short period, averaging 78.3±27.3 min. The range of average arousal bout length per bat was from 38.18 to 180 min (N = 83 bats), while the shortest recorded arousal bout lasted 30 min (the shortest period that could be discerned by our methods) and the longest 330 min. We were unable to test for differences in arousal bout length in relation to WNS status (or severity score) due to the limited data storage capacity of our dataloggers (and thus insufficient resolution for precisely quantifying arousal bout length).

### WNS Status and TBL

Although female bats were in significantly greater body condition than males at the start of hibernation (BMI: 0.2284±0.0283 g/mm (N = 32) vs. 0.2073±0.0210 g/mm (N = 51); t = −3.633, adjusted df = 52.2, p = 0.001), there were no detectable influences of sex on TBL ($F_{(1,76)} = 0.031$, p = 0.861; partial eta squared = 0.000, power = 0.053). Likewise, we did not detect a relationship between BMI at the start of hibernation and TBL ($F_{(1,76)} = 0.140$, p = 0.710; partial eta squared = 0.000, power = 0.066). Our BMI analyses were not biased by recapture dynamics as there was no significant difference in BMI at the time of datalogger attachment between bats for which loggers were retrieved and bats that were not recovered (Mann-Whitney U = 3.339, Z = 1.259, p = 0.208). However, both WNS-status and site identity significantly influenced TBL. Site identity heavily influenced the model ($F_{(1,78)} = 25.027$, p<0.001) as two of the sites contained only one category of bat (site 1 had only 'WNS dead at time of datalogger removal' bat, and site 6 had only 'unaffected' bats). Despite the strong influence of site identity, a significant WNS status main effect was still apparent ($F_{(1,78)} = 7.569$, p = 0.007).

Unaffected bats had a mean TBL of 16.32±6.65 days (Fig. 2). Limited data collected from an additional 12 unaffected bats from field sites where dataloggers were deployed for only eight weeks late in the hibernation season in 2009 are similar with a mean TBL of 15.62±8.07 days (sites 2 and 5, Fig. 1). As predicted, having WNS was associated with decreased TBL (Fig. 2). Bats that were affected by WNS but still alive at the collection of dataloggers (March) had shorter TBLs than unaffected bats, although the difference was small and not statistically significant (13.96±4.30 days vs. 16.32±6.65 days; $F_{(1,69)} = 1.491$, p = 0.226, partial eta squared = 0.021, power = 0.226). However, these affected but alive bats had significantly longer TBLs than WNS-affected bats that were found dead at the time of datalogger collection (7.93±2.49 days; $F_{(1,24)} = 17.191$, p<0.0001). Limited data collected from an additional four WNS-affected bats found dead from a field site where dataloggers were deployed for only eight weeks late in the hibernation season in 2010 are similar with a mean TBL of 6.17±1.79 days (site 4, Fig. 1).

### TBL and Date of Death

Within the 12 WNS-affected bats found dead at the time of datalogger collection, there was a very strong positive relationship between TBL and the number of days that a bat lived (Fig. 3; PPMC, r = 0.763, corrected p = 0.012). Based upon the calculated coefficient of determination ($r^2 = 0.582$), TBL significantly predicted the date of death, explaining 58% of the variance. Similar to the findings of our full ANCOVA, we did not detect a relationship between BMI at the start of hibernation and TBL (PPMC, r = 0.178, p = 0.580) or between BMI at the start of hibernation and date of death (PPMC, r = −0.026, p = 0.936). While the power to detect significant differences at these low effect sizes (correlation coefficients of 0.178 and 0.026) is extremely low (<0.05), even if they were statistically significant, they are not biologically significant. In each bat, mortality was observed immediately after the last arousal to euthermic temperatures. While several bats (Fig. 2C) displayed frequent arousals just before death, most did not, and arousals were spread throughout their hibernation period.

### TBL and WNS Severity Score

In the subset of animals for which the WNS severity score could be calculated (N = 26), TBL was not related to BMI ($F_{(1,21)} = 0.111$, p = 0.743, partial eta squared = 0.005, power = 0.062) or site identity ($F_{(2,22)} = 2.515$, p = 0.104, partial eta squared = 0.186, power = 0.045), but was related to severity score ($F_{(1,24)} = 6.509$, p = 0.018). Bats with more severe fungal infections had significantly shorter torpor bouts (gamma correlation statistic = −0.383, p = 0.022; Fig. 4).

## Discussion

Our results support the hypothesis that WNS causes alterations in bat torpor patterns that likely contribute to death. Our prediction that increased mortality/disease state is associated with abnormally short torpor bouts due to frequent arousal episodes was supported by our larger dataset, in which bats were placed into the WNS status categories of 'unaffected,' 'WNS-affected and alive at time of datalogger collection at the end of hibernation,' and 'WNS-affected and dead at the time of datalogger collection.' While our 'unaffected' bats had an average TBL that falls within the previously documented range for this species (16.32 days) [16,17], TBL was shortened (at the low end of previously described TBLs) in WNS-affected bats (13.96 days), and significantly reduced in WNS-affected bats that died between mid-December and late-February (7.93 days). An average torpor bout length of 7.93 days is presumably not sustainable. In fact, within those WNS-affected bats found dead at the time of datalogger removal, TBL was a very strong predictor of the date of death, explaining 58% of the variance in timing of mortality. The distribution of death dates for these bats (Fig. 3) is earlier than that reported in the USA [7] and earlier than seasonal changes in Gd prevalence reported for Europe [27,28]. However, this was at least the second year of infection at this site, which might shift the

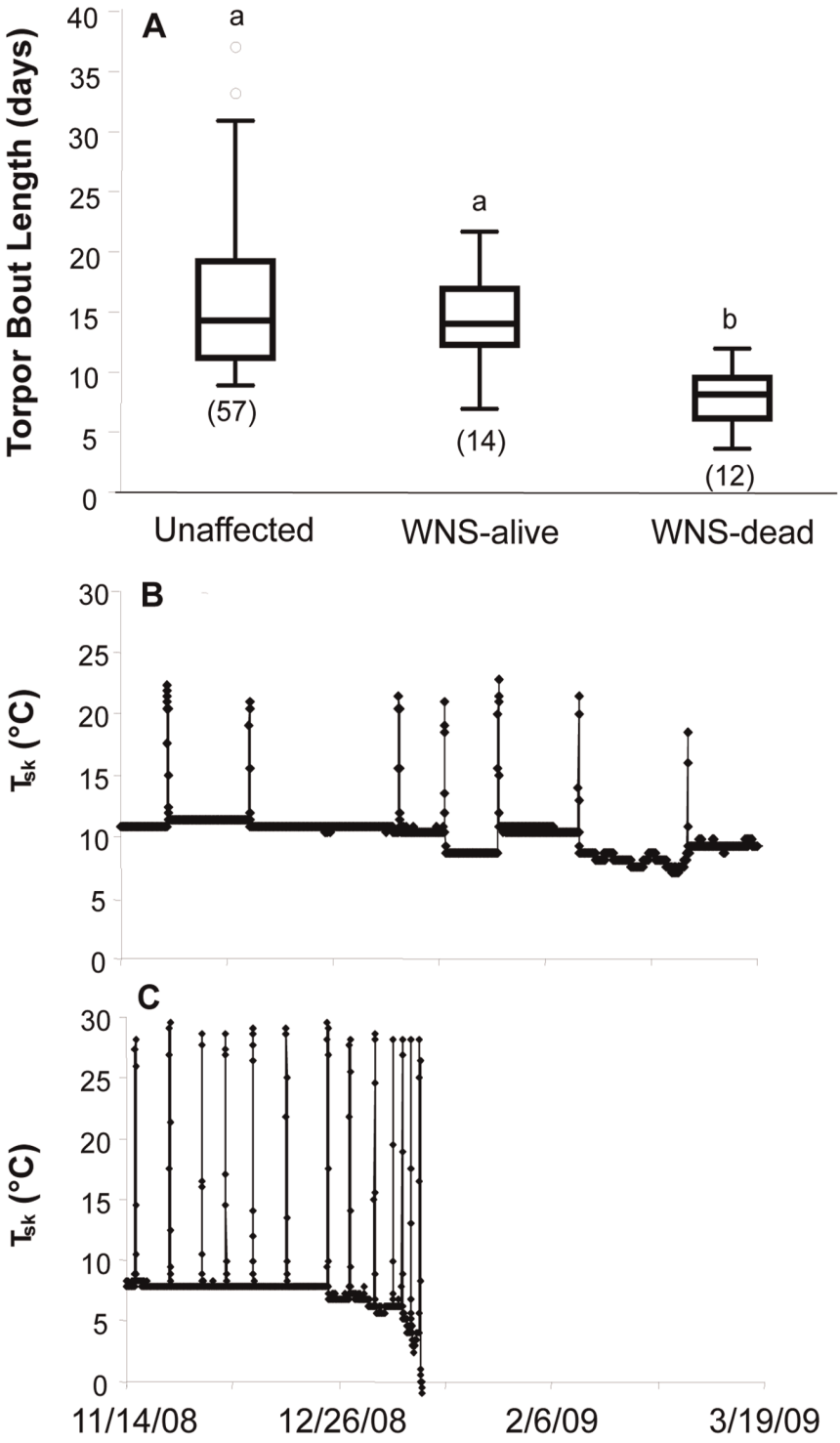



**Figure 2. Torpor bout length (TBL) in days by WNS status.** WNS was associated with decreased TBL: bats that were affected by WNS but still alive at the collection of dataloggers (March) had shorter TBLs than unaffected bats (but this difference was not significant). Significantly shorter TBLs were seen in WNS-affected bats that were found dead at the time of datalogger collection compared to affected but alive bats (2A). Bats were categorized as: unaffected, WNS-affected and alive at time of datalogger removal ('WNS-alive'), and WNS-affected and dead when loggers were removed in the spring ('WNS-dead'). Numbers in brackets indicate sample size and boxes sharing the same letter are not significantly different from each other. Boxes depict the 25th and 75th percentiles, lines within boxes mark the median, and whiskers represent 95th and the 5th percentiles. Outliers are indicated with open circles. Additional panels illustrate sample temperature profile of an unaffected (B) and an affected (C) bat, during the winter of 2009. The bat illustrated in C displayed daily arousals at the end of its life, which was seen in several of these animals. Each of the 'WNS-dead' bats died at the end of their last arousal.
doi:10.1371/journal.pone.0038920.g002

