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Behaviour of hibernating little brown bats experimentally inoculated with the pathogen that causes white-nose syndrome

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Pathogens can affect host behaviour in ways that influence disease transmission as well as survival and fitness for both host and pathogen. Hibernating bats with white-nose syndrome (WNS) show a number of unusual behaviours including increased frequency of arousal from torpor, altered roosting behaviour and premature emergence. However, mechanisms underlying these patterns are not understood, and the behaviour of bats with WNS has not been examined systematically. Three hypotheses could explain increased arousal frequency. Bats may arouse to (1) groom in response to skin infection, (2) drink to offset dehydration or (3) increase activity, possibly in an attempt to access resources, avoid a source of infection or limit the risk of infecting relatives. We tested these hypotheses with captive little brown bats, *Myotis lucifugus*, inoculated with *Pseudogymnoascus destructans*, the fungus that causes WNS. In contrast to predictions of all three hypotheses, bats inoculated with the fungus tended to be less active than controls during arousals from torpor and did not increase grooming or visits to the water source in their enclosures. However, bats showed a dramatic reduction in clustering behaviour as infection progressed. Reduced activity and clustering could represent adaptive, maladaptive or pathological responses. Reduced activity could be an energy-saving mechanism or a pathological consequence of infection while reduced clustering could have beneficial or detrimental consequences for transmission, energetics, water balance and survival. Our results highlight the need for studies of host behaviour to understand dynamics of wildlife infectious diseases.

Emerging wildlife diseases can threaten populations and have large ecological and economic impacts (Boyles, Cryan, McCracken, & Kunz, 2011; Daszak, Cunningham, & Hyatt, 2000; Kilpatrick, Briggs, & Daszak, 2010). Many wildlife diseases are associated with behavioural changes in their hosts (Bos, Lefèvre, Jensen, & d'Ettorre, 2011; Weary, Huzzey, & von Keyserlingk, 2009) that can be adaptive if they improve host survival or reduce transmission to genetically related individuals (Hart, 1988; Loehle, 1995; Moore, 2002; Rueppell, Hayworth, & Ross, 2010). For example, caribou relocate during parasite outbreaks to avoid becoming infected (Downes, Theberge, & Smith, 1986) and infected individuals of some colonial insects self-isolate in the presence of disease to reduce transmission to relatives (Bos et al., 2011; Moore, 2002; Rueppell et al., 2010). Alternatively, changes in host behaviour can be maladaptive if they reduce host survival. For instance, behavioural isolation of infected individuals can increase their risk of predation (Hart, 1988; Loehle, 1995) and, in some cases, pathogens appear to actively manipulate host behaviour to increase predation risk and complete their own life cycle (Libersat, Delgado, & Gal, 2009; Møller, 1993). Understanding behavioural changes of hosts following disease emergence can shed light on mechanisms underlying disease processes and pathogen transmission, and potentially aid management and conservation efforts (Hawley & Altizer, 2010).

Since it was the first photo-documented in Howes Cave in New York State in 2006, white-nose syndrome (WNS) has spread to 22 U.S. states and five Canadian provinces (Blehert et al., 2009; U.S. Geological Survey, 2012). WNS affects seven bat species (Reeder & Turner, 2008; U.S. Fish and Wildlife Service, 2012b) and causes mortality rates as high as 99% in some hibernacula, with millions of bats killed to date and regional extinctions predicted (Frick et al., 2010; U.S. Fish and Wildlife Service, 2012a). The disease is caused by the fungus *Pseudogymnoascus destructans* (*Pd*; formerly

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Geomyces destructans; Minnis & Lindner, 2013) that damages skin by invading and eroding the epidermis, connective tissue and glands (Gargas, Trest, Christensen, Volk, & Blehert, 2009; Lorch et al., 2011; Meteyer et al., 2009; Warnecke et al., 2012). The fungus infects bats throughout Europe but without the mass mortality observed in North America (Martínková et al., 2010; Puechmaille et al., 2011; Wibbelt et al., 2010). This suggests that European bats may exhibit characteristics that aid their survival, or that they evolved behavioural or physiological mechanisms of resistance to *Pd* (Puechmaille et al., 2011; Wibbelt et al., 2010). The hypothesis that *Pd* is an invasive pathogen to North America is supported by experimental evidence demonstrating that North American bats are similarly susceptible to *Pd* from Europe and North America (Warnecke et al., 2012), and genetic evidence demonstrating that isolates of North American *Pd* represent a single, clonal genotype that is not found outside of the known range in North America (Lorch et al., 2012; Rajkumar et al., 2011).

Pseudogymnoascus destructans is cold adapted and infects bats during hibernation. Infection is associated with a number of changes in behaviour. Normal hibernation consists of repeated bouts of torpor (during which body temperature and metabolic rate are dramatically reduced) interspersed with brief periodic arousals to normothermic body temperature that occur on average between every 2–3 weeks in healthy bats (Jonasson & Willis, 2012; Warnecke et al., 2012). One of the most pronounced behavioural changes in bats infected with *Pd* is an increase in the frequency of periodic arousals as infection progresses, resulting in premature depletion of fat stores (Boyles & Willis, 2010; Reeder et al., 2012; Warnecke et al., 2012). Bats with WNS are also often found roosting closer to the entrances of hibernacula than normal (McAlpine, Vanderwolf, Forbes, & Malloch, 2011) and emerging from hibernacula prematurely during winter (Cryan, Meteyer, Boyles, & Blehert, 2010). In addition to affecting arousal frequency and emergence, WNS may also influence social behaviour and clustering. Two of the species most affected by WNS, little brown bats, *Myotis lucifugus*, and Indiana bats, *Myotis sodalis*, typically cluster in groups during hibernation (Brack & Twente, 1985; Clawson, LaVal, LaVal, & Claire, 1980). However, since WNS was first detected, the proportion of individuals of these species observed roosting individually has increased (Langwig et al., 2012). This suggests that either WNS is selecting for bats that tend to roost individually, or infection leads to a behavioural change in the tendency of individual bats to cluster (Langwig et al., 2012). Such a behavioural change could be adaptive if it reduces disease transmission (Langwig et al., 2012) or maladaptive if it increases water loss or energetic costs (Boyles, Storm, & Brack, 2008).

