Effect of Humidity on Development of *Pseudogymnoascus destructans*, the Causal Agent of Bat White-nose Syndrome

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Abstract - The invasive fungal pathogen that causes white-nose syndrome (WNS), *Pseudogymnoascus destructans*, has decimated bat populations in the United States, causing significant mortality since the winter of 2006–2007. Temperatures inside many bat hibernacula are ideal for fungal growth, yet data are limited on the effects of humidity on the development of the fungus. The aim of our study was to determine optimum relative humidity (RH) levels for vegetative growth and sporulation of *P. destructans* growing at optimal temperatures. We cultivated *P. destructans* isolate MYA-4855 at 13 °C in individual humidity chambers where RH was maintained between 70.5 and 96.5% using supersaturated salt solutions. We quantified vegetative growth and conidia formation for 3 weeks and implemented single linear regression and ANCOVA analyses to ascertain the effects of RH. Mycelial growth increased significantly with increasing RH by 2 and 3 weeks post inoculation ($P < 0.001$, $r = 0.49$, 0.61). This effect was most pronounced up to 81.5% RH, after which no significant increases in growth were detected. Conidiation increased linearly with increasing RH by 3 weeks post inoculation ($P < 0.05$, $r = 0.33$). Similar to mycelial growth, there was no difference in conidia production once RH exceeded 81.5%. The RH range permissive for significant mycelial development is fairly wide, and RH levels at 81.5% and above at 13 °C support similar levels of vegetative growth. However, our results indicate that at 13 °C, RH of 70% or lower impedes mycelial growth, which could restrict infection severity and/or colonization of organic matter. Finally, lower RH does not restrict production of conidia, which serve as important transmission propagules. Our study suggests that lowering RH could stem infection serverity but may be of limited value as a means of mitigating pathogen dispersal from infected to healthy bats.

Introduction

Recent mass declines in wildlife populations attributable to pathogens are disproportionately due to mycoses (Fisher et al. 2012). White-nose syndrome (WNS) of bats, caused by the fungus *Pseudogymnoacus destructans*, is but an example of this disconcerting trend (Blehert et al. 2009, Frick et al. 2010, Gargas et al. 2009). To date, WNS has caused precipitous declines in bat populations, and the prognosis for *Myotis lucifugus* (LeConte) (Little Brown Myotis) is precarious, with regional extinction predicted by the year 2020 should declines continue at an unabated rate (Frick et al. 2010, US Fish and Wildlife Service 2015).

P. destructans is classified in the family *Pseudeurotiaceae* (Minnis and Lindner 2013), and evidence points to the fungus reproducing asexually in North America because the population is clonal in nature (Chaturvedi et al. 2010, Khanket et al.

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2014, Ren et al. 2012). Given that *P. destructans* is clonal, WNS provides an opportunity to explore factors besides pathogen genotypic variation that may contribute to lethal epidemics among bat species such as Little Brown Myotis (Langwig et al. 2012). A classical concept from plant pathology, the disease triangle, predicates that disease outcomes are dependent on the interaction between hosts, the pathogen, and the environment (Johnson et al. 2014, Scholthof 2007). Pathogen virulence is not an invariant trait disconnected from host physiology, and the disease triangle aptly takes this into account (Casadevall and Pirofski 1999, Scholthof 2007).

Temperature is an environmental factor that has profound impacts on both bat and *P. destructans* physiology, so much so that it has been argued that *P. destructans* virulence co-varies with temperature (Chaturvedi et al. 2010, Gargas et al. 2009, Langwig et al. 2012). The fungus can grow at temperatures as low as 3 °C, and the upper maximum limit for growth ranges from 19.0 to 19.8 °C depending on the isolate tested (Verant et al. 2012). The temperature optima for the *P. destructans* type isolate from NY (MYA-4855) range from 13.1 to 17 °C (Blehert et al. 2009, Gargas et al. 2009, Verant et al. 2012). Bats that are susceptible to WNS typically hibernate at temperatures within the growth range of *P. destructans*. As an example, Little Brown Myotis hibernacula temperatures range from -4 to 13 °C (Brack, 2007, Langwig et al. 2012 and references therein, Twente 1955). At these lower temperatures, bat metabolism is depressed and consequently, the immune system is also down-regulated (Luis and Hudson 2006, Prendergast et al. 2002). Further evidence corroborating the influence of temperature on virulence of *P. destructans* can be observed in the seasonal dynamics of bat infections. Both pathogen prevalence and infection intensity significantly increase throughout the hibernation period as low roosting temperatures prevail, to peak in late winter (Johnson et al. 2014, Langwig et al. 2015).