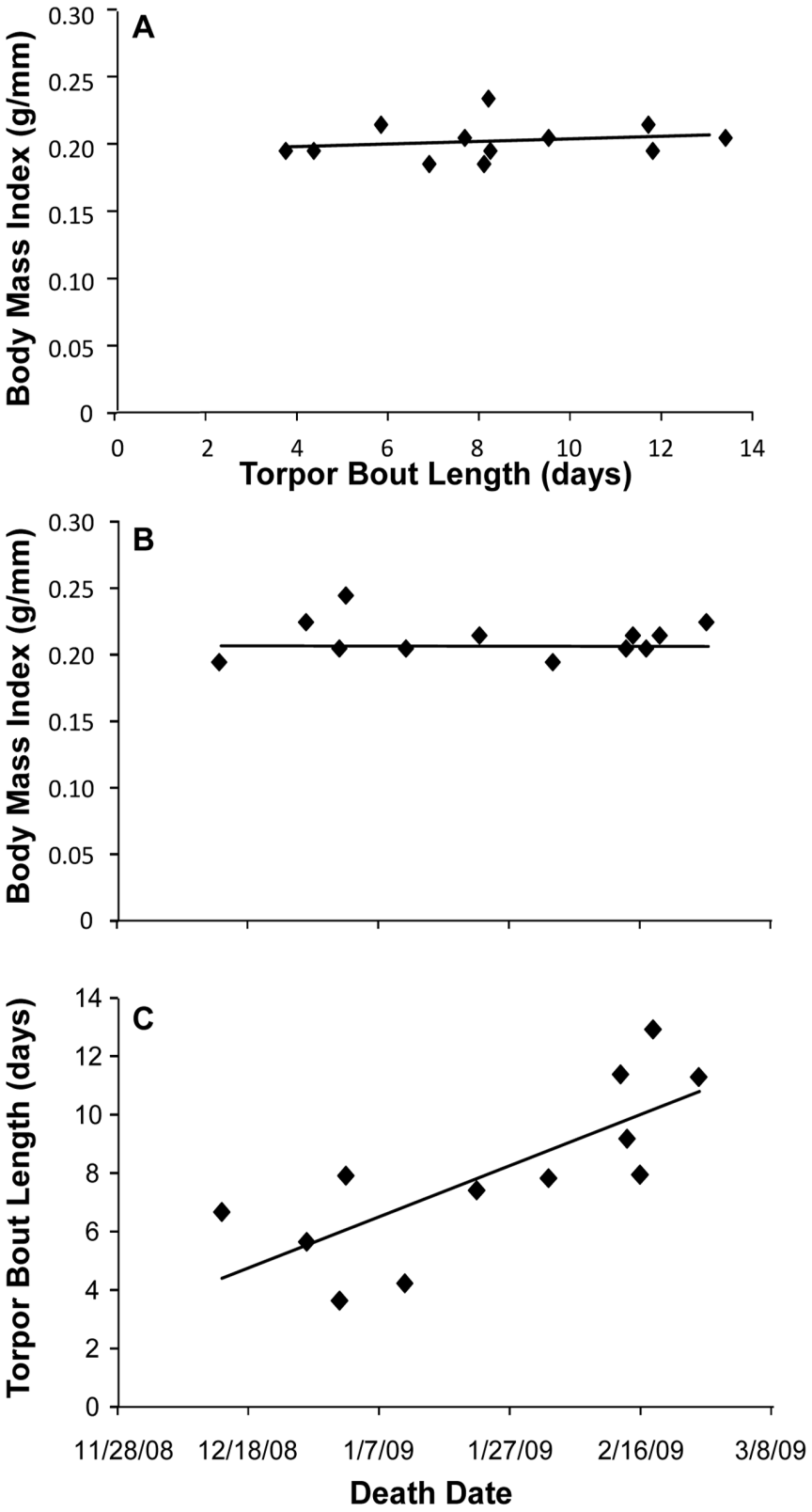



**Figure 3. Torpor bout length (TBL) as a function of date of death and BMI.** For the 12 bats that died from WNS, BMI at the beginning of hibernation was not related to TBL (3A), nor was BMI predictive of the date of death (3B). However, TBL significantly predicted date of death in WNS-affected bats that were found dead at the time of datalogger retrieval (3C) ($r^2 = 0.58$). Bats that died sooner were arousing to euthermic temperatures much more frequently than those that lived longer.
doi:10.1371/journal.pone.0038920.g003

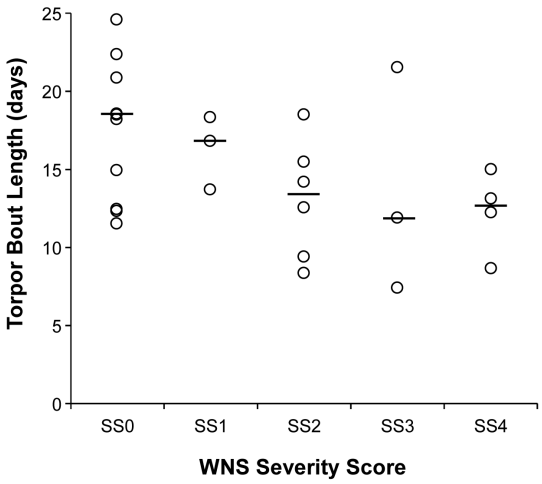



**Figure 4. Torpor bout length (TBL) as a function of WNS severity score.** Wing tissue was assigned a disease severity score (SS0 to SS4) based upon histology, as follows: SS0 = no fungi suggestive of WNS; SS1 = occasional but limited superficial fungal infection; SS2 = more extensive superficial fungal infection with limited invasion; SS3 = more extensive fungal infection with frequent cupping erosions; and SS4 = severe fungal infection with deep tissue invasion. Details of the scoring system can be found in Appendix S2 and scores 1 through 4 were identified as WNS. Individual data points are shown as open circles, the median is indicated by a line. As severity of infection increased, torpor bout length significantly decreased (bats aroused more frequently from torpor.
doi:10.1371/journal.pone.0038920.g004

distribution of death dates earlier relative to compiled data from multiple sites [7,27,28]. Recapture of bats for datalogger removal in March of each year (Table 1), the time when peak mortality has been noted in the field [7], may have prevented us from detecting other mortality events within our study animals.

Our analysis of WNS severity based upon histological confirmation of the degree of fungal invasion and infection further supported and strengthened our conclusion – as the severity of infection increased, so did the frequency of arousals from torpor. Our data mirror the independently derived mathematical model of Boyles and Willis [9], for which an estimated shift in TBL to every 8.33 days resulted in a prediction of 81.9% mortality. Relative to this model, our finding of a TBL of 7.93 days for WNS-affected bats found dead, and field observations of 91% mortality support the linkage between TBL and death, as significant body fat is lost with each arousal [13,18]. Boyles and Willis [9] also proposed that significant changes in arousal bout duration in WNS-affected bats could lead to mortality. Bats are unlike other hibernators [13,18] in that their arousal bouts are typically measured in minutes rather than hours (or even days). Thus, an increase in the duration of euthermy would incur significant energetic costs. Although we were unable to statistically validate differences in arousal bout length in bats of variable WNS status, our finding of an average arousal bout of $78.3 \pm 27.3$ minutes for all bats tested indicate that biologically important shifts in arousal bout length do not occur in WNS-affected animals.

We also predicted that relationships between WNS and torpor patterns would be influenced by the amount of energy stores available to the bat. In a previous study of little brown bats, BMI significantly influenced hibernation energetics such that bats with lower body masses at the beginning of hibernation selected colder roosting sites, which allows for decreased metabolic rates and thus lower energy expenditure [29]. Other studies have demonstrated that bats roosting at colder temperatures arouse from torpor less often, allowing them to conserve even more energy [19,30,31]. Thus, it is reasonable to expect that bats with lower BMIs would display greater TBL and expend less energy.

These energetic arguments underlay the model of Boyles and Willis [9] that our data so closely match. However, contrary to our predictions, we did not find a relationship between BMI and TBL or BMI and 'WNS status', death date, or 'severity score'. As the power for BMI effects in our models was low (driven by the strong site effects), BMI may still play a role in hibernation patterns and in a bat's ability to withstand Gd infection. However, even within a site (WNS-affected bats that were found dead at the time of datalogger attachment from site 1 in Vermont), we failed to find a relationship between BMI and WNS. If a higher BMI could 'buffer' a bat from the effects of WNS by allowing it to withstand more arousals to euthermy, then we should have detected a relationship between BMI and the number of arousals prior to death – but we did not.