The trigger for increased arousal frequency in bats with WNS is still not understood. However, this pattern could be explained by three nonmutually exclusive hypotheses. The first hypothesis suggests that, even though infection with *Pd* causes little inflammation (Gargas et al., 2009; Meteyer et al., 2009), irritation due to fungal invasion and damage to the epidermis could trigger arousal. This hypothesis predicts that infected bats should increase grooming during arousals. Second, wing damage due to fungal infection could increase cutaneous evaporative water loss, loss of fluids and dehydration, forcing bats to arouse more frequently (Cryan et al., 2010; Cryan et al., 2013; Warnecke et al., 2013; Willis, Menzies, Boyles, & Wojciechowski, 2011). This predicts that infected bats should drink more than normal during arousals to replenish water stores and should devote a greater proportion of their time to drinking. Third, bats may arouse more frequently as they become increasingly motivated to leave the hibernaculum or as a consequence of a mechanism to limit pathogen transmission. This hypothesis is consistent with a number of possible scenarios. Increased activity could reflect the energetic or hygric consequences of infection and increasing demand for food or water as fat stores decline or bats become dehydrated. Increased activity could also reflect a behavioural tendency for individual bats to reduce their own risk of pathogen exposure by moving away from unhealthy roostmates, as occurs in other wildlife species (Downes et al., 1986). On the other hand, if bats tend to cluster with relatives during hibernation, increased activity or self-isolation by badly infected individuals could be a mechanism to avoid infecting kin (Bos et al., 2011; Rueppell et al., 2010). If the reduced rate of clustering observed in WNS-affected hibernacula represents a behavioural change rather than selection for solitary bats, this could reflect attempts by individuals to minimize pathogen exposure or transmission to roostmates (Bos et al., 2011; Langwig et al., 2012; Rueppell et al., 2010). Therefore this hypothesis also predicts that infected bats should show heightened activity during arousals and reduced clustering as a mechanism to slow pathogen transmission (Langwig et al., 2012).

Despite the apparent behavioural changes associated with WNS, and the potential for behavioural studies to shed light on disease processes (Moore, 2002), so far only one study has quantified behavioural observations of hibernating bats infected with *Pd* (Brownlee-Bouboulis & Reeder, 2013). This partly reflects the fact that behaviour of free-ranging bats is extremely difficult to observe during hibernation. Bats are highly sensitive to disturbance and hibernacula are often remote and usually too large for effective video recording of the behaviours of individuals. Although some factors experienced in the wild by hibernating bats cannot be simulated in captivity (e.g. freedom to leave the hibernaculum, size/volume of a typical hibernaculum), many conditions can be replicated and, under the right environmental conditions, some bat species, including those susceptible to WNS, hibernate normally in captivity (e.g. Warnecke et al., 2012; Warnecke et al., 2013). Moreover, in the field, all individuals in an affected hibernaculum must be assumed to have been exposed to *Pd*. As a result, comparisons between WNS-affected and unaffected bats may be confounded by site-specific characteristics that do not reflect infection (e.g. microclimate, volume of the hibernacula, possible genotypic and phenotypic difference between bats in different sites) and effects of infection on behaviour cannot be isolated from effects of 'site'. In captivity, environmental variables like microclimate as well as the origin of the bats can be readily controlled. Thus, while results must be interpreted carefully, captive studies of behaviour have strong potential to shed light on disease mechanisms in WNS.

To better understand behavioural changes associated with WNS, and to test hypotheses about mechanisms underlying the disease, we analysed infrared video recordings of captive bats experimentally inoculated with *Pd* by Warnecke et al. (2012). We quantified a range of behaviours associated with each of the hypotheses explaining increased arousal frequency and tested the hypothesis that observations of reduced clustering by bats in affected hibernacula reflect behavioural change rather than natural selection by the disease for solitary bats. Given recent physiological evidence for the dehydration hypothesis (Cryan et al., 2010; Cryan et al., 2013; Warnecke et al., 2013; Willis et al., 2011), we predicted that bats would increase visits to the water source in their enclosure. Bats with WNS show little to no inflammation during hibernation (Meteyer et al., 2009), so we did not expect to observe more grooming by infected bats. We did predict an increase in activity during arousals, consistent with the hypothesis that bats become motivated to access resources and/or move away from roostmates as the disease progresses. In keeping with the hypothesis that reduced clustering represents a behavioural change by individuals, rather than selection (Langwig et al., 2012), we also predicted a reduction in clustering throughout hibernation.

METHODS

Methods for capture and inoculation of bats are described by Warnecke et al. (2012). Briefly, 54 male little brown bats were collected from a WNS-negative cave in central Manitoba, Canada (53°21′N, 99°30′W) in November 2010. Bats were transported to the Western College of Veterinary Medicine at the University of Saskatchewan and randomly assigned to one of three treatment groups: a sham-inoculated control group, a group inoculated with a North American isolate of *Pd* (NA*Pd*) and a group inoculated with a European isolate of *Pd* (EU*Pd*). Each treatment group was inoculated with 20 μl of a conidial suspension containing approximately 500 000 conidia suspended in PBS-Tween 20, while individuals in the control group were inoculated with sham-inoculum containing only PBS-Tween 20. Bats were housed from 27 November 2010 to 5 March 2011 (99 days) in a 37 × 37 × 104 cm nylon mesh enclosure (Reptarium, Apogee, Dallas, TX, U.S.A.) contained in temperature- and humidity-controlled environmental chambers (VWR BOD 2020; VWR International, Mississauga, ON, Canada). Chambers were maintained at 7 °C and more than 97% relative humidity to simulate ambient conditions in hibernacula used by little brown bats (Thomas, Dorais, & Bergeron, 1990). All procedures were approved by the University of Saskatchewan Committee on Animal Care and Supply (Protocol No. 20100120) and animals were collected under the Manitoba Conservation Wildlife Scientific Permit (WB11145).

