

# Efficacy of a probiotic bacterium to treat bats affected by the disease white-nose syndrome

Tina L. Cheng<sup>1\*</sup>, Heather Mayberry<sup>2,3</sup>, Liam P. McGuire<sup>2,4</sup>, Joseph R. Hoyt<sup>1</sup>, Kate E. Langwig<sup>1,5</sup>, Hung Nguyen<sup>1</sup>, Katy L. Parise<sup>6</sup>, Jeffrey T. Foster<sup>6</sup>, Craig K. R. Willis<sup>2</sup>, Auston Marm Kilpatrick<sup>1</sup> and Winifred F. Frick<sup>1,7</sup>

<sup>1</sup>Department of Ecology and Evolutionary Biology, University of California, 1156 High Street, Santa Cruz, CA 95064, USA; <sup>2</sup>University of Winnipeg, Winnipeg, MB R3B 2E9, Canada; <sup>3</sup>University of Toronto, 3359 Mississauga Road, Mississauga, ON L5L 1C6, Canada; <sup>4</sup>Texas Tech University, Lubbock, TX 79409, USA; <sup>5</sup>Harvard T.H. Chan School of Public Health, Boston, MA 02115, USA; <sup>6</sup>University of New Hampshire, Durham, NH 03824, USA; and <sup>7</sup>Bat Conservation International, PO Box 162603, Austin, TX 78716, USA

## Summary

1. The management of infectious diseases is an important conservation concern for a growing number of wildlife species. However, effective disease control in wildlife is challenging because feasible management options are often lacking. White-nose syndrome (WNS) is an infectious disease of hibernating bats that currently threatens several North American species with extinction. Currently, no effective treatments exist for WNS.

2. We conducted a laboratory experiment to test the efficacy of probiotic treatment with *Pseudomonas fluorescens*, a bacterium that naturally occurs on bats, to reduce disease severity and improve survival of little brown bats *Myotis lucifugus* exposed to *Pseudogymnoascus destructans*, the fungal pathogen that causes WNS.

3. We found that application of the probiotic bacteria at the time of fungal infection reduced several measures of disease severity and increased survival, whereas bacterial treatment prior to pathogen exposure had no effect on survival and worsened disease severity.

4. *Synthesis and applications.* Our results suggest that probiotic treatment with *Ps. fluorescens* has potential for white-nose syndrome disease management, but the timing of application is critical and should coincide with natural exposure of bats to *P. destructans*. These results add to the growing knowledge of how natural host microbiota can be implemented as a biocontrol treatment to influence disease outcomes.

**Key-words:** bats, conservation, disease management, *Geomyces destructans*, microbial biocontrol, *Myotis lucifugus*, probiotics, *Pseudogymnoascus destructans*, *Pseudomonas fluorescens*, white-nose syndrome

## Introduction

Emerging infectious diseases can cause dramatic population declines and extinctions (De Castro & Bolker 2004). Effective tools for controlling wildlife diseases are often lacking and disease management can be challenging to implement (Langwig *et al.* 2015a). However, development of viable pathogen control tools allows for targeted treatment of endangered populations to prevent disease-induced extinction (Haydon *et al.* 2006; Chang *et al.* 2007).

White-nose syndrome (WNS) is a recently emerged multihost disease that has caused severe declines in hibernating bat populations throughout eastern and midwestern North America and threatens several bat species with extinction (Frick *et al.* 2010, 2015; Langwig *et al.* 2012, 2015b). WNS is characterized by epidermal invasion by the fungal pathogen, *Pseudogymnoascus destructans* (*Pd*) (Meteyer *et al.* 2009; Lorch *et al.* 2011; Warnecke *et al.* 2012), which causes mortality by disrupting physiological processes (electrolyte imbalance, hypotonic dehydration and increased arousals from torpor) and depleting energy reserves during hibernation (Reeder *et al.* 2012; Warnecke *et al.* 2012; Verant *et al.* 2014). *Pd* infection and host mortality occur primarily during hibernation when bat

\*Correspondence author. E-mail: tinalcheng@gmail.com  
Auston M. Kilpatrick, Craig K. R. Willis, and Winifred F. Frick contributed equally to the work.

hosts are torpid for extended periods (Langwig *et al.* 2015c). Impacts are highly variable, with some species experiencing severe population declines and/or site level extirpations (e.g. *Myotis lucifugus* and *Myotis septentrionalis*), while other species are far less impacted (e.g. *Eptesicus fuscus*, *Myotis leibii*) (Frick *et al.* 2010, 2015; Langwig *et al.* 2012). Currently, no effective treatments for WNS have been demonstrated. Given the rapid declines caused by the disease, a treatment or control strategy for WNS is urgently needed.

The use of naturally occurring microbiota to treat disease is widespread in agriculture (Weller 1988) and has increased recently for both human (Borody & Khoruts 2012) and wildlife disease (Bletz *et al.* 2013). For example, several species of bacteria have recently been examined as possible treatment agents for the amphibian pathogen, *Batrachochytrium dendrobatidis* (*Bd*), including the bacterium, *Janthinobacterium lividum*, which was isolated from a *Bd*-resistant salamander, *Plethodon cinereus* (Brucker *et al.* 2008). Application of these bacteria in a laboratory study prevented fungal infection and the disease, chytridiomycosis, and eliminated the severe mortality (85%) normally caused by *Bd* in a highly susceptible frog species, *Rana muscosa* (Harris *et al.* 2009). More broadly, the skin microbiome has the potential to provide an important protective community for hosts against pathogens (Belden & Harris 2007), and this barrier could be particularly important for bats exhibiting downregulated immune function during hibernation (Meteyer *et al.* 2009; Moore *et al.* 2013).