Although statistical analyses confirmed the significance of our findings, studies of behavior and physiology in free-ranging animals are often fraught with unknowns and potential biases, which likely underlie the significant site effects in our statistical models. One potential source of bias in our dataset is BMI at the start of the hibernation season. While one could predict that bats in poorer body condition would find datalogger attachment more physiologically stressful than bats in greater body condition (and thus be less likely to be recaptured), there was no difference in starting BMI between bats that were recaptured and those that were not. Another source of bias in our WNS-affected bats could have been ambient temperature of hibernacula, because TBL generally decreases with increased ambient temperature [30]. Although the exact ambient temperature at the exact roosting site of each individual studied during hibernation was unknown, our WNS-affected field sites were generally colder than our unaffected sites (e.g., 7.29°C vs. 9.77°C). This would presumably bias bats with WNS toward longer TBLs, but we observed the opposite pattern. Within our unaffected bats, TBLs varied greatly (Fig. 2A), likely due to a number of site-, individual-, and population-specific factors. However, these factors appear to be overridden in the WNS affected bats, especially those found dead at the time of datalogger removal – as variability decreased and all bats exhibited shortened TBLs.

Collectively, our data indicate that one proximate mechanism of the mortality associated with WNS is decreased TBL. Warnecke et al. [21], in a study of captive bats experimentally infected with Gd during the third year of our field study, found a similar TBL shift. The challenge that lies before us is to determine how infection by Gd induces altered torpor patterns and why significant variation in TBL between affected bats occurs. While too-frequent arousal is clearly associated with WNS, not all bats that died displayed the severely shortened TBL characteristic of some that died, and some bats that displayed very short TBL did not die.

In other mammalian hibernators, mechanisms associated with immunity are reduced during hibernation, when the conservation of energy is critical [32,33], and the periodic arousals from hibernation may activate the dormant immune system. Thus, immunological responses to fungal infection may be triggering arousals more frequently than normal [34]. Additionally, physical damage to wing skin caused by fungal infection may disrupt other physiological functions, such water balance, resulting in dehydration, another trigger for arousal from torpor in hibernating animals [8]. Equally important to understanding how Gd infection leads to altered torpor patterns is the need to understand how

these too-frequent arousals to euthermy may be contributing to death – in ways that are not clearly related to energy balance, but are potentially related to the disruption of other homeostatic mechanisms [8].

A detailed understanding of the mechanism(s) by which infection with Gd causes mortality in hibernating bats may provide insights to develop interventional strategies to mitigate this unprecedented wildlife disease. Insectivorous bats perform significant ecosystem services because they are primary predators of nocturnal insects [35–37]. As such, we believe that the loss of cave-dwelling hibernating bats in North America will be ecologically significant.

## Supporting Information

**Appendix S1** Instructions for producing temperature sensitive dataloggers for attachment to bats, including figures.
(PDF)

**Appendix S2** Description of WNS histopathology and assignment of wing damage severity scores (SS), including figures.
(PDF)

## Acknowledgments

For field assistance, we thank R. Arndt, L. DeWolski, B. Douglas, R. Doyle, J. Fregonara, C. Hauser, H. Kaarakka, J. Hajenga, J. Kobilis, M. Kurta, K. Langwig, K. O'Malley, C. Patterson, D. Redell, B. Roelle, C. Rockey, A. Rolfe, B. Scullon, P. Sewell, B. Smith, A. Stauffer, J. Wallace, and P. White. We also thank the individual site owners for access to hibernacula. C. Musante, N. White, K. Weaver, C. Meade, M. Furze, P. Reamey, S. Wade, and S. Alfano assisted in the construction of dataloggers. W. M. Ford provided a review of an early version of the manuscript; M. J. Behr reviewed and provided comments for Appendix S2 (Description of WNS histopathology and assignment of wing damage severity scores (SS)). D. R. Dougherty, P. T. Reamey, J. Glathar, and C. Butchkoski assisted with Figure 1. Use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the US Government.

## Author Contributions

Conceived and designed the experiments: DMR CLF GGT AK ACH ERB SRD CWS DSB. Performed the experiments: DMR CLF GGT AK ACH ERB SRD CWS DSB CUM RJ LEG SAB MEV LKM DSB. Analyzed the data: DMR CLF DSB CUM RJ SAB MEV LEG. Contributed reagents/materials/analysis tools: DMR CLF GGT AK ACH SRD CWS DSB CUM DSB. Wrote the paper: DMR CLF GGT DSB CUM MEV.

## References

1. US Fish and Wildlife Service (2012) North American bat death toll exceeds 5.5 million from white-nose syndrome. Press Release (January 17, 2012). Available: http://www.fws.gov/whitenosesyndrome/pdf/WNS_Mortality_2012_NR_FINAL.pdf. Accessed 2012 Apr 02.
2. Turner GG, Reeder DM, Coleman JTH (2011) A five-year assessment of mortality and geographic spread of white-nose syndrome in North American bats, with a look at the future. Bat Research News 52: 13–27.
3. Blehert DS, Hicks AC, Behr MJ, Meteyer C, Berlowski-Zier BM, et al. (2009) Bat white-nose syndrome: an emerging fungal pathogen? Science 323: 227.
4. Frick WF, Pollock JF, Hicks AC, Langwig KE, Reynolds DS, et al. (2010) An emerging disease causes regional population collapse of a common North American bat species. Science 329: 679–682.
5. Meteyer CU, Buckles EL, Blehert DS, Hicks AC, Green DE, et al. (2009). Histopathologic criteria to confirm white-nose syndrome in bats. Journal of Veterinary Diagnostic Investigation 21: 411–414.
6. Gargas A, Trest MT, Christensen M, Volk TJ, Blehert DS (2009) *Geomyces destructans* sp nov associated with bat white-nose syndrome. Mycotaxon 108: 147–154.
7. Lorch JM, Meteyer CU, Behr MJ, Boyles JG, Cryan PM, et al. (2011) Experimental infection of bats with *Geomyces destructans* causes white-nose syndrome. Nature 480: 376–378.
8. Cryan PM, Meteyer CU, Boyles JG, Blehert DS (2010) Wing pathology of white-nose syndrome in bats suggests life-threatening disruption of physiology. BMC Biology 8: 135. Available: http://www.biomedcentral.com/1741-7007/8/135. Accessed 2011 Jul 28.
9. Boyles JG, Willis CKR (2010) Could localized warm areas inside cold caves reduce mortality of hibernating bats affected by white-nose syndrome? Frontiers in Ecology and the Environment 8: 92–98.
10. Kunz TH, Wrazen JA, Burnett CD (1998) Changes in body mass and body composition in pre-hibernating little brown bats (*Myotis lucifugus*). Ecoscience 5: 8–17.
11. Reynolds DS, Kunz TH (2000) Changes in body composition during reproduction and postnatal growth in the little brown bat, *Myotis lucifugus* (Chiroptera: Vespertilionidae). Ecoscience 7, 10–17.
12. Jonasson KA, Willis CKR (2011) Changes in Body Condition of Hibernating Bats Support the Thrifty Female Hypothesis and Predict Consequences for Populations with White-Nose Syndrome. PLoS ONE 6(6): e21061. Available: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0021061. Accessed 2011 Aug 25.
13. Kayser C (1965) Hibernation. In: Mayer W, Van Gelder R, editors. Physiological Mammalogy. New York: Academic Press. 180–296.
14. Barnes BM (1989) Freeze avoidance in a mammal - body temperatures below 0°C in an arctic hibernator. Science 244: 1593–1595.
15. Geiser F (2004) Metabolic rate and body temperature reduction during hibernation and daily torpor. Annual Review of Physiology 66: 239–274.
16. Brack V Jr, Twente JW (1985) The duration of the period of hibernation of 3 species of vespertilionid bats.1. Field studies. Canadian Journal of Zoology 63: 2952–2954.
17. Thomas DW (1995) The physiological ecology of hibernating bats. In: Racey PA, Swift SM, editors. Ecology, Evolution, and Behaviour of Bats. Oxford: Clarendon Press. 233–244.
18. Thomas DW, Dorais M, Bergeron J-M (1990) Winter energy budgets and cost of arousals for hibernating little brown bats, *Myotis lucifugus*. Journal of Mammalogy 71: 475–479.
19. Humphries MM, Speakman JR, Thomas DW (2005) Temperature, hibernation energetics, and the cave and continental distributions of little brown myotis. In: Zubaid A, McCracken GF, Kunz TH, editors. Functional and Evolutionary Ecology of Bats. Oxford: Oxford University Press. 23–37.
20. Britzke ER, Sewell P, Hohmann MG, Smith R, Darling SR (2010) Use of temperature-sensitive transmitters to monitor the temperature profiles of hibernating bats affected with white-nose syndrome. Northeastern Naturalist 17: 239–246.
21. Warnecke L, Turnera JM, Bollingerb TK, Lorch JM, Misra V, et al. (2012) Inoculation of bats with European *Geomyces destructans* supports the novel pathogen hypothesis for the origin of white-nose syndrome. PNAS Early Edition. Available: www.pnas.org/cgi/doi/10.1073/pnas.1200374109. Accessed 2012 Apr 20.
22. Willis CKR, Brigham RM (2003) Defining torpor in free-ranging bats: experimental evaluation of external temperature-sensitive radiotransmitters and the concept of active temperature. Journal of Comparative Physiology B 173: 379–389.
23. Lovegrove BG (2009) Modification and miniaturization of Thermochron iButtons for surgical implantation into small animals. Journal of Comparative Physiology B 179: 451–458.
24. Lorch JM, Gargas A, Meteyer CU, Berlowski-Zier BM, Green DE, et al. (2010) Rapid polymerase chain reaction diagnosis of white-nose syndrome in bats. Journal of Veterinary Diagnostic Investigation 22: 224–230.
25. Rice WR (1989) Analyzing tables of statistical tests. Evolution 43: 223–225.
26. Siegel S, Castellan NJ (1988) Nonparametric statistics for the behavioral sciences, 2nd ed. New York: McGraw-Hill.
27. Martínková N, Bačkor P, Bartonička T, Blažková P, Červený J, et al. (2010) Increasing incidence of *Geomyces destructans* fungus in bats from the Czech Republic and Slovakia. PLoS One 5(11): e13853. Available: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0013853. Accessed 2012 Apr 20.
28. Puechmaille SJ, Wibbelt G, Korn V, Fuller H, Forget F, et al. (2011) Pan-European distribution of white-nose syndrome fungus (*Geomyces destructans*) not associated with mass mortality. PloS One 6(4): e19167. Available: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0019167. Accessed 2012 Apr 20.
29. Boyles JG, Dunbar MB, Storm JJ, Brack V Jr (2007) Energy availability influences microclimate selection of hibernating bats. Journal of Experimental Biology 210: 4345–4350.
30. Dunbar MB, Tomasi TE (2006) Arousal patterns, metabolic rate, and an energy budget of eastern red bats (*Lasiurus borealis*) in winter. Journal of Mammalogy 87: 1096–1102.
31. Twente JW, Twente J, Brack V Jr (1985) The duration of the period of hibernation of 3 species of vespertilionid bats. 2. Laboratory studies. Canadian Journal of Zoology 63: 2955–2961.
32. Luis AD, Hudson PJ (2006) Hibernation patterns in mammals: A role for bacterial growth? Functional Ecology 20: 471–477.