We recorded behaviour using infrared security cameras (VL650IRVFS; Speco Technologies, Amityville, NY, U.S.A.) attached at the top of each chamber. Cameras allowed for continuous behavioural observations within a given chamber without disturbance to the bats. Videos were recorded using a motion-activated digital video recorder (SHR-3040; Samsung Techwin, Ridgefield Park, NJ, U.S.A.). The video recorder was triggered by movement of the bats at the onset of arousals from torpor, and a single observer later analysed these videos. The observer watched videos at the minimum fast-forward speed (i.e. 30:1; 30 s of footage played back every second). All behaviours (i.e. locomotion, travel to the water dish, grooming) involved readily obvious movements by the bats and were easily detected in the higher-speed playback. Whenever any movement or a change in position by the focal bat was observed, the recording was stopped, rewound to a time-point prior to the onset of the behaviour and viewed in real-time to quantify its duration. We only scored arousals during which a single bat was active to avoid the influence of social interactions on our results and because it was not possible to identify individuals (based on their skin temperature, T_{sk}, recordings; see below) when more than one bat was active. We assessed behaviours associated with each of the three hypotheses that could explain increased arousal: latency to groom and duration and frequency (i.e. the number of times the behaviour occurred during an arousal) of grooming; latency to visit the water dish and duration and frequency of visits to the water dish; latency to locomotion and duration and frequency of locomotion (i.e. activity); latency to fly and frequency of flight (i.e. activity); and changes in clustering behaviour. In cases where a given behaviour was not observed during a particular arousal we set latency as the maximum duration of that arousal following Budaev (1997).

Numbered forearm bands were too small to be identified in the videos. Therefore, to identify individual bats, we used skin temperature (T_{sk}) recordings. As outlined by Warnecke et al. (2012), prior to inoculation, each bat was outfitted with a temperature datalogger (DS1922L-F5 Thermochron iButton; Maxim, Sunnyvale, CA, U.S.A., modified according to Lovegrove (2009), or iBBat; Alpha Mach, Mont-St-Hilaire, QC, Canada) or a temperature-sensitive radiotransmitter (LB-2NT; Holohil Systems Ltd, Carp, ON, Canada). By aligning the T_{sk} profiles of known individuals with the timing of arousals in video recordings, we were able to identify individual animals and quantify their behaviours. Using this method we were able to reliably assign multiple arousals to identified individuals (mean ± SE arousals per bat: control: 2.8 ± 0.27; NA*Pd*: 3.2 ± 0.31; EU*Pd*: 3.9 ± 0.69).

To quantify changes in clustering behaviour as infection progressed, we quantified the number of discrete clusters of individuals, with a cluster defined as an isolated individual or isolated group of bats. We also superimposed a grid of 162 cells, with a 5 × 6 cell base and a single central vanishing point over the video image (Fig. 1) and then quantified the number of grid cells occupied by bats. To standardize our sampling interval, these analyses were conducted for the first video recorded each week throughout hibernation.

To account for temporal autocorrelation and avoid pseudoreplication (Bolker et al., 2009), we used generalized linear mixed models (GLMM) in Proc GLIMMIX of SAS version 9.2. We only included arousals for which we could reliably identify individuals based on their T_{sk} recording. The variable of interest (e.g. duration of grooming) was included as a response variable in all models. To determine whether infected bats used a greater proportion of their time in locomotion to access the water dish, when we analysed duration of visits to the water dish as the response variable, we included the duration of locomotion as a fixed effect. In addition, for all models, we included the duration of the arousal and the treatment (i.e. NA*Pd*, EU*Pd* or control) as fixed effects. We also included, as a fixed effect, the number of days between a given arousal and the occurrence of moribund status (or termination of the treatment) for that individual to account for variation in relative infection status between individual bats at different times during the study. The identity of the individual bats was included as a random effect. We used a spatial power (sp(POW)) covariance structure to model the unequal sampling intervals between

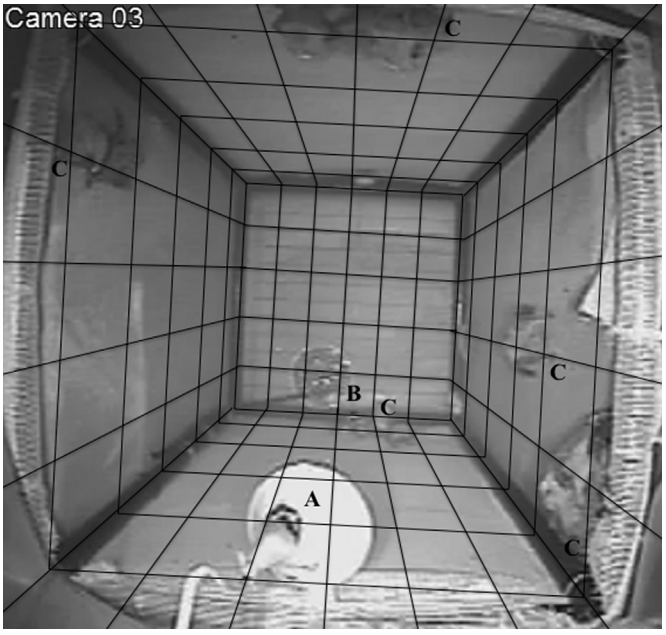

Figure 1. Representative still image from the motion-activated infrared video camera recording from the top of one the experimental enclosures. A grid of 162 cells with a 5 × 6 cell base and a single central vanishing point was superimposed on the image for analysis. A plastic funnel shielding the temperature and humidity sensor is indicated by A, the water dish on the floor of the enclosure is indicated by B, and clusters of bats and individual bats are indicated by C.

repeated measures (Moser, 2004) and applied a logarithmic (log or natural log) or square-root transformation to behavioural variables if it improved model fit (West, Welch, & Galecki, 2007). We specified a Gaussian distribution with an identity link function, with the exception of the frequency of grooming, which fit a Poisson distribution with a log link function. Model fit was assessed using generalized chi-square/degrees of freedom and -2 restricted log likelihood (Schabenberger, 2005). Residual pseudolikelihood was used to estimate parameters and least squares means of the behaviour variables were computed for pairwise comparisons among treatments ($P \leq 0.05$). We assessed 87 days of the trial from 27 November 2010 to 21 February 2011 as the memory capacity of the T_{sk} dataloggers was filled by 21 February, preventing us from identifying specific individuals in the video recordings. However, both treatment groups had clear evidence of advanced WNS and some individuals had already reached moribund status in both groups (Warnecke et al., 2012).

We tested for an effect of inoculation on clustering behaviour using two analyses in R (version 2.14.1; R Core Development Team, 2011). To test for an increase in the space occupied by bats in each enclosure over time we used a generalized least squares model with number of cells occupied by bats as the response variable and date of sampling and treatment as predictors. The number of bats remaining in the chamber was used as a covariate to control for declining group sizes as some bats in treatment groups reached moribund status and were removed from the enclosures. We compared models with and without temporal autocorrelation structures and determined the optimal correlation structure using AIC following the method outlined by Zuur, Ieno, Walker, Saveliev, and Smith (2009). The base model without autocorrelation had higher fit, so we carried through with hypothesis testing using a general linear model for the analysis. We repeated this analysis using the number of discrete clusters of bats observed during the first arousal each week (log transformed) as the response variable. A generalized least squares model with an autoregressive moving average (corARMA) covariance structure was the best-supported model for this analysis.