Bacteria from the *Pseudomonas fluorescens* species complex isolated from bat skin have demonstrated growth inhibition of *Pd* *in vitro*, suggesting that these bacteria could be useful for WNS management (Hoyt *et al.* 2015). These bacteria are also widely used as probiotics in agriculture (Cook & Baker 1983; Weller & Cook 1983; Keel *et al.* 1992) and aquaculture (Gram *et al.* 1999) and have been investigated as a tool for protecting wild amphibian populations against chytridiomycosis (Lauer *et al.* 2007; Myers *et al.* 2012). A related species, *Pseudomonas veronii*-like PAZ1, was recently isolated in Europe and has exhibited strong anti-*Pd* properties *in vitro* (Frtize, Pham & Zaspel 2012). *Pseudomonas* bacteria interact with fungi via multiple pathways including the production of antimicrobial metabolites (e.g. 2-4 DAPG) and competition for iron via siderophore production (Ligon *et al.* 2013; Maschuch *et al.* 2015).

We conducted *in vivo* treatment experiments with captive bats to test the effectiveness of a strain of *Ps. fluorescens* (designated Pf1) isolated from a big brown bat *Eptesicus fuscus* (Hoyt *et al.* 2015) to reduce WNS disease severity and mortality in the highly impacted species, the little brown bat *M. lucifugus*. We examined efficacy of two application periods relative to when bats acquire *Pd* infection to provide greater flexibility for field application. First, we examined effectiveness of applying Pf1 simultaneously with *Pd* inoculation to simulate treatment of bats in early winter when most bats first acquire infection

(Langwig *et al.* 2015c). We also examined applying Pf1 3 weeks prior to *Pd* inoculation to emulate treatment in fall before the onset of hibernation and *Pd* infection (Langwig *et al.* 2015c). We hypothesized that treatment both prior to and simultaneous with *Pd* exposure would reduce disease severity and mortality.

## Materials and methods

### EXPERIMENTAL DESIGN

We randomly assigned 85 wild-collected, male little brown bats into five groups of 17 individuals each (Simultaneous Pf1 application, Pre-exposure Pf1 application, *Pd* Control, Pf1 Control and Sham Control) (see Table S1, Supporting Information). Each treatment group was housed separately in a nylon mesh cage, and all six cages were placed in a single hibernaculum chamber (CARON, Model 6040-1, Marietta, OH, USA) that was maintained at a constant temperature of 7 °C and 98% relative humidity. Little brown bats naturally cluster during hibernation so we housed bats in each treatment group together in a cage to reduce stress. To accommodate clustering as well as constraints on space within the chamber and the number of bats we were permitted to collect from the wild, our design resulted in a single replicate cage per treatment. Previous laboratory infection experiments of bats and WNS have followed a similar design (Lorch *et al.* 2011; Warnecke *et al.* 2012; Johnson *et al.* 2014). In our experiment, co-housing may have influenced survival and arousal patterns if behaviour of individuals within a group influenced co-habiting bats (Turner *et al.* 2015), but was unlikely to confound disease severity metrics measured on individual bats. Replicating cages within treatments would have required many more bats, which was not possible given the severe declines experienced by this species due to WNS.

### ANIMAL COLLECTION AND HANDLING

We collected bats on 29 November 2013 from a hibernaculum in central Manitoba, Canada, where WNS has not been detected. At the time of collection, we swabbed bats for *Pd* by rubbing sterile polyester swabs (Fisherbrand synthetic tipped applicators 23-400-116) five times on the forearm and five times on the muzzle (Langwig *et al.* 2015c) and stored swab samples in RNAlater at -20 °C until testing. We extracted swabs and tested for *Pd* using quantitative polymerase chain reaction (qPCR) (Muller *et al.* 2013) to confirm that collected individuals were negative for *Pd*. Prior to the first treatment, we measured mass ( $\pm 0.1$  g) and forearm length ( $\pm 0.01$  mm) to calculate body condition index (mass/forearm length). We banded each bat with a lipped forearm band (Porzana Ltd., East Sussex, UK) and attached an iButton (Thermochron DS1922L-F5#) to record skin temperature ( $T_{sk}$ ) every 10 min. We modified iButtons following Reeder *et al.* (2012) to reduce their mass and marked each iButton with a unique pattern for individual identification. Bats in each cage were provided with a single water dish (Petri plate filled with water), which was refilled twice each week by a tube extending outside the chamber to prevent disturbance. All bats were handled for the same amount of time and received application of *Ps. fluorescens*, *Pd* or sham inoculum according to their treatment group (Table S1).

#### PREPARATION AND APPLICATION OF SHAM INOCULUM

We prepared 1X PBS (autoclave sterilized at 121 °C for 30 min) to use as the sham inoculum and diluent in *Ps. fluorescens* and *Pd* inocula.

#### PREPARATION AND APPLICATION OF *PS. FLUORESCENS* (PF1) TREATMENT INOCULUM

We used a strain of Pfl that was cultured from a wild-caught big brown bat *E. fuscus* in Virginia in March 2012. This strain exhibited the strongest inhibitory effects against *Pd* growth *in vitro* of the five bacterial strains collected from bats in a prior study (Hoyt *et al.* 2015). To prepare the Pfl treatment inoculum, we prepared 1:10 dilutions of Pfl stock culture (stored at –80 °C in glycerol and defrosted on ice to room temperature) on Luria agar plates. Colony-forming units were counted to determine concentration, and initial stock was diluted in 1X PBS to make a working inoculum at  $1.8 \times 10^7$  CFU mL<sup>-1</sup>. Bats were sprayed with Pfl inoculum on both wings and the tail membrane using a 100-mL generic plastic cosmetic perfume atomizer (ASIN: B018TTEQUS; Amazon.com, Inc., Seattle, WA, USA). Bats received two sprays on each side of each wing and one spray on each side of the tail membrane. Each spray delivered approximately 170 µL of probiotic inoculum, for a total of 1.2 mL total for each bat ( $2.2 \times 10^7$  CFUs). We used a plastic sheet to cover the torso of the bat to prevent dampening the fur while exposing the wings to the spray. Pfl inoculum was sourced from the same stock (stored at –80 °C in 30% glycerol) and prepared identically according to the procedures detailed above for Pre-exposure and Simultaneous Pfl treatments.