33. Bouma HR, Carey HV, Kroese FG (2010) Hibernation: The immune system at rest? Journal of Leukocyte Biology 88: 619–624.
34. Puechmaille SJ, Frick WF, Kunz TH, Racey PA, Voigt CC, et al. (2011) White-nose syndrome: Is this emerging disease a threat to European bats? Trends in Ecology & Evolution 26(11): 570–576.
35. Cleveland CJ, Betke M, Federico P, Frank JD, Hallam TG, et al. (2006) Economic value of the pest control service provided by Brazilian free-tailed bats in south-central Texas. Frontiers in Ecology and the Environment 4: 238–243.
36. Boyles JG, Cryan PM, McCracken GF, Kunz TH (2011) Economic importance of bats in agriculture. Science 332: 41–42.
37. Kunz TH, Braun de Torrez E, Bauer D, Lobova T, Fleming TH (2011) Ecosystem services provided by bats. Annals of the New York Academy of Sciences 1223: 1–38.

**Frequent Arousal from Hibernation Linked to Severity of Infection and Mortality in Bats with White-nose Syndrome.**

DeeAnn M. Reeder[a*], Craig L. Frank[b], Gregory G. Turner[c], Carol U. Meteyer[d], Allen Kurta[e], Eric R. Britzke[f], Megan E. Vodzak[a], Scott R. Darling[g], Craig W. Stihler[h], Alan C. Hicks[i], Roymon Jacob[a], Laura E. Grieneisen[a], Sarah A. Brownlee[a], Laura K. Muller[d], and David S. Blehert[d]

*[a]Department of Biology, Bucknell University, Lewisburg, PA, USA; [b]Department of Biological Sciences, Fordham University, Armonk, NY, USA; [c]Pennsylvania Game Commission, Harrisburg, PA, USA; [d]U.S. Geological Survey - National Wildlife Health Center, Madison, WI, USA; [e]Department of Biology, Eastern Michigan University, Ypsilanti, MI, USA; [f]US Army Engineer Research and Development Center, Vicksburg, MS, USA; [g]Vermont Fish and Wildlife Department, Rutland, VT, USA; [h]West Virginia Division of Natural Resources, Elkins, WV, USA; [i]New York State Department of Environmental Conservation, Albany, NY, USA*

**Supporting Information: Appendix S1. Instructions for producing temperature sensitive dataloggers for attachment to bats.**

Temperature sensitive external dataloggers (Bucknell University Temperature Trackers, BUTTs) were made in from Thermochron DS1922L iButtons (Maxim Integrated Products, Inc., California, USA), which is the same underlying technology found in AlphaMach's iBBat dataloggers (AlphaMach, Inc., British Columbia, Canada). The protocol for creating, programming, and calibrating the iButton components is based upon the modification and miniaturization procedure as described by Lovegrove [S1], but has marked differences.

The DS1922L iButtons are self-contained cylindrical temperature loggers that can measure and record temperatures from -40°C to 85°C. A total of 8,192 8-bit readings can be recorded at periodic intervals ranging from 1 second to 273 hours. Each logger has a unique serial number, which is located on the can and is displayed in the program when missioning the logger. The user-defined mission can start immediately, start upon a specific time delay, or start once a specified temperature has been reached.

**OBTAINING THE INTERNAL COMPONENTS FOR BUTT CONSTRUCTION:**

***De-house the iButton:*** Each iButton weighs 3.3 grams and is made with a silicon chip and battery contained in a stainless steel casing or "can," consisting of a "lid" (part with writing) and "base" (cylinder sides and lipped face) (Fig. S1A). BUTTs only utilize the chip and battery, and thus it is necessary to extract these components. This is accomplished by sawing the casing with a flat metal hand-file approximately one-third to halfway (~ 2:30 and 9:30 on a clock face) on each side of an iButton clamped in a vice (Fig. S1B). File the case at the two points until a black plastic grommet is exposed at the lid edge and there are clear cuts on the lip of the base. While still in the vice, use a set of pliers or the top of the file to bend back the base from the lid (Fig. S1C). After removing the iButton

from the vice, the lid of the canister can be removed and the inside components can be extracted (Fig. S1D).

***Clip Grommet, Circuit Board, and Input/output Prong:*** The inside components consist of the silicon chip and battery surrounded by a black plastic grommet. On the chip a three-pin terminal is found consisting of battery negative, battery positive, and input/output (I/O) terminals. To reduce the weight of the final datalogger, some of these components can be trimmed. Separate the circuit board from the battery by sliding it away from the pins. Use clippers to cut and remove the black plastic grommet to the left and right of the terminal (Fig. S1E-F). Small sharp scissors may be used to trim the chip about 2 mm and 0.5mm from the left and right, respectively, as pictured (Fig. S1G), taking special care not to clip off important components.

To further reduce weight and to prevent future failures when extracting data from the loggers, it is possible to cut the longest prong (I/O) (as shown in Fig. S1H). Use a pair of small sharp scissors and cut it to a length similar to the other prongs. The length of this prong often makes the circuit board detach from the battery when extracting the internal components from their plastic coating for data downloading (see below).

***Glue Circuit Board to Battery and Epoxy the Pins:*** If at any point a programmed circuit board loses its connection with the battery via the three prongs, the mission and all of its recorded data will be lost. To reduce the likelihood of data loss, glue the chip to the battery with a dab of fast setting five minute epoxy (e.g., Locite Instant Mix Epoxy, Loctite® Brand Consumer Products, Henkel Corporation, Westlake, Ohio, USA), and connect the pins to the circuit board with a special flexible silver-filled conductive epoxy (McMaster-Carr, Cleveland, OH, USA; item # 7661A13). This glue comes in small easy-mix packets (2.5 grams).

To glue the circuit board to the battery, mix a small amount of equal parts epoxy resin and five minute hardener (dispensed from a dual syringe) with a wooden applicator stick. Use the stick to spread a thin layer in the center of the battery, making sure not to spread too close to the black grommet that holds the terminal pins. Slide the chip onto the battery to align with the prongs, and then lightly press the circuit board to battery to ensure a bond will form (Fig. S1H). Allow the glue to dry in a fume hood or other ventilated area.

For connecting the pins to the circuit board, mix a small amount of equal parts of the two components of the silver-filled epoxy with a syringe needle and apply a tiny, but sufficient, dot on the far left and right connection points, avoiding the middle prong (shown in Fig. S1H). This is best done under a dissection scope, in order to ensure that the applied silver glue does not touch between any two connection points (which would stop the logger from functioning). Lovegrove [S1] recommends soldering the pins to the circuit board, but we find the use of electrically conductive glue sufficient, and easier.