RESULTS

Grooming

Infected bats did not increase grooming behaviour. There was no effect of inoculation on the latency to groom ($F_{2,38} = 1.4$, $P = 0.26$), or the duration ($F_{2,38} = 0.3$, $P = 0.74$; Fig. 2) or frequency ($F_{2,38} = 1.8$, $P = 0.18$) of grooming. There was a positive effect of arousal duration on latency to groom ($F_{1,17} = 9.4$, $P = 0.007$) and duration of grooming ($F_{1,17} = 5.9$, $P = 0.03$), but the frequency ($F_{1,17} = 2.2$, $P = 0.15$) of grooming was not affected. Similarly, there was a positive effect of the number of days before a bat reached moribund status on latency to groom ($F_{70,17} = 4.7$, $P < 0.001$) and duration ($F_{71,17} = 3.2$, $P = 0.005$) of grooming, while the frequency of grooming was not affected ($F_{71,17} = 1.6$, $P = 0.14$).

Visits to the Water Dish

Infected bats did not increase visits to the water source in their enclosures. Inoculation had no effect on latency to visit the water dish ($F_{2,38} = 1.8$, $P = 0.17$), or duration ($F_{2,38} = 0.6$, $P = 0.53$; Fig. 3) or frequency ($F_{2,38} = 0.7$, $P = 0.50$) of visits to the water dish. The duration of the arousal bout positively affected the latency to visit to the water dish ($F_{1,16} = 14.0$, $P = 0.002$) but did not affect the duration ($F_{1,17} = 0.07$, $P = 0.80$) or frequency ($F_{1,17} = 0.87$, $P = 0.36$) of visits. The number of days before a bat reached moribund status positively affected the latency to visit to the water dish ($F_{71,16} = 2.7$, $P = 0.01$) but did not affect the duration ($F_{71,17} = 1.0$, $P = 0.52$) or frequency ($F_{71,17} = 0.8$, $P = 0.71$) of visits. The duration of locomotion had a positive effect on the duration of visits to the water dish ($F_{1,16} = 5.6$, $P = 0.03$). In other words, during arousals in which the focal bat was active for longer, it spent more time at the water dish. However, this effect did not differ between treatments ($F_{2,38} = 0.9$, $P = 0.42$). Arousal duration ($F_{1,16} = 0.9$, $P = 0.37$) and days before a bat reached moribund status ($F_{71,16} = 0.9$, $P = 0.59$) also had no effect on drinking behaviour.

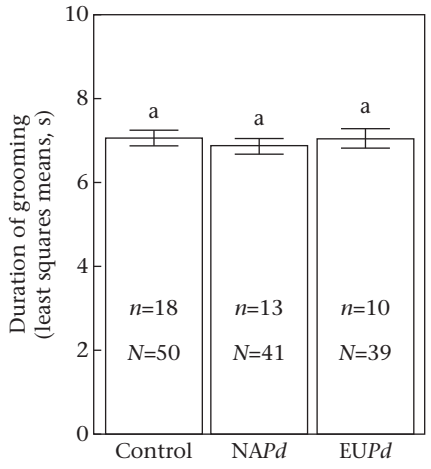

Figure 2. The duration of grooming during periodic arousals throughout hibernation (least squares means ± SE) for sham-inoculated little brown bats (control) versus bats inoculated with either a North American (NA*Pd*) or European (EU*Pd*) isolate of *Pseudogymnoascus destructans*. Number of bats is indicated by *n*; *N* indicates number of arousals.

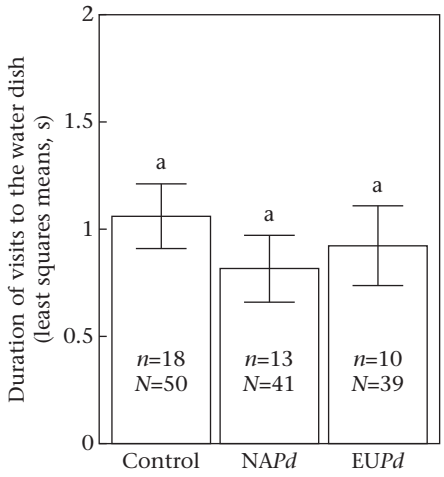

Figure 3. The duration of visits to the water dish during periodic arousals throughout hibernation (least squares means ± SE) for sham-inoculated little brown bats (control) versus bats inoculated with either a North American (NA*Pd*) or European (EU*Pd*) isolate of *Pseudogymnoascus destructans*. Number of bats is indicated by *n*; *N* indicates number of arousals.

Activity and Clustering

The level of activity in bats inoculated with *Pd* was similar, but not identical, to activity levels of controls. Inoculation did not affect latency to become active ($F_{2,38} = 0.1$, $P = 0.92$) or frequency ($F_{2,38} = 0.7$, $P = 0.49$) of locomotion. However, there was an effect of treatment on duration ($F_{2,38} = 3.5$, $P = 0.04$; Fig. 4) of locomotion

with NA*Pd*-inoculated bats being active for less time than controls ($t_{38} = 2.6$, $P = 0.01$; Fig. 4) and a nonsignificant tendency for reduced locomotion in bats inoculated with EU*Pd* ($t_{38} = 1.7$, $P = 0.11$; Fig. 4) with no difference between EU*Pd* and NA*Pd* ($t_{38} = -0.4$, $P = 0.69$; Fig. 4). Arousal duration had a positive effect on latency to locomotion ($F_{1,17} = 11.5$, $P = 0.004$) and frequency of locomotion ($F_{1,17} = 5.7$, $P = 0.03$), but had no effect on duration of locomotion ($F_{1,17} = 3.5$, $P = 0.08$). The number of days before a bat reached moribund status did not affect latency to locomotion ($F_{71,17} = 0.6$, $P = 0.91$), or duration ($F_{71,17} = 1.0$, $P = 0.49$) or frequency ($F_{71,17} = 1.9$, $P = 0.07$) of locomotion. There was no effect of inoculation on latency to fly ($F_{2,38} = 0.4$, $P = 0.68$) or frequency of flight attempts ($F_{2,38} = 0.2$, $P = 0.83$). Arousal duration had a positive effect on latency to flight ($F_{1,17} = 22.5$, $P = <0.001$), but no effect on flight frequency ($F_{1,17} = 2.6$, $P = 0.12$). The number of days before a bat reached moribund status did not affect latency to flight ($F_{70,17} = 1.1$, $P = 0.41$), but bats that were closer to moribund status and the end of the experiment were more likely to undertake flight attempts ($F_{71,17} = 3.0$, $P = 0.006$).