Another important environmental characteristic for disease development is ambient moisture, but there is a paucity of data on impacts of water vapor on *P. destructans* and/or bat physiology. Water vapor pressure deficit (WVPD) is associated with bat hibernacula selection in *Mytotis* in the Upper Peninsula of Michigan, where bats were found to preferentially use hibernacula with lower WVPD (Kurta and Smith 2014). Although measuring WVPD is preferable to measuring RH, the latter is more commonly reported. Hibernacula conditions for WNS-susceptible bats ranges from 65 to 100% RH (Langwig et al. 2012, Perry 2013, Twente 1955), and correlative analysis indicates that increasing RH is associated with population declines of *Myotis sodalis* (Miller and Allen) (Indiana Myotis) in hibernacula where *P. destructans* is present (Langwig et al. 2012). However, Langwig et al. (2012) could not disentangle the effect of RH on *P. destructans* and/or the bats. Bat species that experience higher evaporative water losses (EWL) during hibernation are more likely to select roost sites with high moisture levels (Cryan et al. 2010). It is not unusual for bats to develop condensation on their pelage (Brack 2007, Cryan et al. 2010). Bats with condensation on the fur that also cluster together would generate a saturated atmosphere immediately surrounding the bats as equilibrium water pressure is established (Kurta 2014). Generally, bats that tend to cluster during hibernation are more susceptible to WNS than bats that do not (Cryan et al. 2010),

and bats in Northeast hibernacula post-WNS have been found to roost alone at a higher frequency when compared to pre-WNS (Langwig et al. 2012). Together, these findings suggest that higher moisture levels are more supportive of *P. destructans* pathogenesis.

There are numerous examples of phytopathogenic fungi where infection (Abawi and Grogan 1975, Canihos et al. 1999, Obanor et al. 2008, Quinn and Powell 1982) and production of both asexual and sexual propagules are significantly related to the amount of, as well as the duration of exposure to those levels of, moisture in the air (Oh 1997, Sosa-Alvarez et al. 1995, Zhao and Shamoun 2006). It is conceivable that animal pathogenic fungi like *P. destructans* are similarly impacted by RH. However, most studies on WNS have been carried out at either saturation/near saturation or unspecified humidity conditions, making it challenging to deduce exactly how RH influences pathogen development (Blehert et al. 2009, Chaturvedi et al. 2010, Gargas et al. 2009, Johnson et al. 2014, Khankhet et al. 2014, Lorch et al. 2011). *P. destructans* growth does decrease under matric potential stress, and in the absence of any surface-tension–reducing compounds (such as lipoidal secretions on bat wing membranes), biomass production is decreased and completely ceases at -2.5 MPa and -5 Mpa, respectively (Raudabaugh and Miller 2013).

Even though *P. destructans* grows well under high humidity levels (Chaturvedi et al. 2010), it is not clear if lower humidity levels would impose a significant restriction on mycelial growth and/or conidiation. Some plant pathogenic fungi grow better and/or produce more propagules as RH or moisture duration increases (Sosa-Alvarez et al. 1995, Zhao and Shamoun 2006), while others have peak RH optima well below 90% RH (Oh 1997). The purpose of our study was to determine the influence of RH on *P. destructans* growth and development. Using humidity levels reflective of those normally encountered in bat hibernacula, we hypothesized that RH would significantly impact mycelial expansion and conidia formation, and predicted that humidity levels at the higher range (>90% RH) would support the most vegetative growth and conidiation.

Methods

Freeze-dried conidia of *P. destructans* MYA-4855 (American Type Culture Collection, Manassas, VA) were rehydrated for a period of about 6 hours and then spread-plated onto Sabouraud dextrose agar (SDA, Remel, KA) plates amended with 50 µg × ml^{-1} chloramphenicol and incubated at 8 °C for 4 weeks. We regularly subcultured *P. destructans* at 8 °C and used a 5-mm–diameter cork-borer to punch out inoculum agar plugs from the edge of the mycelial colony from 4-week–old cultures. We used agar plugs to inoculate 35-mm–diameter SDA plates for humidity bioassays.