**FINAL BUTT PREPARATIONS:**

***Program:*** The modified chip and battery now can be prepared for programming and coating. BUTTs are constructed from the pieces of the iButton and are thus programed using the same software (One Wire Viewer: http://www.maxim-ic.com/products/ibutton/software/1wire/OneWireViewer.cfm). The iButton reader does not work without the canister, however, so a specially modified lead system must be made. As Lovegrove [S1] explained, the connecting lead should be created from a 6P2C modular (telephone) cord or Ethernet cord that is appropriate for the 1-Wire RJ11 port reader. Instead of using crocodile clips, we have found that splicing probe leads to the cable eliminates the risk of pulling the leads off of the circuit board. For initial programming, one person touches the probe leads to the I/O and ground pins on the circuit board while a second person types missioning specifications into the computer. Alternatively, for the initial programming (but not later downloading), the probe leads can be propped in place with larger clips and balanced on the table, allowing a single person to program the loggers (Fig. S1I). After the logger is programmed and before it is covered in plastic coating, it is important to not allow de-housed iButtons to touch each other as this can cause a loss of the programming.

***String:*** In order to facilitate coating in plastic, pass about 250 cm total length of thread through the gap between the circuit board and battery on the terminal end of the modified iButton (Fig. S1J). Place a piece of lab tape folded onto itself on one end of the string to record either the serial code of the circuit board or another unique number assigned to the datalogger. The string can be put in place before or after programming. The serial code of the circuit board can be found on the original can that housed it, or is displayed in the missioning software.

***Plastic Cling Wrap:*** The internal components must be wrapped in plastic wrap before they can be coated with a thick plastic dip. The piece of wrap used must cover the components completely, but not add excess surface for the dip to cling. Using a small square piece (about 3.8 X 3.8 cm) of plastic cling wrap, place the logger components face down with the strings up and one corner of wrap between the two strings. Fold the left and right corners to cover the battery first, followed by the bottom corner. Finally, fold the top corner down. Make sure that the strings are pulled taught and the plastic wrap does not bunch on them. By folding the top down last, the chances of the dip pulling the plastic off are minimized (Fig. S1K).

***Plasti-Dip:*** The logger components are now ready to be coated in a synthetic rubber, Plasti Dip, which is available in a variety of colors (Plasti Dip International, Blaine, MN, USA). The number of coats of Plasti Dip is based on lab or field use (thicker for enduring field conditions) and the starting thickness of the liquid Plasti Dip, which thickens in the jar after opening. Pour about 40 mL of Plasti Dip into a

plastic 50 mL beaker. Mix small amounts of turpentine into the Plasti Dip until the appropriate consistency, approximating that of warm honey, is reached. Multiple thin coats are preferable to a single thick coat. Holding onto the strings, dip the logger in its plastic wrap into the Plasti Dip and then hold it over the Dip container to allow excess to rubber to drop off. Hang the logger to dry in a fume hood or other highly ventilated area by both strings (Fig. S1.1L). Before applying additional coats of Plasti Dip, excess plastic can be trimmed (e.g., at the bottom corners).

***Label:*** Once BUTTs are fully dipped and dry, carefully remove them from the fume hood. Clip the strings from the BUTTs, but make sure to keep the ID tag with its logger. Use an indelible marker to write the ID number on the flat, battery side of the BUTT (Fig. S1M), as the side of the logger with the leads is attached to the bat. The number also can be written on the edge of the logger, where the strings were. Unlike the unprotected de-housed iButton components that cannot touch, BUTTs after coating can now touch anything.

***Calibrate:*** Because of the significant alterations that have been made to the iButton components, and because of variable levels of plastic coating on the final BUTTs, each logger must be individually calibrated to ensure accurate measurements. To calibrate loggers, BUTTs are placed in an airtight container with a thermocouple set to record temperature every 15 s, and the container is submerged into 2°C, 23°C, and 37°C water baths for a minimum of 4 hours each. After downloading the values from the thermocouple, an average of at least 20 temperature recordings should be taken from the timepoints in which temperature was stable at each of the calibration temperatures. These averaged values are $T_1$, $T_2$, and $T_3$, respectively. The average temperature recorded when the thermocouple was at each calibration temperature ($T_1$, $T_2$, and $T_3$) can then be compared to the values from identical timepoints for each individual logger to generate three deviations (temperature of logger - temperature of thermocouple; $D_1$, $D_2$, and $D_3$). These deviations are used to find the equation of the quadratic curve, from which additional temperature readings (from the loggers) can be corrected. If the three deviations are $D_1$, $D_2$, and $D_3$ and the temperatures measured by the thermocouple are $T_1$, $T_2$, and $T_3$, the roots of the quadratic equation (α (alpha) and ß (beta)) can be calculated, as can be the constants A, B, and C, as follows:

$$\alpha = (D_1 - D_3) + ((D_2 - D_1)*(T_3 - T_1))/(T_2 - T_1)$$

$$ß = T_3^2 - T_2*T_3 + T_2*T_1 - T_3*T_1$$

$$A = D_1 - B*T_1 - C*T_1^2$$

$$B = (D_2 - D_1 - C*(T_2^2 - T_1^2))/(T_2 - T_1)$$

$$C = - \alpha/ ß$$

From these values, corrected temperatures are calculated as follows, where $T_M$ is the uncorrected (measured) temperature and $T_C$ is the corrected (calculated) temperature:

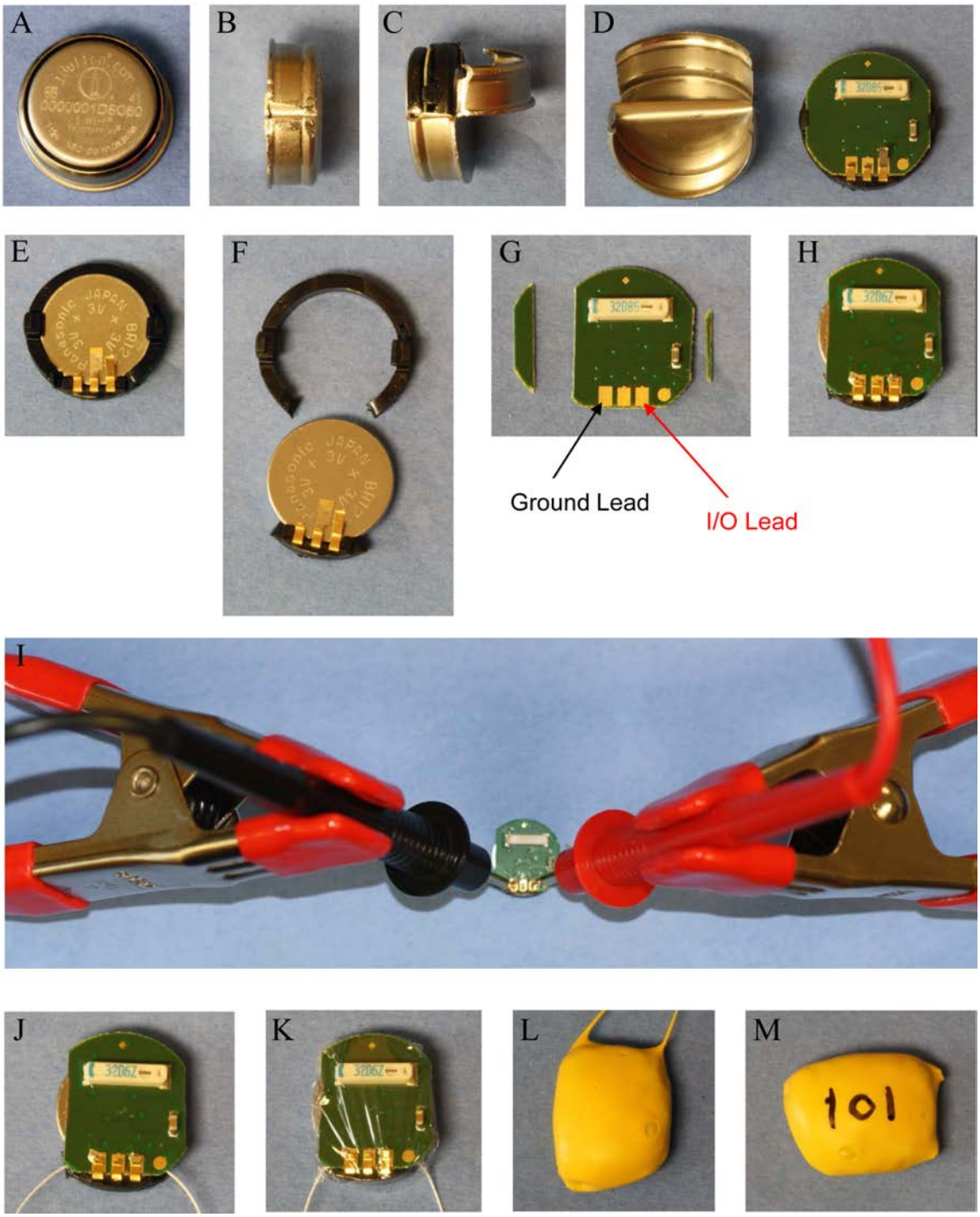
$$T_C = T_M - A - B*T_M - C*T_M^2$$

While data from the thermocouple should be downloaded immediately after calibration to ensure that the thermocouple recorded periods of stable temperature at each of the three calibration temperatures, the remainder of the calibrations must be performed after data from the loggers are downloaded (after removal from a bat).

***Download:*** To re-access the terminal pins needed to download the information from the loggers, carefully make an incision at the top edge of the logger (where the remaining portion of the grommet is). Peel back the PlastiDip and plastic wrap just enough to expose the pins. Establish a connection with the computer and software using the leads as described above in the programming section.