The numbers of bats remaining in each chamber, as individuals in treatment groups reached moribund status and were removed, did not affect the space occupied by bats ($F_{1,26} = 0.9$, $P = 0.35$) or the number of clusters of bats ($F_{1,26} = 0.1$, $P = 0.83$). Therefore, we excluded this covariate from further analyses. The NA*Pd* and EU*Pd* treatments did not differ in terms of space occupied ($F_{1,25} = 0.2$, $P = 0.66$) or number of clusters ($F_{1,25} = 0.7$, $P = 0.41$), and both treatments exceeded controls, so we also pooled the treatment groups for subsequent analysis. There was no change in numbers of cells occupied by control bats throughout the winter ($F_{1,15} = 0.3$, $P = 0.61$; Fig. 5), but there was an increase in the number of cells occupied by the treatment groups as infection progressed ($F_{1,27} = 40.2$, $P < 0.001$; Fig. 5). Similarly, there was no change in the number of clusters of control bats ($F_{1,15} = 3.3$, $P = 0.09$), but there was an increase in the number of clusters for the infected groups ($F_{1,27} = 25.3$, $P < 0.001$).

DISCUSSION

We observed a number of changes in the behaviour of experimentally inoculated bats that could have important consequences for survival, recovery from infection and pathogen transmission. In contrast to our prediction, *Pd* infected bats reduced activity during arousals from torpor. Bats inoculated with NA*Pd* showed a significant reduction in activity and, while nonsignificant ($P = 0.11$), activity of EU*Pd*-inoculated bats was also qualitatively lower. In the wild, more bats roost alone in sites affected by WNS (Langwig et al., 2012) and, in our study, both treatment groups also reduced clustering behaviour as infection progressed. We found no evidence that inoculation with *Pd* influenced grooming or drinking behaviour.

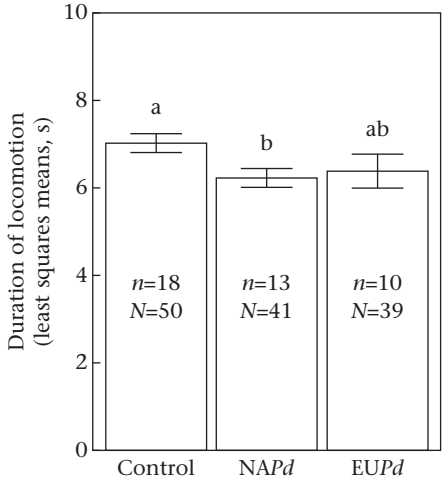

Figure 4. The duration of locomotion during periodic arousals throughout hibernation (least squares means ± SE) for sham-inoculated little brown bats (control), or bats inoculated with either a North American (NA*Pd*) or European (EU*Pd*) isolate of *Pseudogymnoascus destructans*. Number of bats is indicated by *n*; *N* indicates number of arousals. Test statistics and *P* values refer to the results for the whole model; results for the multiple comparisons are presented in the Results.

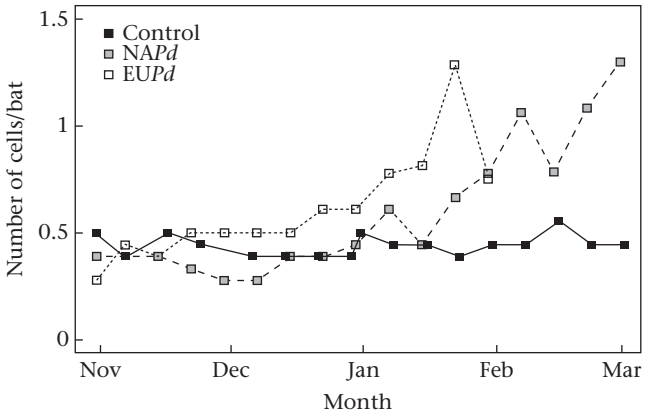

Figure 5. The change in the number of cells occupied by bats in environmental chambers by month for hibernating little brown bats inoculated with either a North American (NA*Pd*) or European (EU*Pd*) isolate of *Pseudogymnoascus destructans*, or sham-inoculated (controls).

Grooming

Bats increase grooming in response to increasing ectoparasite loads (Giorgi, Arlettaz, Christie, & Vogel, 2001; Kerth, Almasi, Ribi, Thiel, & Lüpold, 2003). As *Pd* hyphae proliferate and damage exposed skin, we predicted that bats would increase grooming in response to the infection and in an attempt to remove the fungus. Excessive grooming can negatively impact fitness by decreasing energy reserves (Giorgi et al., 2001), so in addition to explaining why infected bats arouse more often from torpor, this hypothesis could also help explain why infected bats are emaciated. However, we found no evidence that infected bats groom more often or for longer than controls despite advanced infection and substantial fungal growth confirmed via histology for these individuals by Warnecke et al. (2012). This is consistent with reports that there is little inflammation occurring at sites of *Pd* infection during hibernation (Gargas et al., 2009; Meteyer et al., 2009), possibly due to down-regulation of the immune system during hibernation (Maniero, 2002; Moore et al., 2011). Thus, despite the fact that bats are suffering from invasion of their skin by the fungus, excessive grooming or scratching did not appear to contribute to increased arousal frequency in our experiment (but see Brownlee-Bouboulis & Reeder, 2013).