#### PREPARATION AND APPLICATION OF *P. DESTRUCTANS* INOCULUM

We obtained a wild strain of *Pd* from an infected *Perimyotis subflavus* in New Brunswick, Canada, isolated on 4 April 2013 (Karen Vanderwolf, Canadian Wildlife Federation). We cultured *Pd* on Sabouraud Dextrose Agar with chloramphenicol and gentamicin at 7 °C for 30–60 days until cultures produced characteristic curved conidia. *Pd* cultures were harvested and diluted with 1X PBS to make an inoculum of concentration  $2.5 \times 10^4$  conidia µL<sup>-1</sup> (Lorch *et al.* 2011). We inoculated each bat with 20 µL of *Pd* inoculum containing  $5 \times 10^5$  conidia. Inoculum was applied and spread onto the dorsal side of both wings with a pipette. We selected this dose and method of application because it produced skin lesions, increased arousal frequencies and behavioural changes characteristic of WNS in previous experiments (Lorch *et al.* 2011; Warnecke *et al.* 2012).

#### EXPERIMENTAL PROTOCOL

We examined five metrics of WNS severity that characterize different stages of disease (McGuire *et al.* 2016). These include *Pd* load based on qPCR of swabs (Muller *et al.* 2013), surface area of the wing covered in *Pd* by histological examination, the per cent of visible ultraviolet (UV) fluorescence from *Pd* invasion on the wing (Turner *et al.* 2014), number of invasive tissue lesions by histological examination (Meteyer *et al.* 2009; Reeder *et al.* 2012) and changes in hibernation torpor patterns (Reeder *et al.* 2012; Warnecke *et al.* 2012).

We continuously monitored bats using motion-sensitive infra-red cameras mounted inside the hibernation chamber. Individuals were identified based on symbols on each iButton datalogger. We removed and examined individuals within 24 h of observing the following behaviours: (i) arousal on three consecutive days, (ii) shaky and/or lethargic movements during arousal, (iii) roosting solitarily at the bottom of the cage or on the floor or (iv) roosting with wings spread out (Warnecke *et al.* 2012). We weighed bats and assessed health status based on active or lethargic response to handling. If individuals were not responsive during handling, bats were humanely euthanized by decapitation under isoflurane anaesthesia.

At the termination of the experiment or when bats were removed and euthanized, individuals were swabbed using the protocol described above. We photographed the left ventral wing using UV transillumination (368-nm wavelength and 2-s exposure) to measure fluorescence. UV photographs were analysed using the colour-matching application in Photoshop (Appendix S1). For histology, we removed the wing section from the fifth digit to the body, rolled the tissue around dental wax dowels and stored it in neutral-buffered formalin. Histological sections were prepared at the Wisconsin Veterinary Diagnostic Laboratory. Rolled wing sections were trimmed in six cross sections per bat and stained with periodic acid–Schiff. Prepared sections were examined under a light microscope at 400–600×. We identified and counted WNS lesions, defined as cup-like skin erosions filled with *Pd* conidia (Meteyer *et al.* 2009) for each tissue section of each bat (Fig. S1). We also approximated, to the nearest 5%, the fraction of the epidermal surface covered with *Pd* on each tissue section per bat (Fig. S1). We scored all histology slides blind to the treatment group. We quantified torpor patterns based on data recorded by iButtons attached to each bat. We defined torpor bout duration (TBD) as the time in days that a bat was torpid between arousals. Arousals, in which  $T_{sk}$  was within 10 °C of the maximum  $T_{sk}$  recorded for each individual, were used to distinguish long bouts of steady-state torpor (Reeder *et al.* 2012).

Bats in this region enter hibernation between early September and late October (Norquay & Willis 2014). Therefore, we monitored bats for ≤ a 210-day hibernation period beginning on 1 October 2014 halfway through this period. Morbidity increased significantly in our Sham Control group after 185 days (>6 months) into hibernation, indicating that survival was compromised after 185 days in captivity regardless of WNS status (all Sham Control bats tested negative for *Pd*). Therefore, we examined differences in survival among groups up to day 185 to exclude mortality unrelated to WNS. We defined mortality as the proportion of bats in each treatment group that had died or been euthanized by day 185.

#### STATISTICAL ANALYSES

All statistical analyses were performed using R v. 3.0.2 (R Core Team 2016). We considered individual bat as the experimental unit for all analyses. For each metric of WNS severity, we included initial body mass index, treatment group, number of days in hibernation and an interaction between treatment group and number of days in hibernation as fixed effects. There were a total of ten candidate models in each model set (Table S2), and we selected the best-supported model by the Akaike Information Criterion (AIC) (Burnham & Anderson 2002). We used a

generalized linear model with a binomial distribution to compare disease prevalence, defined as the proportion of bats exhibiting a metric of WNS (*Pd* load, Surface *Pd*, UV fluorescence, Lesions), among treatment groups. We analysed variation in TBD over time for 9–12 bats in each treatment group (i.e. those with functioning iButtons) using linear mixed models (function *lme* in package *NLME*) and included bat ID as a random effect to account for multiple measurements from each bat. We determined whether an individual exhibited decreased TBD as a symptom of WNS by comparing the slope of TBD vs. time between Sham Control bats and all other bats (i.e. we assessed the interaction between time and treatment group). If this slope was significantly lower for a given bat compared to Sham Control bats, we considered this evidence of disrupted torpor behaviour. We used pairwise log-rank survival analyses to compare survival between treatment groups using function *survdiff* in package *SURVIVAL*. We compared the number of lesions among treatment groups by fitting generalized linear mixed models with a zero-inflated geometric distribution using function *zeroinfl* in package *pscl* to the total number of lesions in 5–6 tissue sections per bat (tissue sections were included as an offset). We also fit models with a negative binomial distribution, but the estimated theta parameter was 1.04, and since the geometric distribution is a special case of the negative binomial distribution with theta (or  $k$ ) = 1, the geometric distribution was preferred based on AIC;  $\Delta\text{AIC} = 1.98$ . We used linear mixed models to compare the fraction of the skin's surface area infected with *Pd*, after normalizing the data with an arcsine square-root-transformation, and we included bat ID as a random effect. We used linear models (function *lm*) to compare arcsine square-root-transformed UV fluorescence and  $\log_{10}$ -transformed *Pd* loads among treatment groups.