**References**

S1. Lovegrove BG (2009) Modification and miniaturization of Thermochron iButtons for surgical implantation into small animals. Journal of Comparative Physiology B 179: 451-458.




**Appendix S1, Figure S1. Construction of Bucknell University Temperature Tracker dataloggers (BUTTs) from iButtons.** An iButton prior to modification (A), iButton showing filing to remove casing or 'can' (B), peeling back of can (C), iButton can and internal components separated (D), battery with black plastic grommet intact (E), battery with most of grommet removed to decrease weight (F), circuit board with sides trimmed to decrease weight (G), circuit board reattached to battery, with I/O (input/output) lead trimmed to decrease weight and with leads secured to the circuit board with silver-filled conductive epoxy (H), programming the datalogger via the ground and I/O leads (I), attaching string to facilitate coating in plastic (J), wrapping logger in plastic wrap to protect circuit board from coating rubber (K), first coating in rubber 'Plasti Dip' (L), completed logger (M).

**Frequent Arousal from Hibernation Linked to Severity of Infection and Mortality in Bats with White-nose Syndrome.**

DeeAnn M. Reeder[a*], Craig L. Frank[b], Gregory G. Turner[c], Carol U. Meteyer[d], Allen Kurta[e], Eric R. Britzke[f], Megan E. Vodzak[a], Scott R. Darling[g], Craig W. Stihler[h], Alan C. Hicks[i], Roymon Jacob[a], Laura E. Grieneisen[a], Sarah A. Brownlee[a], Laura K. Muller[d], and David S. Blehert[d]

*[a]Department of Biology, Bucknell University, Lewisburg, PA, USA; [b]Department of Biological Sciences, Fordham University, Armonk, NY, USA; [c]Pennsylvania Game Commission, Harrisburg, PA, USA; [d]U.S. Geological Survey - National Wildlife Health Center, Madison, WI, USA; [e]Department of Biology, Eastern Michigan University, Ypsilanti, MI, USA; [f]US Army Engineer Research and Development Center, Vicksburg, MS, USA; [g]Vermont Fish and Wildlife Department, Rutland, VT, USA; [h]West Virginia Division of Natural Resources, Elkins, WV, USA; [i]New York State Department of Environmental Conservation, Albany, NY, USA*

**Supporting Information: Appendix S2. Histologic severity scoring (SS) of white-nose syndrome (WNS) using wing membrane.**

Wing membrane was used to score the damage associated with WNS. The skin of the muzzle may or may not be affected in bats with WNS, and if infected, may not be as physiologically important as the damage the fungal agent *Geomyces destructans* (Gd) causes to the wing membrane.

All bats used for this classification system were *Myotis lucifugus* that were part of this study or euthanized for state surveillance for WNS, shipped chilled for overnight arrival, and processed the day they arrived to avoid postmortem changes that might interfere with lesion interpretation. It may be difficult to wrap all of the wing membrane on two dowels for bats that are much larger than M. lucifugus. If this is not possible, as much of the leading edge and trailing edge of the wing should be included for histologic evaluation as these margins can be the primary areas infected.

**ASSUMPTIONS:**

Both wings are equally affected by Gd. Using only one wing for histopathology allows the second wing to be removed aseptically from the body for PCR and culture.

Rolling all of the membrane from one wing onto 2 dowels and trimming each into 3, 0.5 cm segments, embedding and sectioning all segments, mounting on a slides and observing the microscopic sections provides a reasonable representation of severity of wing damage.

Biological systems rarely fit exactly into the round holes we carve out for them, but they can be placed in general categories that can help us better understand disease progression.

**METHOD FOR PREPARING WING MEMBRANE:**

Dowels are rolled to 0.25 cm diameter from unflavored and uncolored dental orthodontic wax, and cut to 2.5 cm lengths. A piece of orthodontic paraffin rolled to 10 cm long and cut into 4 equal lengths will provide the appropriate length and diameter for the dowels. All membrane from one wing is removed, cut into 1cm strips, rolled in overlapping spirals around the dowel so that all

membrane is wrapped onto 2 dowels resulting in multiple layers of membrane. These paraffin dowels are placed into a labelled cassette to maintain the arrangement of the membrane on the paraffin, and this cassette is placed in formalin for at least 24 hrs. The entire 'membrane roll', inclusive of paraffin dowel, is trimmed to approximately 0.5 cm cross sections yielding approximately 6 whorls of tissue (2 dowels, 3/dowel). These cross-sections of rolls of wing membrane with the central paraffin dowel are placed cut side down in a cassette, processed and embedded in paraffin, sectioned at 4 um, placed on a glass slide and stained using PAS [S1]. Six rolls of wing membrane will be visible on the slide.

**METHOD FOR FIGURES S1-S4:**

One prototype bat was chosen from this study for each grade; mild, moderate, moderately severe, and severe. The digital images were taken using an Insight Firewire Spot camera and software. One field of view was used for the set of pictures that represent a grade of severity 1 through 4. A set of 4 images was taken at different magnifications to illustrate both distribution (low magnification) and invasion (higher magnifications).

**CLASSIFICATION OF SEVERITY SCORES:**

Cupping erosions filled with dense aggregates of fungal hyphae are currently used as the criteria to diagnose WNS. Severity scores from 0 (unaffected), to 4 (severe) depend on presence, extent, and distribution of these cupping erosions. The cupping erosions form a discrete interface with the skin. As these erosions progress, the thin, pigmented epidermis is no longer visible at the 'front' of the invading aggregate of fungal hyphae.

Grading the severity of WNS histopathology considers the presence of typical cupping erosions, the depth and surface area of these erosions and the extent to which these erosions cover the observable wing membrane on the slide. If some of the rolls are more severely affected than others on the same slide, the most severely affected wing rolls are used to establish the severity score. It is difficult to assess severity until you have seen a bat wing membrane that truly fits the designation of 'severe'. It is then easier to put the other degrees of severity in perspective.

The degree of fungal surface colonization and production of conidia are not included in the criteria for diagnosing WNS or in the severity scoring system. Colonization of superficial skin with fungal hyphae and production of conidia are quite variable within and between severity grades although, in general, the density and extent of hyphae on the surface and the production of conidia increase with severity.

The presence and degree of inflammation and bacterial infection of wing membranes are not included in the criteria for diagnosing WNS or in the severity scoring system. Bacteria and inflammation are inconsistent findings and are not necessary for full manifestation of WNS and mortality. However, both can be present in some bats, particularly in spring near the end of hibernation.

**CRITERIA USED TO ASSIGN SEVERITY SCORES**

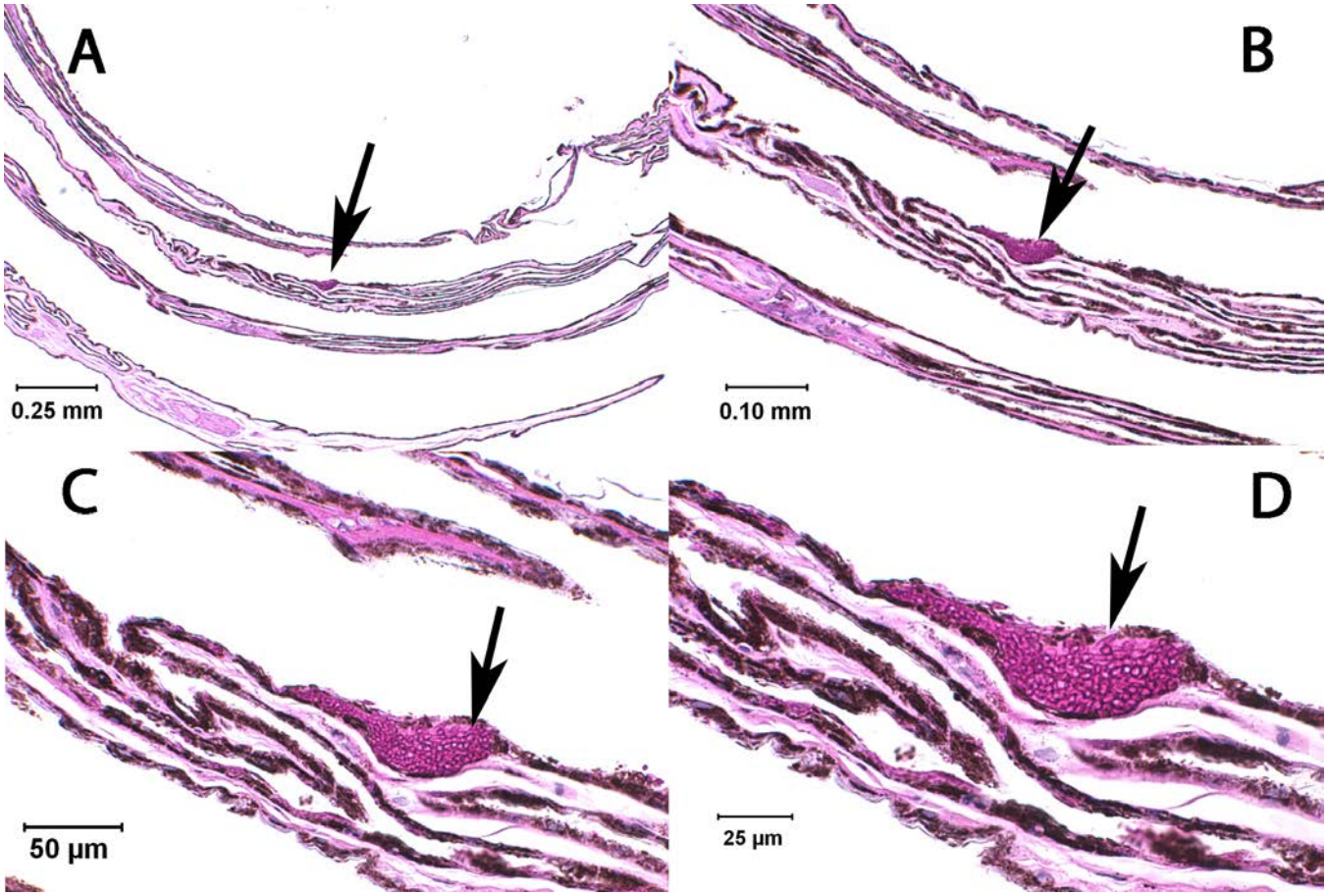
**Severity Score 0 (SS0)**
No fungal cupping and erosion; the wing membrane is considered negative for WNS.