Visits to the Water Dish

A growing body of evidence supports the hypothesis that bats with WNS are dehydrated due to fluid loss across skin damaged by infection with *Pd* and that this may be causing an increase in arousal frequency (Cryan et al., 2010; Cryan et al., 2013; Warnecke et al., 2013; Willis et al., 2011). We found no behavioural evidence to support this hypothesis, as bats inoculated with *Pd* spent no more time visiting the water dish at the bottom of their enclosure than controls. This suggests that the increased arousal frequency of

infected bats may be triggered by some factor other than water or fluid loss (Cryan et al., 2010). On the other hand, one possibility is that we missed quantifying drinking behaviour that did not involve visiting the water dish at the bottom of a cage. In the wild, little brown bats hibernate in humid sites where condensation may accumulate on substrates and on their fur. This condensation and dripping water in hibernacula can provide an alternative water source that bats may exploit in the wild (Codd, Clark, & Sanderson, 1999). We replicated these humid conditions in our artificial hibernacula and, although condensation did accumulate, the resolution of our cameras was not high enough to conclusively determine whether bats drank from condensation. Thus, we cannot rule out the possibility that bats drank from sources other than their water dish, and we recommend that future studies use higher resolution IR cameras to account for drinking from other sources. If bats infected with *Pd* are dehydrated, they may also be more likely to devote a greater proportion of their locomotion time to accessing water, but we found no support for this hypothesis.

Activity and Clustering

In contrast to the hypothesis that activity levels might increase with infection, NA*Pd*-inoculated bats were less active than controls, and bats inoculated with EU*Pd* also showed a nonsignificant tendency for reduced activity. Reduced activity is characteristic of many diseases and may be adaptive if lethargy results in energy conservation and the potential reallocation of energy reserves (Hart, 1988; Weary et al., 2009). Bats infected with *Pd* suffer from dramatically increased energy expenditure during hibernation due to increased arousal frequency (Boyles & Willis, 2010; Warnecke et al., 2012). Therefore, reduced activity by infected bats during arousals could be an adaptive response to infection if it helps bats partially compensate for the increased energetic costs of more frequent arousal (but see Brownlee-Bouboulis & Reeder, 2013). On the other hand, reduced activity and lethargy could simply be a pathological response to severe infection (Moore, 2002).

We found reduced rates of clustering in bats inoculated with *Pd*. Infected bats divided into more clusters with fewer bats per cluster as hibernation progressed, and many roosted by themselves towards the end of the experiment. This is consistent with observations that a larger proportion of bats in infected hibernacula roost solitarily compared to bats in uninfected sites (Langwig et al., 2012). Although our observations do not rule out the possibility that WNS selects for solitary roosting bats, it is more consistent with Langwig et al.'s (2012) hypothesis that the reduced clustering observed in the wild reflects a behavioural change by individuals rather than selection. This behaviour could be maladaptive if solitary roosting increases energetic costs. Thomas et al. (1990) estimated that a solitary roosting little brown bat expends 84% of its winter energy budget on arousals if arousals occur every 12–15 days. By clustering, bats may decrease the energetic cost of these arousals by decreasing heat loss (Boyles et al., 2008). Clustering may also reduce evaporative water loss and, therefore, indirectly reduce energy requirements because high rates of water loss during torpor are thought to increase arousal frequency (Thomas & Cloutier, 1992). Alternatively, hibernation energy expenditure could increase with clustering if a rise in body temperature caused by the cluster increases torpid metabolic rate (Boyles et al., 2008; McNab, 1974). Solitary roosting could also have direct survival benefits for infected bats if it slows fungal growth, perhaps by altering the microclimate in which *Pd* must grow. Thus, changes in clustering behaviour have potentially important consequences for energy balance and survival. In addition, the tendency to self-isolate could have implications for pathogen transmission. Isolation could represent an adaptive response by relatively healthy individuals to the presence of a pathogen. Healthy individuals of many species will avoid infected conspecifics, if possible, to reduce risk of contracting pathogens (Behringer, Butler, & Shields, 2006; Kennedy, Endler, Poynton, & McMinn, 1987; Kiesecker, Skelly, Bears, & Preisser, 1999). Thus, individuals isolating themselves from clusters of bats in our study, or in WNS-hibernacula in the wild, could be relatively healthy individuals motivated to avoid a possible source of infection. On the other hand, in colonial systems driven strongly by kin selection, infected individuals may isolate themselves from healthier colonymates to avoid infecting relatives (Bos et al., 2011; Heinze & Walter, 2010; Rueppell et al., 2010). In this scenario, if bats roost with relatives during hibernation, relatively sick individuals may be most likely to self-isolate. More work is needed to determine the influence of clustering on *Pd* growth and transmission, and the genetic relatedness of clustermates within bat hibernacula. In addition to energetic consequences, reduced locomotion combined with self-isolation by infected bats may have implications for transmission of *Pd* by reducing contact rates between individuals in the later stages of infection.

Captive versus Natural Hibernacula

Our laboratory hibernacula were artificial environments that could have influenced the behaviour of bats compared to free-ranging individuals. The smaller size of our hibernacula and the fact that bats could not emerge at will were different from the situation for most free-ranging bats. On the other hand, there are reasons to be confident that our results reflect normal behaviour by both infected and uninfected bats. For one, particularly in northern sites with cold temperature and where snow cover may block cave or mine entrances, bats are effectively captive inside hibernacula for months at a time. Warnecke et al. (2012) showed that the torpor/arousal patterns of control bats used in this study were virtually identical to free-ranging animals studied by Jonasson and Willis (2012) and Czenze, Park, and Willis (2013). Moreover, the fact that a similar change in clustering behaviour was observed in our study and by Langwig et al. (2012) suggests our captive bats behaved in a similar way to free-ranging individuals. Importantly, captive inoculation experiments also allow for control of numerous factors (e.g. hibernaculum microclimate, population of origin for study animals), which could strongly affect comparisons of healthy and WNS-affected bats in the wild. Thus, while results from captive studies of animals should be interpreted cautiously, we argue that our results provide useful data on the effects of WNS on bats that would be impractical if not impossible to collect in the wild.

Conclusions

We quantified behavioural consequences of infection with *Pd* in hibernating bats during a period of their life cycle that is difficult to observe in the wild. We found no behavioural evidence that infected bats arouse to drink or groom, but we did observe decreased activity in infected bats, as well as reduced clustering behaviour. Solitary roosting could increase energetic costs and evaporative water loss but could help reduce pathogen transmission. The observed reduction in activity could reflect attempts by individuals to reduce energy expenditure, avoid additional contact with infected individuals or substrates, or avoid infecting genetically related roostmates. The analysis of behaviour is a useful tool to complement pathophysiological studies and can improve understanding of mechanisms underlying wildlife diseases. We encourage more detailed studies of the behaviour of bats with WNS in both the laboratory and the field, particularly studies aimed at understanding the influence of the behavioural changes we have observed on survival and disease transmission.