Humidity chambers were constructed by placing plexiglass platforms (20 cm × 28 cm) supported by glass cylinders in clear polystyrene boxes (31 cm × 23 cm × 10 cm; Potomac Display, Bunn, NC), and all components of the chambers were either surface disinfected with 70% ethanol or autoclaved after being washed in a 10% bleach solution. We added 400 ml of supersaturated $Mg(NO_3)_2 \times 6H_2O$, NaCl, KCl, or distilled sterile H_2O to the bottom of each humidity chamber, resulting

in an average 70.6, 81.7, 89.5, and 96.5% RH, respectively, in the different chambers (Table 1; Winston and Bates 1960). We then placed uncovered SDA plates inoculated with *P. destructans* on the plexiglass platforms and individual LogTag HAXO-8 loggers (MicroDaq, Contoocook, NH) in each chamber to allow for continuous measure of RH (%) and temperature (°C). A humidity chamber for each RH was incubated at 13 °C for 3 weeks, and we collected and destructively analyzed 4 replicate plates from each humidity chamber every week. We selected the bioassay temperature on the basis of it being identified as the optimum growth temperature for MYA-4855 (Verant et al. 2012). The experiment was run to completion for 3 weeks for a total of 3 times independently.

We estimated vegetative growth by measuring the colony diameter to the nearest mm. When there was no confluent growth from *P. destructans* satellite colonies established by dispersed spores, we measured the diameter twice in directions perpendicular from each other and averaged the 2 values. Sporulation was assessed by taking a tape mount and staining conidia using lactophenol cotton blue. We counted conidia in a random field of view (FOV) at 1000× magnification and used a micrometer (American Optics, Burlington, ON, Canada) to determine the FOV diameter. The number of conidia × FOV^{-1} was used to extrapolate the number of spores per colony by the following formula, where r_c and r_{FOV} equals the radius of the colony and FOV, respectively:

$$spores \times colony^{-1} = ([\pi \times r_c^2] / [\pi \times r_{FOV}^2]) \times spores_{FOV}$$

Both the area of the colony and FOV were expressed in mm^2. We replicated the experiment 3 times, and upon concluding each experimental replicate, downloaded the logger data in order to verify the actual RH and temperature in each chamber (Table 1).

Minitab Express (Minitab, Inc., State College, PA) and SAS v9.4 (SAS Institute, Inc., Cary, NC) were used for all statistical analyses. First, we ran a 2-way ANOVA using either conidia count or colony diameter as the response variable and time and humidity as predictor variables. Means were separated and Tukey's pairwise comparisons were performed. Second, we performed simple linear regressions to determine if there was a significant relationship between either conidia count or colony diameter and time or humidity. For the former predictor variable, we assayed each humidity level in isolation and for the latter predictor, we analyzed each week singly. Each experimental replicate was analyzed separately, and then the

Table 1. Humidity levels and temperatures in humidity chambers averaged across the 3-week incubation period and all 3 experimental replicates. Numbers after the ± denotes the standard error.

Salt	RH (%)	Temperature (°C)
$Mg(NO_3)_2$	70.6 ± 0.07	13.2 ± 0.007
NaCl	81.7 ± 0.06	12.9 ± 0.010
KCl	89.5 ± 0.05	11.9 ± 0.008
No salt[A]	96.5 ± 0.03	13.1 ± 0.008

[A]Distilled water used.

pooled dataset was subjected to the same analysis. Finally, we employed a regression analysis using the PROC GLM procedure and an ANCOVA analysis on the pooled data set to compare the slopes of colony diameter or conidia count versus time for different humidity levels. We created and assessed diagnostic plots (normality and equal variances) to ensure that assumptions to tests were not violated as well as to check for influential data points. Unless otherwise noted, significance means P-value ≤ 0.05 for treatment comparisons.

Results

Vegetative growth increased significantly with increased RH levels at 2 and 3 weeks post inoculation (WPI) on SDA plates, while there was no difference in growth after 1 week (Table 2, Fig. 1A). The r-value increased from 0.49 to 0.61 by 2 and 3 weeks of growth, respectively, indicating an increased strength in the linear relationship between humidity and mycelial growth diameter (Table 2). Examining mycelial growth for each individual humidity level over time revealed that radial expansion significantly increased over time for all RH levels, but the strength of the relationship was greater at 81.5% RH and above (r = 0.87–0.93) compared to 70% RH (r = 0.49; Table 3). The slope of the mycelial growth over time was significantly higher at 81.5 to 96.5% RH compared to 70% RH (Table 3).