We report one-tailed *P*-values for comparisons between Simultaneous Pfl, Sham Control and *Pd* Control groups based on our *a priori* hypothesis that Simultaneous Pfl treatment or no infection would be beneficial compared to *Pd*-infected bats. However, we report two-tailed *P*-values for all other comparisons, including comparisons between Pre-exposure Pfl treatment and *Pd*-infected bats because differences did not support our hypothesis and were in the opposite direction of our *a priori* prediction.

## Results

### DISEASE SEVERITY AND SURVIVAL

Disease severity increased over time, differed among treatments and was not correlated with initial body mass index (Figs 1 and 2; Table S3). Severity in Simultaneous Pfl

bats was significantly reduced compared to *Pd* Control bats in four out of five disease severity metrics (number of lesions, surface fungi, UV fluorescence and TBD) (Figs 1 and 2; Table S4; Fig. S2). In contrast, severity in Pre-exposure Pfl bats was significantly greater than *Pd* Control bats for all five metrics (number of lesions, surface fungi, UV fluorescence, *Pd* load and TBD) (Figs 1 and 2; Table S4; Figure S2), and thus, the data did not support our *a priori* predictions about the impact of this treatment. The zero-inflated model indicated that Simultaneous Pfl treated bats with lesions had marginally fewer lesions than *Pd* Control bats ( $P = 0.06$ ; Table S4), but were not significantly different in the probability of having at least one lesion compared to *Pd* Control bats ( $P = 0.25$ ; Table S4). In contrast, Pre-exposure Pfl bats had a marginally greater probability of acquiring a lesion compared to *Pd* Control ( $P = 0.06$ ; Table S4), whereas those that had lesions did not differ significantly in the number of lesions compared to *Pd* Control bats ( $P = 0.65$ ; Table S4).

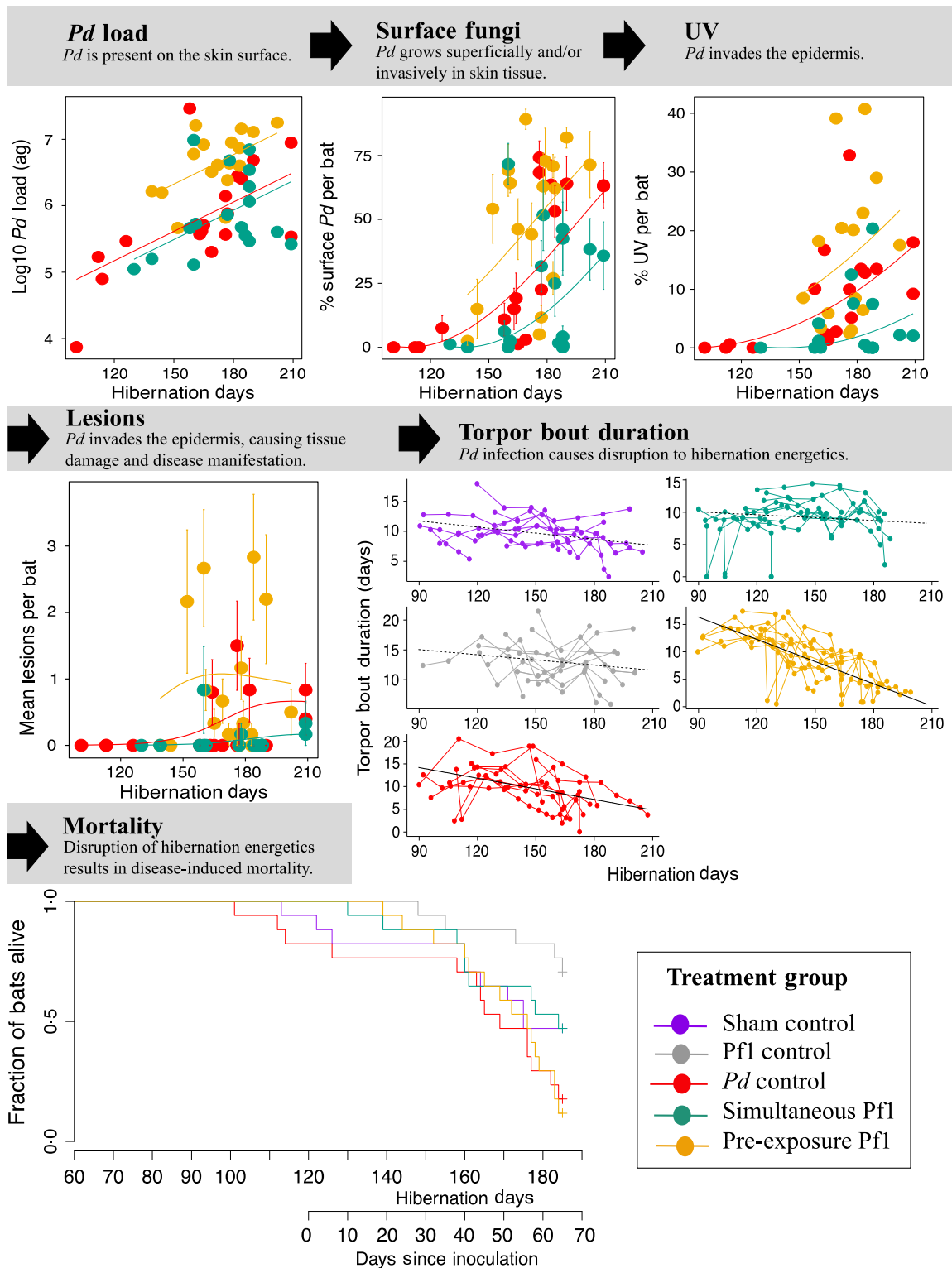
Survival varied between treatments (Fig. 1; Table S2). The Pfl Control group had the highest survival, and bats treated with Pfl at the time of exposure to *Pd* had significantly higher survival compared to untreated *Pd*-infected bats and equal or higher survival than the Sham Control group. Most other comparisons were not significant, including the comparison of Pre-exposure Pfl bats and untreated infected bats (*Pd* Control group). Although Simultaneous Pfl bats had higher survival than *Pd* and Sham Control bats for much of the experiment, substantial mortality in the final 10 days eliminated these differences.