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References

Behringer, D. C., Butler, M. D., & Shields, J. D. (2006). Avoidance of disease by social lobsters. *Nature, 441*, 421.

Blehert, D. S., Hicks, A. C., Behr, M., Meteyer, C. U., Berlowski-Zier, B. M., Buckles, E. L., et al. (2009). Bat white-nose syndrome: an emerging fungal pathogen? *Science, 323*, 227.

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H., et al. (2009). Generalized linear mixed models: a practical guide for ecology and evolution. *Trends in Ecology & Evolution, 24*, 127–135.

Bos, N., Lefèvre, L., Jensen, A. B., & d'Ettorre, P. (2011). Sick ants become unsociable. *Journal of Evolutionary Biology, 25*, 1–10.

Boyles, J. G., Cryan, P. M., McCracken, G. F., & Kunz, T. H. (2011). Economic importance of bats in agriculture. *Science, 332*, 41–42.

Boyles, J. G., Storm, J. J., & Brack, V., Jr. (2008). Thermal benefits of clustering during hibernation: a field test of competing hypotheses on *Myotis sodalis*. *Functional Ecology, 22*, 632–636.

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.

Brack, J. R., & Twente, J. W. (1985). The duration of the period of hibernation of three species of vespertilionid bats. I. Field studies. *Canadian Journal of Zoology, 63*, 2952–2954.

Brownlee-Bouboulis, S. A., & Reeder, D. M. (2013). White-nose syndrome affected little brown bats (*Myotis lucifugus*) increase grooming and other active behaviors during arousals from hibernation. *Journal of Wildlife Diseases, 49*, 850–859.

Budaev, S. V. (1997). The statistical analysis of behavioural latency measures. *International Society for Comparative Psychology Newsletter, 14*, 1–4.

Clawson, R. L., LaVal, R. K., LaVal, M. L., & Claire, W. (1980). Clustering behavior of hibernating *Myotis sodalis* in Missouri. *Journal of Mammalogy, 61*, 245–253.

Codd, J., Clark, B., & Sanderson, K. (1999). Drinking by the common bent-wing bat *Miniopterus schreibersii* and calcium in cave water. *Bat Research News, 40*, 9–10.

Cryan, P. M., Meteyer, C. U., Blehert, D. S., Lorch, J. M., Reeder, D. M., Turner, G. G., et al. (2013). Electrolyte depletion in white-nose syndrome bats. *Journal of Wildlife Diseases, 49*, 398–402.

Cryan, P., Meteyer, C. U., Boyles, J., & Blehert, D. (2010). Wing pathology of white-nose syndrome in bats suggests life-threatening disruption of physiology. *BioMed Central Biology, 8*, 135.

Czenze, Z. J., Park, A. D., & Willis, C. K. R. (2013). Staying cold through dinner – cold-climate bats rewarm with conspecifics but not sunset during hibernation. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 183*, 859–866.

Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife: threats to biodiversity and human health. *Science, 287*, 443–449.

Downes, C. M., Theberge, J. B., & Smith, S. M. (1986). The influence of insects on the distribution, microhabitat choice, and behavior of the Burwash caribou herd. *Canadian Journal of Zoology, 64*, 622–629.

Frick, W. F., Pollock, J. F., Hicks, A. C., Langwig, K. E., Reynolds, D. S., Turner, G. G., et al. (2010). An emerging disease causes regional population collapse of a common North American bat species. *Science, 329*, 679–682.

Gargas, A., Trest, M. T., Christensen, M., Volk, T. J., & Blehert, D. S. (2009). *Geomyces destructans* sp. nov. associated with bat white-nose syndrome. *Mycotaxon, 108*, 147–154.

Giorgi, M., Arlettaz, R., Christie, P., & Vogel, P. (2001). The energetic grooming costs imposed by a parasitic mite (*Spinturnix myoti*) upon its bat host (*Myotis myotis*). *Proceedings of the Royal Society B, 268*, 2071–2075.

Hart, B. L. (1988). Biological basis of the behavior of sick animals. *Neuroscience & Biobehavioral Reviews, 12*, 123–137.

Hawley, D. M., & Altizer, S. M. (2010). Disease ecology meets ecological immunology: understanding the links between organismal immunity and infection dynamics in natural populations. *Functional Ecology, 25*, 1–13.

Heinze, J., & Walter, B. (2010). Moribund ants leave their nests to die in social isolation. *Current Biology, 20*, 249–252.

Jonasson, K. A., & Willis, C. K. R. (2012). Hibernation energetics of little brown bats. *Journal of Experimental Biology, 215*, 2141–2149.

Kennedy, C. E. J., Endler, J. A., Poynton, S. L., & McMinn, H. (1987). Parasite load predicts mate choice in guppies. *Behavioral Ecology and Sociobiology, 21*, 291–295.

Kerth, G., Almasi, B., Ribi, N., Thiel, D., & Lüpold, S. (2003). Social interactions among wild female Bechstein's bats (*Myotis bechsteinii*) living in a maternity colony. *Acta Ethologica, 5*, 107–114.

Kiesecker, J. M., Skelly, D. K., Bears, K. H., & Preisser, E. (1999). Behavioral reduction of infection risk. *Proceedings of the National Academy of Sciences of the United States of America, 96*, 9165–9168.

Kilpatrick, A. M., Briggs, C. J., & Daszak, P. (2010). The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends in Ecology & Evolution, 25*, 109–118.

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.

Libersat, F., Delgado, A., & Gal, R. (2009). Manipulation of host behavior by parasitic insects and insect parasites. *Annual Review of Entomology, 54*, 189–207.

Loehle, C. (1995). Social barriers to pathogen transmission in wild animal populations. *Ecology, 76*, 326–335.

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*, 1–4.

Lorch, J. M., Muller, L. K., Russell, R. E., O'Connor, M., Lindner, D. L., & Blehert, D. S. (2012). Distribution and environmental persistence of the causative agent of white-nose syndrome, *Geomyces destructans*, in bat hibernacula of the eastern United States. *Applied and Environmental Microbiology, 79*, 1293–1301.

Lovegrove, B. G. (2009). Modification and miniaturization of thermochron iButtons for surgical implantation into small animals. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology, 179*, 451–458.