Table 2. Regression analysis of *P. destructans* vegetative growth and conidia production in response to humidity, using a pooled data set comprised of 3 experimental replicates. r = regression coefficient for response variable ("vegetative growth" and "conidia production") in response to humidity. R^2 = coefficient of determination followed by the significance of the F-test for the regression where NS = nonsignificant; *, $P \leq 0.05$: **, $P < 0.01$; ***, $P < 0.001$.

	Vegetative growth (mm)		Conidia production (log n × colony^{-1})	
Time (week)	r	R^2	r	R^2
1	0.17	0.0483 (NS)	0.14	0.0194 (NS)
2	0.49	0.2384***	0.01	0.0002 (NS)
3	0.61	0.3689***	0.33	0.1069*

Table 3. Regression and ANCOVA analysis of *P. destructans* vegetative growth and conidia production in response to time, using a pooled data set including data from 3 experimental replicates. r = regression coefficient for response variable ("vegetative growth" and "conidia production") in response to time. R^2 = coefficient of determination followed by the significance of the F-test for the regression where NS = nonsignificant; *, $P \leq 0.05$: **, $P < 0.01$; ***, $P < 0.001$. LS mean = least-squares mean estimates from ANCOVA comparing treatment slopes, where estimates followed by different letters are significantly different.

	Vegetative growth (mm)			Conidia production (log n × colony^{-1})		
RH(%)	r	R^2	LS mean	r	R^2	LS mean
70	0.49	0.2435**	18.1c	0.27	0.0706 (NS)	5.4a
81.5	0.87	0.7655***	22.1ab	0.51	0.2638**	5.5a
89.5	0.93	0.8613***	21.2b	0.54	0.2928***	5.3a
96.5	0.88	0.7720***	23.1a	0.59	0.3488***	5.4a

Fungal conidia production was less influenced by variation in RH within the range tested (70.5 to 96.5%; Table 3, Fig. 1). There was no significant linear relationship between humidity and sporulation level by 2 WPI (Table 2, Fig. 1B). By 3 WPI, higher RH levels were correlated with increased conidia production (r = 0.33). It was found that increasing the humidity from 70 to 81.5% was primarily responsible for this linear relationship (Table 2, Fig. 1B). Conidia production in-