### DISEASE PREVALENCE

There were no differences between Pfl-treated and *Pd*-infected groups in the fraction of bats detected with *Pd* based on qPCR or those with superficial surface fungi based on histology (Fig. S3; Table S3). In contrast, fewer Simultaneous Pfl bats had detectable sign of WNS by UV fluorescence, disrupted torpor patterns, or mortality compared to *Pd* Control bats, whereas more Pre-exposure Pfl bats had detectable WNS by UV fluorescence and disrupted torpor patterns compared to *Pd* Control bats (Fig. S3; Table S4).

**Fig. 1.** Comparison of white-nose syndrome (WNS) severity among treatment groups along a gradient in disease progression. WNS disease severity is shown along a progression of increasing disease manifestation (indicated by the right-facing arrow): exposure to *Pd* (*Pd* load) leads to superficial (Surface Fungi) and dermal invasion (UV/Lesions), which leads to increased arousals [torpor bout duration (TBD)], which results in fat depletion and death (Mortality). WNS disease severity for Sham Control (purple), Pfl Control (grey), *Pd* Control (red), Simultaneous Pfl (orange) and Pre-exposure Pfl (blue-green) are shown for six disease metrics. *Pd* load, Surface Fungi, UV fluorescence and Lesions: Circles show the average ( $\pm 1$  SE) number of lesions, per cent surface fungi on each tissue section, per cent area with fluorescing *Pd* and  $\log_{10}$ (*Pd* load) for each of 17 individual bats per treatment group. Lines show the best-fitting model (Table S4). TBD: Points indicate the duration of time since last arousal and segments connect torpor bouts of individual bats in each treatment group. Lines show the predicted TBD over time from the best-fit model (Table S4). The slope of the dashed lines was not significantly different from zero, and the slopes of solid lines formed three groups which were significantly different from each other: Simultaneous Pfl = Sham Control = Pfl Control < *Pd* Control < Pre-exposure Pfl (Table S4). Survival curves: The fraction of each group of 17 bats remaining alive for the first 185 days of hibernation (125 days post-*Pd* inoculation) is shown. Shared letters (A, B) at the end of each line indicate treatment groups that do not differ significantly in pairwise log-rank survival tests (Table S2).





## Discussion

Our results indicate that *Ps. fluorescens* application is non-detrimental to bats and that treatment with strain

Pfl at the same time bats are exposed to *Pd* could reduce WNS severity and mortality and, therefore, reduce impacts of WNS on bat populations. Bats treated with Pfl and not infected with *Pd* had the highest survival and

WNS metric	Simultaneous Pfl	Pre-exposure Pfl
WNS mortality	↓ (P = 0.04)	█ (P = 0.87)
Lesions (Histology)	↓ (P = 0.06)	↑ (P = 0.06)
Surface <i>Pd</i> (Histology)	↓ (P = 0.01)	↑ (P = 0.05)
UV	↓ (P < 0.001)	↑ (P = 0.08)
<i>Pd</i> load (qPCR)	█ (P = 0.27)	↑ (P < 0.0001)
Torpor bout length	↓ (P < 0.001)	↑ (P < 0.001)

**Fig. 2.** Summary of the degree of white-nose syndrome (WNS) disease severity relative to *Pd* Control for Pre-exposure Pfl and Simultaneous Pfl treatment groups. Red upward facing arrows represent increased WNS disease severity; blue downward facing arrows represent decreased WNS disease severity. Associated *P*-values compare the treatment group to *Pd* Control (Table S4). Gradient in red and blue colour represents significance levels, with solid blue/red indicating significance at  $\alpha < 0.05$  and muted colours representing marginal significance at  $\alpha < 0.10$ . Black bars represent no significant difference compared to *Pd* Control.

fat stores, suggesting that Pfl treatment did not have any pathological effects in the absence of *Pd*. In addition, bats treated with Pfl at the same time as *Pd* exposure had higher survival compared to untreated infected bats (*Pd* Control) and lower disease severity across most of the disease metrics we examined.

The disparity in disease outcomes between Pre-exposure and Simultaneous Pfl bats suggests that the mechanism for successful Pfl application is dependent on treatment timing. Neither Pre-exposure nor Simultaneous Pfl treatment prevented *Pd* colonization since *Pd* was detected on all bats in these groups and fungal loads were similar on Simultaneous Pfl and *Pd* Control bats. However, bats infected with *Pd* and treated with Pfl simultaneously had significantly reduced *Pd* invasion (assessed by UV and histology) and showed no increase in arousal frequency, which has been proposed as a precursor to disease-induced mortality (Reeder *et al.* 2012; Warnecke *et al.* 2012, 2013; Verant *et al.* 2014). We found no evidence that our application of Pfl persisted or amplified on bats during the experiment because bats in Pfl-treated groups did not differ in prevalence of *Ps. fluorescens* by the end of the experiment (Appendix S2; Table S6). Thus, interaction between Pfl and *Pd* is likely restricted to short-term impacts following application and potentially functions by delaying fungal invasion via production of antifungals (Brucker *et al.* 2008), resource competition (e.g. iron sequestration; Ligon *et al.* 2013; Mascuch *et al.* 2015) or reducing colonization (Warmink *et al.* 2011). In contrast, pre-exposure application of Pfl appears to compromise some degree of natural resistance, potentially by disrupting the natural skin microbiome, and resulted in higher severity of *Pd* invasion (although without a detectable increase in mortality). Given the importance of treatment timing, application of Pfl should take place in early hibernation at sites where *Pd* has established and when *Pd* prevalence is near 100% (Langwig *et al.* 2015c).

Our study suffers from pseudoreplication (Hurlbert 1984), in that we had a single cage for each of our treatments. We grouped bats in a single cage for ethical reasons because little brown bats are highly social and cluster during hibernation. Replication of experimental groups (cages) was also limited by the number of hibernation chambers available, space within each chamber and, most importantly, the number of bats we could collect from the wild. Cage effects are most likely to influence comparisons of torpor patterns, which could in turn affect mortality, if an arousal by one bat disturbed other individuals in that cage (Turner *et al.* 2015). However, differences among groups in other metrics of severity (*Pd* load by qPCR, UV fluorescence, lesions, surface fungi) are less likely to be caused by cage effects because all infected bats were inoculated with a single high dose of *Pd*. Results from all five WNS metrics were very similar; thus, it is more likely that differences in all disease metrics were due to treatments rather than cage effects, but cage effects cannot be ruled out.