**Severity Score 1 (SS1) -** Mild wing membrane damage with cupping and erosions diagnostic of WNS are present but few (Fig. S1).
*Degree of fungal erosion*: The cupping erosions are discrete but relatively shallow.
*Extent of fungal erosion*: Erosions are few and widely scattered over the rolled sections of wing membrane. Even if infection is limited to only one visible 'cupping erosion' in the 6 whorls of wing membrane, it is considered positive for WNS.

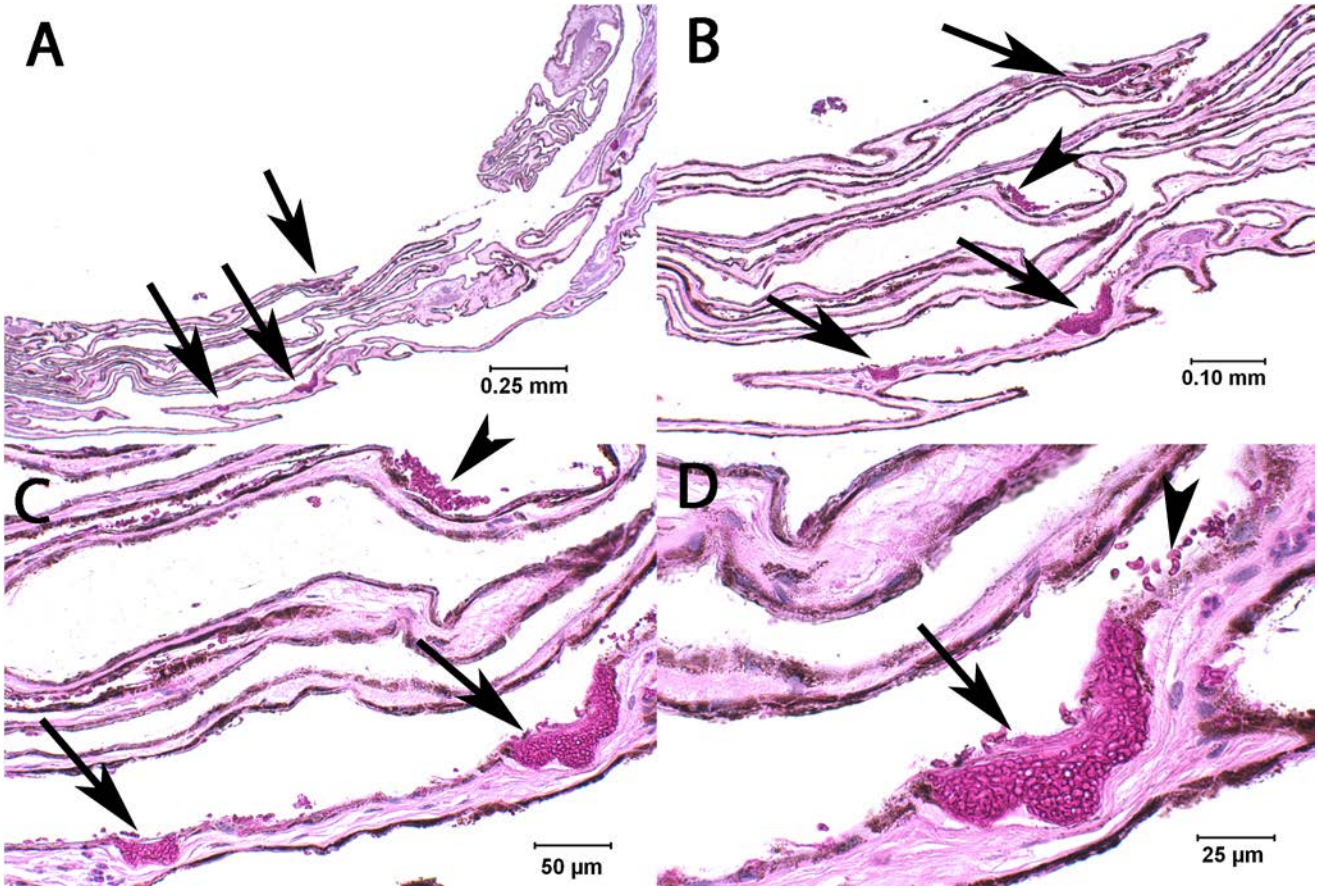
**Severity Score 2 (SS2) -** Moderate wing membrane damage (Fig. S2).
*Degree of fungal erosion:* Cupping erosions are still separate and relatively discrete, but individual erosions involve tissues deeper in the dermis, can be considered ulcers, and can begin to replace regional adnexa.
*Extent of fungal erosion:* Usually all rolls of wing have at least some cupping erosions. A minimum of 4 of the 6 wing rolls should have the characteristic erosions. The majority of individual wing rolls usually have approximately 10 or more cupping erosions

**Severity Score 3 (SS3) -** Moderately severe wing membrane damage (Fig. S3).
*Degree of erosion:* The dense aggregates of fungal hyphae invade wing membrane replacing the components of dermis, including adnexa. This invasion can become almost trans-membrane and individual erosions and ulcers begin to coalesce, resulting in larger regions of wing membrane that are eroded and ulcerated. Individual hyphae penetrate the deeper dermis beyond the discrete interface of the dense aggregate.
*Extent of erosion:* All rolls of wing (6/6) have characteristic erosions/ulcers. The majority of individual wing rolls have more than 10 cupping erosions/ulcers and at least 2 rolls should have more than 20.

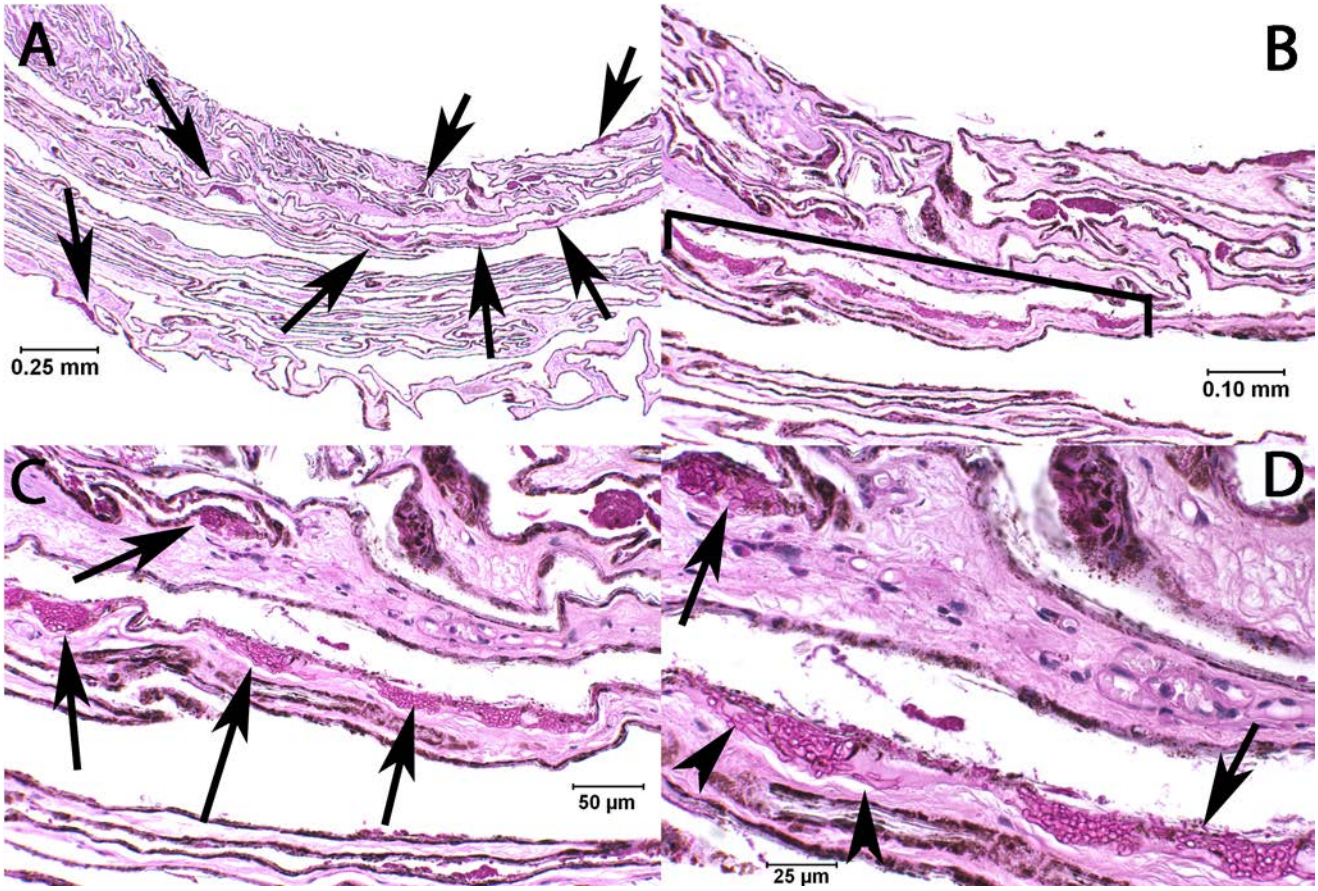
**Severity Score 4 (SS4) -** Severe wing membrane damage (Fig. S4).
*Degree of erosion:* There is extensive tissue invasion. The fungal aggregates coalesce and erode deeper, some almost trans-membrane, and individual hyphae penetrate randomly into the dermis beyond the interface of the fungal aggregate. The morphology of the wing membrane becomes multifocally distorted in response to the extensive fungal invasion. Adnexa can be completely effaced by fungal hyphae and regions of membrane can have changes suggesting infarcts with hypereosinophilia and loss of all identifiable vital structures in the dermis [S2].
*Extent of erosion:* All of the wing rolls (6/6) have cupping erosions. Most of the rolls have more than 20 erosions and some can have as many as 100 or more.