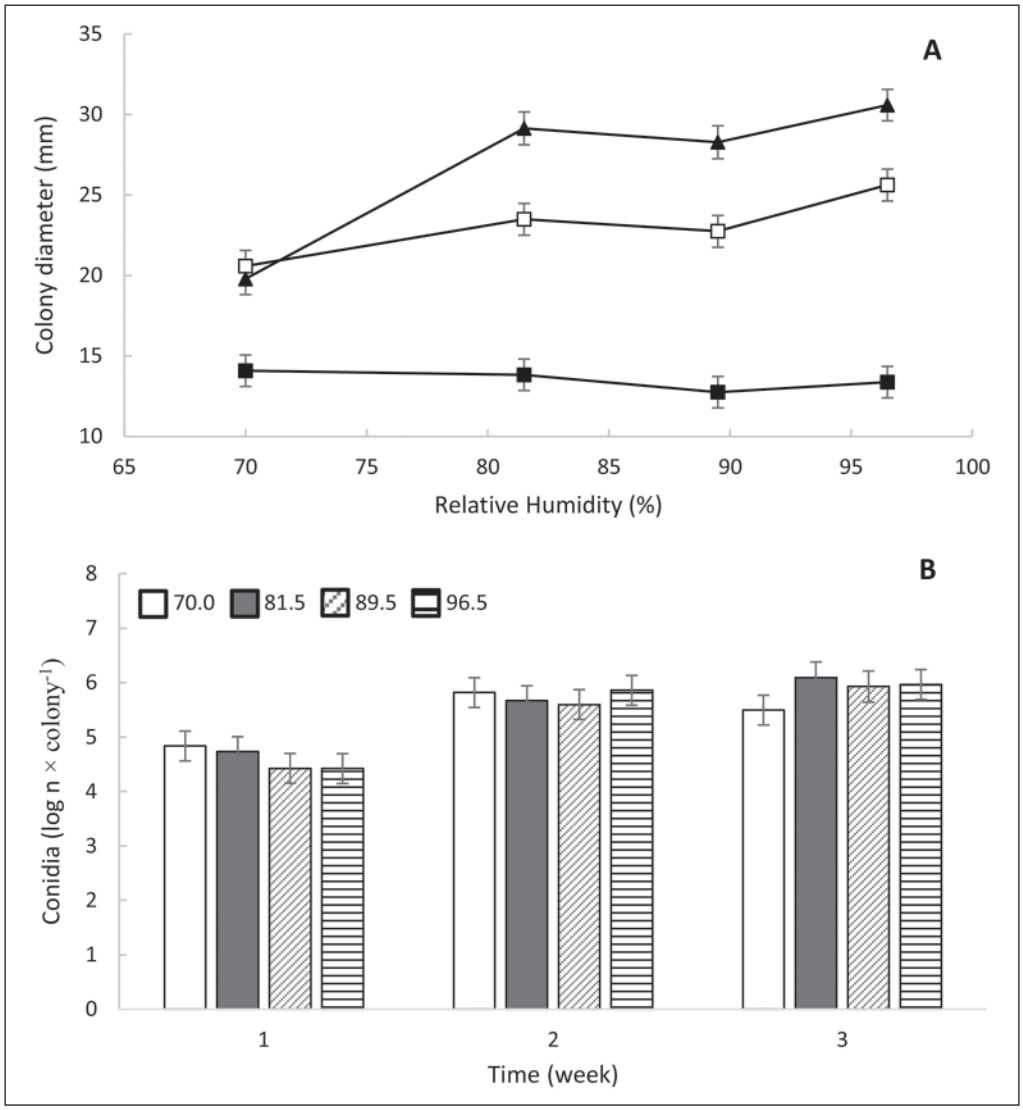

Figure 1. Vegetative growth and conidia production of *P. destructans* over time at different humidity levels. (A) The fungal colony diameter in response to humidity (%) assessed at 1 (■), 2(□), and 3 (▲) weeks post-inoculation. (B) Fungal conidia production (log n × colony^{-1}) at 70-96.5% humidity at 1, 2, and 3 WPI. The figure is based on a pooled data set from 3 experimental replicates. Each marker or bar is the mean of 12 replicates (except for 3 where 1 data point was omitted due to contamination), and error bars are the standard error of the mean.

creased significantly over time at 81.5 to 96.5% RH, whereas no such relationship was denoted for 70% RH (Table 3). Despite these differences, the slope of conidia increases were similar for all relative humidities, and the strength of the linear response was low at 81.5 to 96.5% RH (r = 0.51–0.59).

Discussion

Increasing moisture levels yielded a concomitant increase in the mycelial expansion of *P. destructans*, as initially predicted (Fig. 1A, Table 2). However, RH levels of 81.5% result in similar vegetative growth rates to the rates at 89.5 and 96.5% RH (Table 3). This was somewhat surprising, as our prediction was that RH >90% would be most supportive of growth. Also contrary to our hypothesis, conidiation was not affected by the RH, at least not using the range of moistures at 13 °C in this study (Fig. 1B, Table 3).

Mycelial expansion is an important determinant for *P. destructans* pathogenesis, and reduced mycelial growth may impart a reduced risk for bats developing substantial infections. Most caves in the Northeast where bats hibernate have relative humidities that range from 60 to 100% (Langwig et al. 2012, Perry 2013), which means that some hibernacula or microclimates therein would be less supportive of mycelial growth than others. Our data suggests that microclimates of 70% RH and 13 °C are significantly less supportive of *P. destructans* growth than those with higher RH at that temperature (Table 3, Fig. 1). Bats can hibernate over a range of temperatures, but prefer temperatures <13 °C, which hold less ambient moisture than air at 13 °C, even at the same RH (Brack 2007, Kurta 2014, Kurta and Smith 2014). Thus, 70% RH at temperatures below 13 °C would likely support even less mycelial growth. Water vapor pressure (WVP), which takes into account both temperature and ambient moisture, is a metric that is reflective of absolute moisture level in the air, and the resulting saturation water vapor pressure (SWVP) varies with temperature (Kurta 2014). As temperatures in hibernacula are expected to vary over the course of hibernation, SWVP will also fluctuate. Hence, WVP and SWVP will be more appropriate measures in future studies seeking to examine the role of moisture on *P. destructans* development in situations reflective of a natural setting. In this study, *P. destructans* was held at a constant temperature, allowing us to directly compare impact of differing moisture levels, as reflected in RH readings, on fungal growth and development.