Our results suggest that *Ps. fluorescens* could be a useful tool for disease management of wild bat populations affected by WNS. *Pseudomonas* bacteria are commonly found on hibernating bats throughout eastern North America (Hoyt *et al.* 2015) (Appendix S2; Table S5). Thus, treatment with this bacterium effectively represents bio-augmentation, which is beneficial in reducing impacts of non-target effects. Due to the rapid spread of WNS and the urgency for action, we recommend a small-scale field trial as the next step in development of *Pseudomonas* as a treatment for WNS. Such a trial should be conducted in a human-made habitat (e.g. a mine, to prevent potential non-target effects on cave-dwelling organisms) with treatment timed to coincide with *Pd* infection. These trials should furthermore be conducted within an adaptive management framework and in compliance with biological control protocols for other systems (e.g. chytridiomycosis; Woodhams *et al.* (2011)). Future studies might also improve treatment efficacy by determining the mechanism of *Ps. fluorescens-Pd* inhibition and by applying different doses and strains of *Ps. fluorescens*. Given the uncertainty associated with the mechanism of action, treatment with *Ps. fluorescens* should be focused at sites where *Pd* has recently arrived and where bats are likely to decline precipitously, and to focus on the most susceptible species, rather than at sites where bats are persisting with *Pd* (Langwig *et al.* 2015a).

The importance of resident microbes in health and disease is becoming increasingly evident (Cho & Blaser 2012). Concurrently, recent advancements in microbial biotechnology and bioinformatics have expanded the potential of probiotics to be developed as tools in disease management (Rebollar *et al.* 2016). The recent emergence of several virulent, multihost and rapidly spreading infectious diseases (e.g. chytridiomycosis, white-nose syndrome, *Batrachochytrium salamandrivorans*, west nile virus) highlights the critical need for rapid development of

disease management tools for conservation (Voyles *et al.* 2014; Langwig *et al.* 2015a). Probiotics represent a promising alternative to traditional antimicrobials. Our experiment provides an important step in the development of *Ps. fluorescens* as a probiotic agent that could be further developed as a disease management tool against WNS. Further progress in the protection of imperilled bat populations in North America will require an adaptive management approach that should include field trials for probiotics and other biological agents (Cornelison *et al.* 2014) and exploration of other interventions, such as habitat decontamination (Bosch *et al.* 2015) and microclimate manipulations (Boyles & Willis 2010; Langwig *et al.* 2012; Johnson *et al.* 2014; Grieneisen *et al.* 2015; Wilcox & Willis 2016). Rapidly leveraging results of these studies is important for directing long-term strategies to halt the rapid decline and extirpation of many bat populations.

## Acknowledgements

Funding was provided by USFWS: F12AP01081; NSF: DEB-1115895 and DEB-1336290; and Bat Conservation International. We thank the many members of the Willis Lab (Ally Menzies, Alana Wilcox, Dylan Baloun, Mary-Anne Collis, Quinn Webber, Kristina Muise and Christina Davy) and the staff from the University of Winnipeg Animal Facility (Dan Wasyliv, Robyn Cole) for their assistance in collecting bats, monitoring and helping to conduct the experiment. We thank Melissa Behr and Kevin Keel for their assistance with histological preparation and training. We acknowledge the technical support from Benjamin Abrams, UCSC Life Sciences Microscopy Center, and Maninderpaul Sidhu for their assistance with microscope photography of histological slides. We thank Karen Vanderwolf for providing *Pseudogymnoascus destructans* culture. We extend our sincere gratitude to Rain Ackerman and the Ackerman family for their support.

## Data accessibility

Data are available from Dryad Digital Repository <http://dx.doi.org/10.5061/dryad.p1bd1> (Cheng *et al.* 2016).