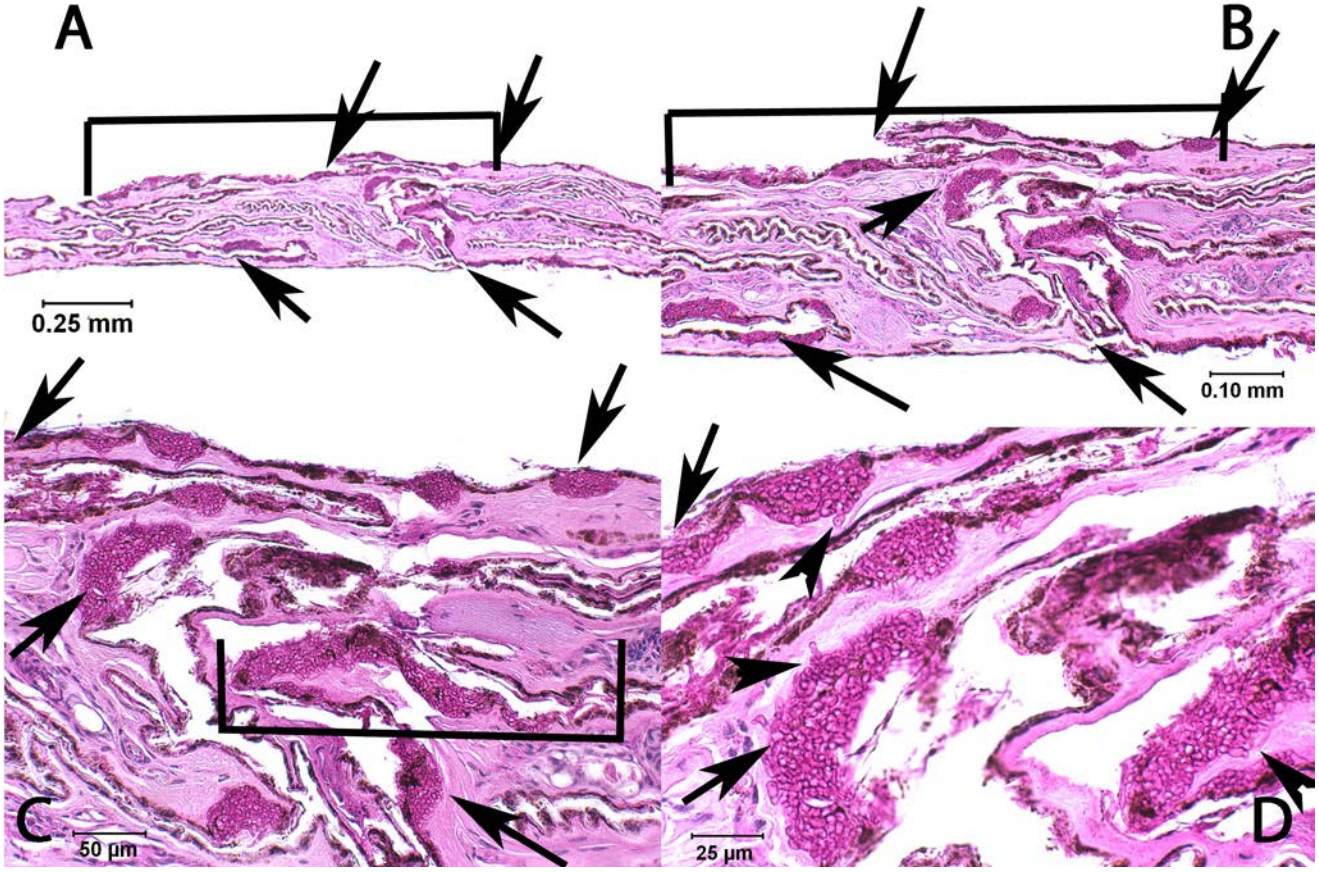

**Appendix S2, Figure S1. Wing membrane damage severity score = 1 (SS1), mild damage due to WNS.** Photomicrographs of periodic acid Schiff-stained 4-µm sections of wing membrane prepared as described above. A portion of a single roll of wing membrane from a little brown bat (*Myotis lucifugus*) contains a single cupping erosion (arrows) fulfilling the diagnostic criteria for WNS. Four magnifications of this single aggregate (A, B, C, D) have calibrations bars embedded in the image.




**Appendix S2, Figure S2. Wing membrane damage severity score = 2 (SS2), moderate damage due to WNS.** Photomicrographs of periodic acid Schiff-stained 4-µm sections of wing membrane prepared as described above. A portion of a single roll of wing membrane from a little brown bat (*Myotis lucifugus*) contains many cupping erosions (arrows). Although more numerous, the cupping erosions are still separate and relatively discrete. Individual erosions are larger than in Fig. S1 and begin to distort the morphology of the wing membrane. Conidia consistent with *Geomyces destructans* are present (arrowheads). Four magnifications of this field of view (A, B, C, D) have calibrations bars embedded in the image.




**Appendix 2, Figure S3. Wing membrane damage severity score = 3 (SS3), moderately severe damage due to WNS.** Photomicrographs of periodic acid Schiff-stained 4-µm sections of wing membrane prepared as described above. A portion of a single roll of wing membrane from a little brown bat (*Myotis lucifugus*) contains numerous cupping erosions; only a subset of these erosions is marked (arrows). The cupping erosions are expanding and coalescing (bracket). Individual fungal hyphae are beginning to move beyond the interface of the fungal aggregate and invade the deeper dermis (arrowheads, C). Four magnifications of this field of view (A, B, C, D) have calibrations bars embedded in the image.




**Appendix 2, Figure S4. Wing membrane damage severity score = 4 (SS4), severe damage due to WNS.** Photomicrographs of periodic acid Schiff-stained 4-µm sections of wing membrane prepared as described above. A portion of a single roll of wing membrane from a little brown bat (*Myotis lucifugus*) containing more numerous and extensive erosions than Fig. S3, and many are approaching transmembrane invasion; only a subset of these erosions and ulcers are marked (arrows). Coalescing fungal aggregates (brackets) expand to cover more surface area of wing membrane. The morphology of the wing membrane becomes multifocally distorted in response to the extensive fungal invasion. Individual fungal hyphae are beginning to move beyond the interface of the fungal aggregate and invade the deeper dermis (arrowheads, C). Four magnifications of this field of view (A, B, C, D) have calibrations bars embedded in the image.

**TABLE SUMMARIZING CRITERIA USED TO SCORE THE SEVERITY OF WNS-ASSOCIATED WING MEMBRANE DAMAGE**

| **Severity Score (SS) or Grade** | **Terminology** | **Number of wing membrane rolls out of the 6 with WNS cupping erosions** | **Number of WNS cupping erosions or ulcerations in the membrane rolls** |
|---|---|---|---|
| 0 | Not WNS | None | None |
| 1 | Mild | At least one | At least one erosion in any of the 'rolls' |
| 2 | Moderate | At least 4/6 | Approximately 10 erosions in each 'roll' |
| 3 | Moderately severe | All affected 6/6 | At least one 'roll' with more than 20 |
| 4 | Severe | All affected 6/6 | Most rolls with more than 20 erosions or ulcerations, some may have more than 100 |

**References**


S1. Meteyer CU, Buckles EL, Blehert DS, Hicks AC, Green DE, et al. (2009) Histopathologic criteria to confirm white-nose syndrome in bats. Journal of Veterinary Diagnostic Investigation 21: 411-414.

S2. Cryan PM, Meteyer CU, Boyles JG, Blehert DS (2010) Wing pathology of white-nose syndrome in bats suggests life-threatening disruption of physiology. BMC Biol 8: 135.