Caves with higher humidities provide *P. destructans* with a greater potential to cause mortality in bat species that exhibit a preference for such relatively humid hibernacula (Wilder et al. 2011). Also, biomass production of *P. destructans* is sensitive to water stress, and lower matric potentials of -5MPa completely abolishes mycelial expansion (Raudabaugh and Miller 2013). Surface-tension–reducing materials, such as fats secreted on wing membranes, can allow the fungus access to water (Raudabaugh and Miller 2013), but paradoxically, some fatty acids present on bat wings can also inhibit *P. destructans* growth (Frank et al. 2016). At temperatures of 10.5 to 13.4 °C, similar to that employed in this study, myristic acid and stearic acids constrained colony expansion (Frank et al. 2016).

It was somewhat surprising that the tested RH levels did not impact *P. destructans* conidiation, as there are numerous examples of phytopathogenic fungi for which altering moisture levels results in concomitant changes in sporulation (Oh 1997, Sosa-Alvarez et al. 1995, Zhao and Shamoun 2006). For example, *Uncinula necator* produced the most conidia at 75.6% RH on grapevine hosts, regardless of the tested temperature (Oh 1997). Conidia serve as important transmission propagules and can initiate infections of healthy bats (Lorch et al. 2011). Our data suggests that mitigating transmission rates during an ongoing WNS outbreak in a hibernaculum by manipulating RH would be of limited value as it would not suppress conidia production substantially unless the humidity is below levels not included in this study. However, the lowest RH tested, 70%, is close to the lower range of RH encountered at bat hibernacula (60–65%; Langwig et al. 2012, Perry 2013); thus it is questionable whether such a small RH reduction would have a significant impact on overall conidia production. Furthermore, considering that many bat species preferentially hibernate in sites with more moisture, presumably as a means to alleviate EWL, reductions in RH would also be harmful for overwintering bats already faced with significant water loss (Cryan et al. 2010, Kurta and Smith 2014). Bats typically do not hibernate at 13 °C, the temperature that was used in this study, and as mentioned above, at lower temperatures the moisture content at the same RH will be lower (Brack 2007, Kurta and Smith 2014, Twente 1955).

Bats are vulnerable to dehydration during torpor due to EWL, which probably explains why humidity is an important factor for bat hibernacula selection (Cryan et al. 2010, Kurta and Smith 2014, Perry 2013). By selecting hibernacula or sites within hibernacula with higher humidity, and by clustering, bats may reduce the amount of water loss through wing and lung membranes (Cryan et al. 2010, Willis et al. 2011). For bats that aggregate in larger clusters and experience high enough EWL to generate condensation on the fur, there will be a saturated atmosphere surrounding such clusters. This effect is due to equilibrium water pressure being reached, and without significant air flow, this atmosphere would be supportive of *P. destructans* mycelia growth, regardless of overall hibernacula RH (Hayman et al. 2016, Kurta 2014, Radabaugh and Miller 2013). Interestingly, a behavioral adaptation and/or selection denoted in post-WNS populations of Little Brown Myotis is an increased frequency of bats hibernating in smaller groups (Wilcox et al. 2014), which could decrease RH within a bat aggregate because fewer bats clustering together would yield a lower volume of condensed water on the fur.

Some Little Brown Myotis populations initially decimated by *P. destructans* seem to be rebounding from the WNS epidemic waves (Lilley et al. 2016). The bats that are remaining in these surviving populations have a lower arousal frequency than bats during peak WNS that is more similar to frequencies observed pre-WNS. Similarly, bat torpor temperatures post-WNS in these renewed populations are also lower than during peak WNS and more similar to pre-WNS temperatures, and lower temperatures does retard *P. destructans* vegetative growth (Chaturvedi et al. 2010, Lilley et al. 2016, Verant et al. 2012). Modeling of WNS impacts in bat populations articulates the importance of both temperature

and moisture in understanding disease outcomes (Hayman et al. 2016). High humidity was anticipated to increase fungal growth and lead to more frequent arousals, which in turn was a predictor of bat mortality (Hayman et al. 2016). Taken together, these more recent findings underscore the importance of considering how environmental parameters influence both bat and fungal physiology and how these conspire to generate disease outcomes.

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