## References

- Belden, L.K. & Harris, R.N. (2007) Infectious diseases in wildlife: the community ecology context. *Frontiers in Ecology and the Environment*, **5**, 533–539.
- Bletz, M.C., Loudon, A.H., Becker, M.H., Bell, S.C., Woodhams, D.C., Minbiole, K.P.C. & Harris, R.N. (2013) Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecology Letters*, **16**, 807–820.
- Borody, T.J. & Khoruts, A. (2012) Fecal microbiota transplantation and emerging applications. *Nature Reviews Gastroenterology & Hepatology*, **9**, 88–96.
- Bosch, J., Sanchez-Tomé, E., Fernández-Loras, A., Oliver, J.A., Fisher, M.C. & Garner, T.W.J. (2015) Successful elimination of a lethal wildlife infectious disease in nature. *Biology Letters*, **11**, 20150874.
- Boyles, J.G. & Willis, C.K.R. (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.
- Brucker, R.M., Harris, R.N., Schwantes, C.R., Gallaher, T.N., Flaherty, D.C., Lam, B.A. & Minbiole, K.P.C. (2008) Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *Journal of Chemical Ecology*, **34**, 1422–1429.
- Burnham, K.P. & Anderson, D. (2002) *Model Selection and Multimodel Inference*. Springer-Verlag, New York, NY, USA.
- Chang, G.-J.J., Davis, B.S., Stringfield, C. & Lutz, C. (2007) Prospective immunization of the endangered California condors (*Gymnogyps californianus*) protects this species from lethal West Nile virus infection. *Vaccine*, **25**, 2325–2330.
- Cheng, T.L., Mayberry, H., McGuire, L.P., Hoyt, J.R., Langwig, K.E., Nguyen, H. *et al.* (2016) Data from: Efficacy of a probiotic bacterium to treat bats affected by the disease white-nose syndrome. *Dryad Digital Repository*, <http://dx.doi.org/10.5061/dryad.p1bd1>.
- Cho, I. & Blaser, M.J. (2012) The human microbiome: at the interface of health and disease. *Nature Reviews Genetics*, **13**, 260–270.
- Cook, R.J. & Baker, K.F. (1983) *The Nature and Practice of Biological Control of Plant Pathogens*. American Phytopathological Society, St. Paul, MN, USA.
- Cornelison, C.T., Keel, M.K., Gabriel, K.T., Barlament, C.K., Tucker, T.A., Pierce, G.E. & Crow, S.A. (2014) A preliminary report on the contact-independent antagonism of *Pseudogymnoascus destructans* by *Rhodococcus rhodochrous* strain DAP96253. *BMC Microbiology*, **14**, 1–7.
- De Castro, F. & Bolker, B. (2004) Mechanisms of disease-induced extinction. *Ecology Letters*, **8**, 117–126.
- Frick, W.F., Pollock, J.F., Hicks, A.C., Langwig, K.E., Reynolds, D.S., Turner, G.G., Butchkoski, C.M. & Kunz, T.H. (2010) An emerging disease causes regional population collapse of a common North American bat species. *Science*, **329**, 679–682.
- Frick, W.F., Puechmille, S.J., Hoyt, J.R., Nickel, B.A., Langwig, K.E., Foster, J.T. *et al.* (2015) Disease alters macroecological patterns of North American bats. *Global Ecology and Biogeography*, **24**, 741–749.
- Fritze, V.M., Pham, T.L.H. & Zaspel, I. (2012) Effekt des bodenbakteriums *Pseudomonas veronii*-like PAZ1 auf das wachstum des white-nose erregers *Geomyces destructans* in antagonistentests. *Nyctalus*, **17**, 104–107.
- Gram, L., Melchiorson, J., Spanggaard, B., Huber, I. & Nielsen, T.F. (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. *Applied and Environmental Microbiology*, **65**, 969–973.
- Grieneisen, L.E., Brownlee-Bouboulis, S.A., Johnson, J.S. & Reeder, D.M. (2015) Sex and hibernaculum temperature predict survivorship in white-nose syndrome affected little brown myotis (*Myotis lucifugus*). *Royal Society Open Science*, **2**, 140470.
- Harris, R.N., Brucker, R.M., Walke, J.B., Becker, M.H., Schwantes, C.R., Flaherty, D.C. *et al.* (2009) Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *The ISME Journal*, **3**, 818–824.
- Haydon, D.T., Randall, D.A., Matthews, L., Knobel, D.L., Tallents, L.A., Gravenor, M.B. *et al.* (2006) Low-coverage vaccination strategies for the conservation of endangered species. *Nature*, **443**, 692–695.
- Hoyt, J.R., Cheng, T.L., Langwig, K.E., Hee, M.M., Frick, W.F. & Kilpatrick, A.M. (2015) Bacteria isolated from bats inhibit the growth of *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome. *PLoS One*, **10**, e0121329.
- Hurlbert, S.H. (1984) Pseudoreplication and the design of ecological field experiments. *Ecological Monographs*, **54**, 187–211.
- Johnson, J.S., Reeder, D.M., McMichael, J.W., Meierhofer, M.B., Stern, D.W.F., Lumadue, S.S. *et al.* (2014) Host, pathogen, and environmental characteristics predict white-nose syndrome mortality in captive little brown myotis (*Myotis lucifugus*). *PLoS One*, **9**, e112502.
- Keel, C., Schnider, U., Marurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D. & Défago, G. (1992) Suppression of root diseases by *Pseudomonas fluorescens* CHAO: importance of the bacterial secondary metabolite 2, 4-diacetylphloroglucinol. *Molecular Plant-Microbe Interactions*, **5**, 4–13.
- Langwig, K.E., Frick, W.F., Bried, J.T., Hicks, A.C., Kunz, T.H. & Kilpatrick, A.M. (2012) Sociality, density-dependence and microclimates determine the persistence of populations suffering from a novel fungal disease, white-nose syndrome. *Ecology Letters*, **15**, 1050–1057.
- Langwig, K.E., Voyles, J., Wilber, M.Q., Frick, W.F., Murray, K.A., Bolker, B.M. *et al.* (2015a) Context-dependent conservation responses to emerging wildlife diseases. *Frontiers in Ecology and the Environment*, **13**, 195–202.
- Langwig, K.E., Hoyt, J.R., Parise, K.L., Kath, J., Kirk, D., Frick, W.F., Foster, J.T. & Kilpatrick, A.M. (2015b) Invasion dynamics of white-nose syndrome fungus, Midwestern United States, 2012–2014. *Emerging Infectious Diseases*, **21**, 1023–1026.
- Langwig, K.E., Frick, W.F., Reynolds, R., Parise, K.L., Drees, K.P., Hoyt, J.R. *et al.* (2015c) Host and pathogen ecology drive the seasonal