Maniero, G. D. (2002). Classical pathway serum complement activity throughout various stages of the annual cycle of a mammalian hibernator, the golden-mantled ground squirrel, *Spermophilus lateralis*. *Developmental and Comparative Immunology, 26*, 563–574.

Martínková, N., Bačkor, P., Bartonička, T., Blažková, P., Červený, J., Falteisek, L., et al. (2010). Increasing incidence of *Geomyces destructans* in bats from the Czech Republic and Slovakia. *PLoS One, 5*, e13853.

McAlpine, D. F., Vanderwolf, K. J., Forbes, G. J., & Malloch, D. (2011). Consumption of bats (*Myotis* spp.) by raccoons (*Procyon lotor*) during an outbreak of white-nose syndrome in New Brunswick, Canada: implications for estimates of bat mortality. *Canadian Field-Naturalist, 125*, 257–260.

McNab, B. K. (1974). The behavior of temperate cave bats in a subtropical environment. *Ecology, 55*, 943–958.

Meteyer, C. U., Buckles, E. L., Blehert, D. S., Hicks, A. C., Green, D. E., Shearn-Bochsler, V., et al. (2009). Histopathologic criteria to confirm white-nose syndrome in bats. *Journal of Veterinary Diagnostic Investigation, 21*, 411–414.

Minnis, A. M., & Lindner, D. L. (2013). Phylogenetic evaluation of *Geomyces* and allies reveals no close relatives of *Pseudogymnoascus destructans*, comb. nov., in bat hibernacula of eastern North America. *Fungal Biology, 117*, 638–649.

Møller, A. P. (1993). A fungus infecting domestic flies manipulates sexual behaviour of its host. *Behavioral Ecology and Sociobiology, 33*, 403–407.

Moore, J. (2002). *Parasites and the behavior of animals*. New York, NY, U.S.A.: Oxford University Press.

Moore, M. S., Reichard, J. D., Murtha, T. D., Zahedi, B., Fallier, R. M., & Kunz, T. H. (2011). Specific alterations in complement protein activity of little brown myotis (*Myotis lucifugus*) hibernating in white-nose syndrome affected sites. *PLoS One, 6*, e27430.

Moser, E. B. (2004). Repeated measures modeling with PROC MIXED. In *Proceedings of the SAS Users Group International, Statistics and Data Analysis, 29*. Paper 188-29. Retrieved from: http://www2.sas.com/proceedings/sugi29/188-29.pdf.

Puechmaille, S. J., Wibbelt, G., Korn, V., Fuller, H., Forget, F., Mühldorfer, K., et al. (2011). Pan-European distribution of white-nose syndrome fungus (*Geomyces destructans*) not associated with mass mortality. *PLoS One, 6*, e19167.

R Development Core Team. (2011). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing, ISBN 3-900051-07-0. http://www.R-project.org.

Rajkumar, S. S., Li, X., Rudd, R. J., Okoniewski, J. C., Xu, J., Chaturvedi, S., et al. (2011). Clonal genotype of *Geomyces destructans* among bats with white-nose syndrome. New York, NY, USA. *Emerging Infectious Diseases, 17*, 1273–1276.

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.

Reeder, D. M., & Turner, G. R. (2008). Working together to combat white-nose syndrome: a report of a meeting on 9–11 June 2008, in Albany, New York. *Bat Research News, 49*, 75–78.

Rueppell, O., Hayworth, M. K., & Ross, N. P. (2010). Altruistic self-removal of health-compromised honey bee workers from their hive. *Journal of Evolutionary Biology, 23*, 1538–1546.

Schabenberger, O. (2005). Introducing the GLIMMIX procedure for generalized linear mixed models. In *Proceedings of the SAS Users Group International, Statistics and Data Analysis, 30*. Paper 196-30. Retrieved from: http://www2.sas.com/proceedings/sugi30/196-30.pdf.

Thomas, D. W., & Cloutier, D. (1992). Evaporative water loss by hibernating little brown bats, *Myotis lucifugus*. *Physiological Zoology, 65*, 443–456.

Thomas, D. W., Dorais, M., & Bergeron, J. (1990). Winter energy budgets and cost of arousals for hibernating little brown bats, *Myotis lucifugus*. *Journal of Mammalogy, 71*, 475–479.

U.S. Fish and Wildlife Service. (2012a). *North American bat death toll exceeds 5.5 million from white-nose syndrome* (Press release, 17 January). Retrieved from: http://static.whitenosesyndrome.org/sites/default/files/files/wns_mortality_2012_nr_final_0.pdf.

U.S. Fish and Wildlife Service. (2012b). *White-nose syndrome confirmed in federally endangered grey bats* (Press release, 29 May). Retrieved from: http://static.whitenosesyndrome.org/sites/default/files/files/gray_bats_2012_nr_final_0.pdf.

U.S. Geological Survey. (2012). *National Wildlife Health Center: White-nose syndrome (WNS)*. Retrieved from: http://www.nwhc.usgs.gov/disease_information/white-nose_syndrome/.

Warnecke, L., Turner, J. M., Bollinger, T. K., Lorch, J. M., Misra, V., Cryan, P. M., et al. (2012). Inoculation of a North American bat 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., et al. (2013). Pathophysiology of white-nose syndrome in bats: a mechanistic model linking wing damage to mortality. *Biology Letters, 9*, 20130177.

Weary, D. M., Huzzey, J. M., & von Keyserlingk, M. A. G. (2009). Board-invited review: using behavior to predict and identify ill health in animals. *Journal of Animal Science, 87*, 770–777.

West, B., Welch, K. B., & Galecki, A. T. (2007). *Linear mixed models: A practical guide using statistical software*. Boca Raton, FL, U.S.A.: Chapman & Hall/CRC.

Wibbelt, G., Kurth, A., Hellmann, D., Weishaar, M., Barlow, A., Veith, M., et al. (2010). White-nose syndrome fungus (*Geomyces destructans*) in bats, Europe. *Emerging Infectious Diseases, 16*, 1237–1242.

Willis, C. K. R., Menzies, A. K., Boyles, J. G., & Wojciechowski, M. S. (2011). Evaporative water loss is a plausible explanation for mortality of bats from white-nose syndrome. *Integrative and Comparative Biology, 51*, 364–373.

Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A., & Smith, G. M. (2009). Violation of Independence. Part II. In *Mixed effects models and extensions in ecology with R* (p. 70). New York, NY, U.S.A.: Springer.