- dynamics of a fungal disease, white-nose syndrome. *Proceedings of the Royal Society B: Biological Sciences*, **282**, 20142335.
- Lauer, A., Simon, M.A., Banning, J.L., Lam, B.A. & Harris, R.N. (2007) Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. *The ISME Journal*, **2**, 145–157.
- Ligon, J.M., Hill, D.S., Hammer, P.E., Torkewitz, N.R., Hofmann, D., Kempf, H.J. & van Pée, K.H. (2013) Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. *Pest Management Science*, **56**, 688–695.
- Lorch, J.M., Meteyer, C.U., Behr, M.J., Boyles, J.G., Cryan, P.M., Hicks, A.C. *et al.* (2011) Experimental infection of bats with *Geomyces destructans* causes white-nose syndrome. *Nature*, **480**, 376–378.
- Mascuch, S.J., Moree, W.J., Hsu, C., Turner, G.G., Cheng, T.L., Blehert, D.S. *et al.* (2015) Direct detection of fungal siderophores on bats with white-nose syndrome via fluorescence microscopy-guided ambient ionization mass spectrometry. *PLoS One*, **10**, e0119668.
- McGuire, L.P., Turner, J.M., Warnecke, L., McGregor, G., Bollinger, T.K., Misra, V. *et al.* (2016) White-nose syndrome disease severity and a comparison of diagnostic methods. *EcoHealth*, **13**, 60–71.
- Meteyer, C.U., Buckles, E.L., Blehert, D.S., Hicks, A.C., Green, D.E., Shearn-Bochsler, V., Thomas, N.J., Gargas, A. & Behr, M.J. (2009) Histopathologic criteria to confirm white-nose syndrome in bats. *Journal of Veterinary Diagnostic Investigation*, **21**, 411–414.
- Moore, M.S., Reichard, J.D., Murtha, T.D., Nabhan, M.L., Pian, R.E., Ferreira, J.S. & Kunz, T.H. (2013) Hibernating little brown myotis (*Myotis lucifugus*) show variable immunological responses to white-nose syndrome. *PLoS One*, **8**, e58976.
- Muller, L.K., Lorch, J.M., Lindner, D.L., O'Connor, M., Gargas, A. & Blehert, D.S. (2013) Bat white-nose syndrome: a Real-Time TaqMan polymerase chain reaction test targeting the intergenic spacer region of *Geomyces destructans*. *Mycologia*, **105**, 253–259.
- Myers, J.M., Ramsey, J.P., Blackman, A.L., Nichols, A.E., Minbiole, K.P.C. & Harris, R.N. (2012) Synergistic inhibition of the lethal fungal pathogen *Batrachochytrium dendrobatidis*: the combined effect of symbiotic bacterial metabolites and antimicrobial peptides of the frog *Rana muscosa*. *Journal of Chemical Ecology*, **38**, 958–965.
- Norquay, K.J.O. & Willis, C.K.R. (2014) Hibernation phenology of *Myotis lucifugus*. *Journal of Zoology*, **294**, 85–92.
- R Core Team (2016) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available at: <https://www.R-project.org/>.
- Rebollar, E.A., Antwis, R.E., Becker, M.H., Belden, L.K., Bletz, M.C., Brucker, R.M. *et al.* (2016) Using 'Omics' and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Frontiers in Microbiology*, **7**, 733.
- Reeder, D.M., Frank, C.L., Turner, G.G., Meteyer, C.U., Kurta, A., Britzke, E.R. *et al.* (2012) Frequent arousal from hibernation linked to severity of infection and mortality in bats with white-nose syndrome. *PLoS One*, **7**, e38920.
- Turner, G.G., Meteyer, C.U., Barton, H., Gumbs, J.F., Reeder, D.M., Overton, B. *et al.* (2014) Nonlethal screening of bat-wing skin with the use of ultraviolet fluorescence to detect lesions indicative of white-nose syndrome. *Journal of Wildlife Diseases*, **50**, 566–573.
- Turner, J.M., Warnecke, L., Wilcox, A., Baloun, D., Bollinger, T.K., Misra, V. & Willis, C.K.R. (2015) Conspecific disturbance contributes to altered hibernation patterns in bats with white-nose syndrome. *Physiology & Behavior*, **140**, 1–8.
- Verant, M.L., Meteyer, C.U., Speakman, J.R., Cryan, P.M., Lorch, J.M. & Blehert, D.S. (2014) White-nose syndrome initiates a cascade of physiologic disturbances in the hibernating bat host. *BMC Physiology*, **14**, 1–10.
- Voyles, J., Kilpatrick, A.M., Collins, J.P., Fisher, M.C., Frick, W.F., McCallum, H.I. *et al.* (2014) Beyond too little, too late: managing emerging infectious diseases requires international action. *EcoHealth*, **12**, 404–407.
- Warmink, J.A., Nazir, R., Corten, B. & van Elsas, J.D. (2011) Hitchhikers on the fungal highway: the helper effect for bacterial migration via fungal hyphae. *Soil Biology & Biochemistry*, **43**, 760–765.
- Warnecke, L., Turner, J.M., Bollinger, T.K., Lorch, J.M., Misra, V., Cryan, P.M., Wibbelt, G., Blehert, D.S. & Willis, C.K.R. (2012) Inoculation of bats with European *Geomyces destructans* supports the novel pathogen hypothesis for the origin of white-nose syndrome. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 6999–7003.
- Warnecke, L., Turner, J.M., Bollinger, T.K., Misra, V., Cryan, P.M., Blehert, D.S., Wibbelt, G. & Willis, C.K.R. (2013) Pathophysiology of white-nose syndrome in bats: a mechanistic model linking wing damage to mortality. *Biology Letters*, **9**, 20130177.
- Weller, D.M. (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology*, **26**, 379–407.
- Weller, D.M. & Cook, R.J. (1983) Suppression of Take-All of wheat by seed treatments with fluorescent *Pseudomonads*. *Phytopathology*, **73**, 463–469.
- Wilcox, A. & Willis, C.K.R. (2016) Energetic benefits of enhanced summer roosting habitat for little brown bats (*Myotis lucifugus*) recovering from white-nose syndrome. *Conservation Physiology*, **4**, 1–12.
- Woodhams, D.C., Bosch, J., Briggs, C.J., Cashins, S., Davis, L.R., Lauer, A. *et al.* (2011) Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. *Frontiers in Zoology*, **8**, 8.

Received 4 April 2016; accepted 28 July 2016

## Supporting Information

Additional Supporting Information may be found in the online version of this article.

**Appendix S1.** Ultraviolet analyses.

**Appendix S2.** *Pseudomonas fluorescens* PCR.

**Fig. S1.** Comparison of histological analyses of WNS disease severity.

**Fig. S2.** Predicted values and standard errors for five WNS disease severity metrics at the end of hibernation.

**Fig. S3.** Comparison in disease prevalence between treatment groups along a gradient of WNS progression.

**Table S1.** Probiotic treatment experimental design.

**Table S2.** Per cent survival in each treatment group and comparison of survival.

**Table S3.** Model selection of candidate models for WNS disease severity metrics.

**Table S4.** Results from best-supported models of PfI treatment experiment for four metrics of WNS disease severity metrics.

**Table S5.** *Ps. fluorescens* prevalence from bat and substrate swab samples in Michigan and Wisconsin.

**Table S6.** Prevalence *Ps. fluorescens* from bat swab samples taken at the end of the experiment by treatment group.